EU-ANSA Research Cluster on 'Innovative 3Rs (Replacement, Reduction and Refinement of animal testing) approaches for the prediction of properties of chemicals, cosmetic ingredients, medicines, environmental contaminants and other regulated products'

Prepared by ECHA, EFSA, EMA and the SCCS/SCHEER in consultation with the JRC-IHCP.

Introduction and current situation

- **Legislative requirements**

Humans, animals and the environment are exposed to chemicals and pollutants in various contexts and by various means. Legislation within the EU addresses this by means of different regulatory schemes covering chemicals (industrial and household), cosmetics and toiletries, medicines (human and veterinary), biocides, plant protection products, food and animal feed additives and food contaminants (i.e. from food packaging or residues from pesticides or veterinary products, or other sources). All these schemes require an assessment of properties on humans and, if applicable, on the environment and animals. This means examining their toxicological and ecotoxicological properties as well as human data, i.e. hazard identification and quantification (or semi-quantification). The exposure to the relevant human (or animal) population and environmental compartment(s) determines whether the hazardous properties will result in harm. The final step in risk assessment is risk characterisation, in which exposure is compared to the results of the hazard identification and quantification, resulting into a qualitative or (semi-)quantitative estimate of the likelihood that adverse effects may occur. (Eco)toxicological studies using experimental animals are traditionally a major source of evidence for hazard and risk assessment. However, non-animal studies such as in silico tools, in vitro methods, and other methods resulting in the reduction of animal testing such as biologically-based computer modelling, are increasingly prominent. There is a high motivation to develop these approaches further and explore their applications in the various regulatory areas. These new approaches have the potential to support screening and priority setting for chemicals of concern, enhance mechanistic understanding of biological and toxicological processes, as well as provide means to move towards quantitative hazard and risk assessment.

The REACH Regulation provides a high level of protection for human health and the environment: the legislator balanced the need for information on properties of chemicals while avoiding unnecessary animal testing. New animal testing must only be done as a last resort and registrants have the possibility to make use of 'alternatives' to fulfil information requirements. Such 'surrogate' data must be good-enough for classification and risk assessment. ECHA's Article 117(3) reports show that the most frequently used alternative methods are read-across/categories and weight of evidence (WoE) adaptations (ECHA, 2017b).

The need for non-animal alternatives is important for hazard assessment and is especially pressing for cosmetics' risk assessment. In Europe testing of cosmetic products and their ingredients on animals, as well as their marketing, are banned under the Cosmetics Regulation (Regulation (EC) No 1223/2009). This means that safety data for cosmetic ingredients can only be drawn from validated alternative methods, and since 11 March 2013 all 3R methods are, for the purpose of cosmetics, restricted to only 1R (i.e. Replacement of animal testing). In this regard, the Scientific Committee on

Consumer Safety (SCCS), takes into account all available toxicological data, including data from *in vitro* tests, *ex vivo assays*, *in silico* (computational) models, read-across, grouping and physiologically-based pharmacokinetics (PBPK) and toxicokinetics (PBTK) modelling (SCCS, 2016a).

The regulatory acceptance of several non-animal approaches has been achieved for some of the so-called lower-tier information requirements of local toxicity and short-term effects, but not for effects that generally become evident over long-term exposure. No validated replacement alternatives are currently available for repeated-dose toxicity (subacute, sub-chronic and chronic toxicity), carcinogenicity, reproductive toxicity and the major part of toxicokinetics.

When assessing toxicity of chemicals for risk assessment, an understanding is needed of what the body does to the xenobiotic (i.e. "toxicokinetics (TK) and metabolism") and what the xenobiotic and its metabolites do to the body (i.e. "toxicodynamics"). The predicted toxicity and realistic exposure scenarios are the basis for the risk characterisation of a chemical. For the pharmaceutical sector, active sub**s**tances in medicinal products are data-rich with clinical knowledge of kinetics and metabolism. PBPK models when available, may further help understanding the effects of the substance in humans (or in animals for veterinary medicines).

- **Alternative methods**

Significant developments have taken place over the last decade to replace vertebrate animal testing for chemical safety assessment with non-animal approaches. These developments have already resulted in a reduced need for animal testing under the EU chemicals legislation and hold promise for enabling the regulators to take further steps towards a stronger implementation of the 3Rs principle of Replacement, Reduction, and Refinement of animal testing (ECHA, 2017a).

In the context of 21st century toxicology, a number of new approaches and methods have been developed to support risk assessment using the latest developments in molecular biology and bioinformatics, which reduce animal testing. These methods are based on a collection of computational, *in chemico*, *in vitro* and "-omics" approaches, as well as other forms of non-standard evidence, not traditionally used for such purpose (e.g. exposure considerations and TK modelling) (EFSA, 2014). Non-animal approaches include all approaches that do not involve new *in vivo* testing. *In silico* methods refer to computational methods such as those based on quantitative or qualitative structure-activity relationships ((Q)SARs), expert systems or physiologically based toxicokinetic (PBTK) modelling. *In vitro* methods usually involve isolated organs, tissues, cells, or biochemical systems. Most common *in vitro* methods are based on cell or tissue cultures. Cell cultures are either monocultures (one cell type) or co-cultures of two or more different cell types and may represent complex in vitro systems). Stem cells are also used in some systems. Most cell cultures have a 2D-configuration. The latest developments, however, aim to create 3D-models including microphysiological systems also called organs-on-a chip, which are more complex and better mimic *in vivo* organ functionality. Examples are 3D skin models and other organs-on-a-chip (liver, gut etc.). The most advanced methods aim to mimic the functions of several organs, in 3D models of multi-organs combination.

The combination of high-throughput (HTP) and high-content methods (HCM) with *in vitro* methods allows the use of multi-analyses and multi-sample test systems compatible with automation and reduced amounts of test substance. Various methods are based on "-*omics*", which are large-scale analytical techniques that can be used to support and understand biological mechanisms, to group

particular sets of biological molecules produced in cells and provide profiles or "fingerprints" that reflect cell responses to a toxicant. *In chemico* assays are abiotic assays that measure chemical reactivity. The term New Approach Methods (NAM) embraces all these non-animal approaches and was coined at ECHA's Topical Scientific Workshop 'New Approach Methodologies in Regulatory Science' (ECHA, 2016).

The field of *in silico* toxicology has undergone a lot of scientific developments over the past few decades with the availability of large property/effect databases, powerful data-mining tools, diverse statistical algorithms and soft-computing techniques that can find relational patterns in complex datasets. As a result, a number of versatile *in silico* methods and tools is now available, including toxicity expert systems that combine rules, structural alerts and/or (Q)SAR models. The available *in silico* models and systems cover a wide variety of chemical types and many of the key toxicological endpoints. Hybrid models have been developed that are based on a combination of knowledge-based rules and statistically derived models. *In silico* models/systems are only considered suitable for regulatory use if they have been developed in accordance with the stringent quality criteria and the validation principles laid down by the OECD (2004). The OECD QSAR Toolbox has been developed continuously for the last 10 years as a collaborative project between ECHA and the OECD.

A single alternative model usually is not sufficient to study all chemical types and all toxicological endpoints. It is more appropriate to use a combination of relevant model/system to increase confidence in the derived toxicity estimates, which should be further integrated with the overall weight of evidence (WoE). Recently examples of in silico modelling including QSAR models have been developed using EFSA's Chemical hazards database OpenFoodTox, other relevant databases (e.g. US-EPA terrestrial database, Fraunhofer RepDose) and the open source VEGA platform. QSAR models were developed for predicting sub-chronic toxicity in rats, for ecological risk assessment using a continuous QSAR model for predicting acute toxicity in rainbow trout and a classification QSAR model for acute contact toxicity data in bees (Como et al., 2017; Toporova et al., 2017).

- **Combining evidence**

Different approaches exist to combine evidence to characterise hazards. The general term integrated approach to testing and assessment (IATA) is used for a pragmatic, science-based approach, consisting of modules or components that are each based on types of evidence (i.e. by type of information or at the mechanistic level). An IATA necessarily includes a degree of expert judgement in weighing the available information.

The adverse outcome pathway (AOP) concept significantly increases the possibilities for constructing non-animal approaches that utilise information on biological pathways and disturbances that relate to adverse effects. An AOP is defined by the OECD as 'an analytical construct that describes a sequential chain of causally linked events at different levels of biological organisation that lead to an adverse health or ecotoxicological effect'. AOPs are the central element of a toxicological knowledge framework being built to support chemical risk assessment based on mechanistic reasoning" (OECD, 2017). An AOP typically starts with a molecular initiating event (MIE), which is the interaction of the substance of interest with its biological targets, e.g. cellular proteins. The MIE triggers a sequential chain of key events (KEs), which are alterations of biological processes each causing a certain downstream effect. This chain eventually leads to an adverse outcome at the tissue/organ level (like liver fibrosis) or at the organism or even population level (for environmental effects). It is generally

assumed that a certain threshold has to be met to provoke the MIE and each of the following KEs. Thus, a certain concentration of the substance is needed to lead to an adverse outcome. It should also be considered that several upstream KEs might lead to the same downstream event. In that sense, there is not a unique AOP for each adverse health or toxicological effect; rather, it can be through several, sometimes inter-linked, AOPs that substances can cause a specific adverse effect. AOPs do not include aspects related to the toxicokinetics of a substance. Therefore, additional information, in particular on metabolism, is essential to understand whether, for instance, it is the substance itself and/or its metabolites that trigger the MIE. Modes of action (MoAs) refer to toxicological pathways leading to effects. MoAs may not describe mechanisms of action but refer to pathways at a more general level. MoAs and AOPs are different frameworks, although there are similarities. As described above, AOPs concern non-substance-specific biological pathways and the outcome of an AOP is an adverse effect. By contrast, MoAs are substance-specific and include elements such as toxicokinetics and metabolism, and adversity is not needed to define a MoA.

- **Summary**

In summary, NAMs have the potential to predict human relevant adverse effects, but so far they can only replace animal tests predicting local and short-term toxicity in humans. Nevertheless, NAMs have the potential to complement and enhance the 'traditional' animal-based (eco)toxicology studies, together with 'read-across'. In addition, non-animal approaches are able to confer classical, whole organism-based toxicology with mechanistic information.

Challenges and current limitations of non-animal approaches

- ***in silico* methods**

It should be noted that for complex toxicological endpoints, QSAR prediction models are currently not considered reliable. The SCCS Memorandum on the use of *in silico* methods for assessment of chemical hazard (SCCS, 2016a) highlights the current limitations and barriers in regard to the use of *in silico* models/systems in regulatory risk assessment. Risk assessors rely on data from 'valid' methods; hence a system for assessing and regarding the *in silico* models/systems as 'valid' (within the bounds of the applicability domain and other statistical parameters) is needed. Further limitations of the methodology include that most of the currently available *in silico* models/systems cannot make precise estimates for toxicity of stereoisomers of a bioactive substance, inorganic substances, and certain other materials (e.g. nanomaterials). A robust framework is also needed to establish the quality and validity of the different available in silico models/systems, as well as a systematic way for the selection and use combined models/systems to overcome some of the limitations associated with the use of a single model/system. This will inevitably also include a framework to resolve any conflicting results and integrate toxicity estimates from different models. Another common limitation is the fact that dose-response information required for risk assessment cannot be derived from the currently available QSAR models.

- ***In vitro* tests**

It should be noted that *in vitro* models are mostly used to predict effects with a known mechanism of action for which these models were designed. Nevertheless, this can help to reduce the use of animals in by informing on how to avoid non-relevant *in vivo* studies. Ideally, results from *in vitro*

studies should be extrapolated to reflect the situation *in vivo* or in humans to allow considerations of hazardous exposure levels. There is still limited knowledge on (quantitative) *in vitro* to *in vivo* extrapolation ((Q)IVIVE) and to correlate the *in vitro* concentration-response curves to equivalent *in vivo* or human dose-response relationships and hence determine threshold exposure values for risk assessment. Another challenge is the fact that cell lines may give a different response compared to cells in the body ("wild type cells"). Moreover, cultured cells may lack metabolic capability or have an unbalanced metabolism: hence, this is often compensated for by the addition of an external source of metabolic enzymes (S9 fraction). More recently, the use of engineered cells expressing metabolic enzymes, the use of enriched culture media, 3D co-cultures and organ-on-a-chip technology are often used to overcome this limits). Furthermore, *in vitro* tests hardly reflect systemic interactions of cells, tissues and organs, which is often lacking, and do not simulate the effects in the whole organism, which is always more complex than a combination of information from several *in vitro* tests. Again new technologies such as the microfluidic systems are under development.

Tests based on "omics"-approaches and the interpretation of these results are still under development and have not yet been standardised for regulatory purposes.

- **Combining evidence**

In order to replace animal studies for risk assessment of individual substances, it is necessary to provide a full account of what data have been generated while using NAMs and other information to further develop approaches to combine different lines of evidence. In this area, EFSA has recently published a guidance document on the use of the WoE approach in scientific assessment: WoE approaches have been discussed for chemical risk assessment including the use of in vivo studies for regulated substances and in silico results for data poor chemicals (e.g. emerging contaminants) (EFSA, 2017).

IATAs may pose a particular challenge because they cannot be validated for regulatory purposes in the conventional way. This is only possible for defined approaches which include a fixed data interpretation procedure (DIP) applied to data generated with a defined set of information sources . IATAs allow the use of flexible approaches and WoE approaches using expert judgement, which are more difficult to standardise and are more complex to evaluate compared to single test methods.

Our actual knowledge of potential MoAs and AOPs is limited, thus restricting the development of AOP-based methods and approaches. It is not possible to develop an AOP without knowledge of the MIE and KEs and how they are linked together. For complex toxicity endpoints, it is challenging to develop AOPs, because many of them actually cover a wide range of mechanisms. Although complex endpoints could in principle be split into several distinct processes that are easier to model, there are still many unknown aspects and many biological events that may not be covered. This makes the prediction using these non-animal approaches currently impossible for complex endpoints.

- **Barriers for NAM use**

The following barriers to the wider use of NAMs in regulatory contexts have been identified:

- Difficulty for industry, risk assessors and regulators in assessing the relevance of the NAM evidence and its associated uncertainty, especially in relation to risk assessment and classification.

- Lack of quality standards on the performance of NAM tests (to demonstrate robustness and reproducibility) and lack of common standards for reporting NAM evidence.
- Lack of a means of getting a seal of 'recommendation/approval' by an authoritative body for some of the approaches (e.g. *in silico*) to enable the NAMs to cross the current barriers for regulatory acceptance.
- So far, NAMs have focused mostly on toxicodynamic aspects (e.g. toxicity) while biokinetic aspects (including absorption, bioavailability, distribution, metabolism, excretion) applied also to in vitro systems are often missing. In comparison, risk assessment of medicines, such as human or veterinary pharmaceuticals, requires very detailed information on both the pharmaco- toxicokinetics, and the pharmaco- toxico-dynamics including human safety data and clinical pharmacology before a medicinal product reaches the market.

Common Research Priorities

- **Stocktaking**

The availability of comprehensive, validated and up-to-date databases (on both human exposure to and effects of various stressors in humans) form the basis for the further development of the new paradigm and for the advancement of (Q)SAR and read-across approaches in risk assessment.

Building an inventory of NAMs and available models predicting different types of effects, and being at different stages of development and regulatory applicability, would clarify their diversity and interrelations, and hence could facilitate their further development and application. A high-level mapping of NAM techniques and perhaps also a more detailed comprehensive NAM inventory would be useful in facilitating research and encouraging identified 'gaps' to be filled. This could complement some existing projects: e.g. COSMOS (www.cosmostox.eu), ANTARES for *in silico tools* (www.antares-life.eu/index.php?sec=modellist), the JRC database of QSAR models (https://eurl-ecvam.jrc.ec.europa.eu/databases/jrc-qsar-model-database) and the OECD Toolbox (http://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm).

Research is needed to further improve and potentially combine these approaches to bring more mechanistic and biologically based approaches as well as statistically sound methods to the risk assessment process.

- **Combining evidence**

The AOP concept, the MoA approach, and IATA's are available research tools that can be further improved for risk assessment and regulatory use in the future. In practice, these integrated approaches apply well-established WoE methods considering the confidence in toxicity prediction and characterising uncertainty and variability.

Within this context, it should be kept in mind that the degree of confidence needed for the prediction depends on data availability and the purpose of the prediction, i.e.:

- As a direct replacement for animal studies.
- As a tool to provide mechanistic understanding.
- As a contributor to assess the human relevance of toxicology studies, especially for higher-tier endpoints.
- As a tool for screening and priority setting.

Research is needed to address the challenges of (a) biokinetics, (b) coverage of biological pathways (i.e. developing relevant AOPs) and (c) quantitative AOPs (i.e. currently, AOPs are only qualitative).

- **Selective versus non-selective chemicals**

A useful starting point could be to distinguish between toxicants that are 'non-selective' (most general chemicals) versus 'selective' with a dominant MoA or AOP (e.g. pharmaceuticals and pesticides as these have an intended biological activity). A screening set of NAMs, based on the ToxCast testing, could provide evidence that the substance has a non-specific MoA. For these chemicals, toxicity may be expressed on multiple organs and may be driven by multiple targets. Moreover, for approaches such as modelling effects in organs, 'general' non-specific adverse effects can be examined in parallel with specific MoA or general chemical activity (e.g. alkylation) to elucidate their mechanism. It is foreseen that in the future designing screening sets of NAMs including transcriptomic profiling 'fingerprinting' may be a supportive tool to provide data on sets of Key Events common to 'non-specific toxicity' and to screen for concern for specific toxicity. Another important aspect is the analysis of standard *in vivo* test guidelines and studies to gain an understanding of which findings are critical for the assessment of adversity e.g. derivation of reference points/points of departure (NOAEL, BMDL etc.) and for the derivation of reference points (e.g. ADI, DNEL etc.). These parameters and observations constitute the *in vivo* consequence of a MoA/AOP to be covered by NAMs including non-testing methods. Finally, the issue of how to validate the predictions needs to be addressed to complete the approach.

- **Other ideas**

Work is also needed on human relevance and toxicokinetic and toxicodynamic differences *in vitro* compared to the *in vivo* situation.

Finally, approaches should be developed on the application of NAMs to assess both complex-composition substances, i.e. multi-constituent substances and unknown or variable composition, complex reaction products or biological materials (UVCBs) and intentional chemical mixtures.

Strategic approach

- **SCHER/SCENIHR/SCCS view**

The general use of NAMs in a regulatory context is very challenging and would require strong international commitment and focussed research initiatives involving all stakeholders.

In the SCs opinion 'Addressing the New Challenges for Risk Assessment (SCHER/SCENIHR/SCCS, 2013) it is clearly stated that the primary changes proposed for the future improvement in risk assessment procedure may be characterised as follows:

- A paradigm shift from a hazard-driven process to one that is exposure-driven,
- A progressive reduction of tests using laboratory animals.
- An increasing importance of mechanistic and kinetics data to be integrated with dynamic data
- Focus on quantitative aspect and human data

The first bullet point allows limiting testing by considering exposure-based waiving, making use of the so-called non-testing approaches, such as Thresholds of Toxicological Concern - TTC, grouping and read-across (supported by *in silico* analysis).

Toxicokinetics can inform on the need for further testing based on bioavailability considerations. Hence, for safety assessment of a cosmetic ingredient, testing for systemic toxicity would in principle only be necessary if the ingredient penetrates into the body following dermal, oral, or inhalation exposure and if the internal exposure potentially exceeds critical levels: this is the concept of the internal Threshold of Toxicological Concern (Adler *et al*, 2011). Therefore, validated methods in order to obtain toxicokinetic information are crucial for an appropriate risk assessment.

The second bullet point can be achieved by the development of new experimental systems (e.g. microfluidic models, 3-D cultures, lab-on-a-chip), or new *in vitro* methods that preserve all the properties of their *in vivo* original source for prolonged periods of time, including the biokinetic parameters in the study design and allowing the establishment of clear relationship between *in vitro* endpoints and adverse effects *in vivo* (differentiation, adaptation and adversity biomarkers).

Since most of such alternative methods cannot be used as standalone, it will be necessary to integrate them into an IATA or intelligent testing strategy (ITS) based on a WoE to integrate several lines of evidence on mode or mechanisms of action. Such IATAs/ITSs need to be informed by mechanistic and biologically based approaches such as toxicokinetic and toxicodynamic modelling, AOPs and MoA information.

Validation of NAMs is extremely challenging. Current validation of tests uses the animal test as the gold standard, but NAMs should preferably be validated on the basis of human biology and using human data. This needs the establishment of generally accepted performance criteria for the elements in the NAMs and for the integrated system.

- **Elements for enhancing NAM development and application**

The following priorities provide elements for a way forward of using NAMs in a regulatory context:

- A step-wise strategy for the integration of new standards in regulatory applications at a horizontal level in EU legislation.
- For application of NAMs on short-term and with maximised impact, the priority should be set on further improvement of read-across methodologies. In particular:
  - For the mechanistic support of read-across applications addressing mechanistic aspects at the toxicity/toxicodynamic and toxicokinetic level.
  - Improving read-across from comparing biokinetics assessment in the ‘source’ and the ‘target’ substances: the question to be answered is whether the difference in chemical structure between the ‘source’ and ‘target’ substances affects the toxicokinetics significantly.
  - Screening to eliminate the possibility of unexpected toxicity in the ‘target’ substance in read-across cases.
  - Improved grouping using NAMs based on bioactivity, i.e. strengthened evidence for biological similarity complementing chemical similarity.
- A consultation of risk assessors from ECHA, EMA and EFSA as well as SCCS/SCHEER could lead to a priority list of chemicals, cosmetic ingredients and pharmaceuticals that are of

regulatory interest. A "research challenge" could be issued to researchers to explore the use of NAM for the shortlisted chemicals.

- Develop structured assessment approaches for weight of evidence approaches of NAMs.
- Besides the role in current risk assessment schemes, in the field of chemicals it should be further elaborated how to:
  - Use NAM-derived hazard information to prioritise further assessment of data-poor chemicals, and
  - Explore novel hazard classification schemes and related criteria based solely on NAM-derived information.

International consensus on the regulatory application of NAMs is essential, and this would be appropriate under the auspices of the OECD.

A degree of validation and standardisation of NAMs, where possible, is desirable in order to encourage routine use and facilitate their application in risk assessment.

Opportunities for Collaboration between Agencies

Publicly available data from the scientific opinions from SCCS/SCHEER as well as ECHA, EFSA and EMA databases could be used to explore case studies using NAMs, particularly for substances common to several regulatory schemes (e.g. pesticides and biocides, food contaminants, cosmetics and pharmaceutical/veterinary residues).

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