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Workshop on alternative in vitro methods to vertebrate studies under CLP

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Abbreviations

AOP	Adverse Outcome Pathway
ECHA	European Chemical Agency
FHAIVE	Finnish Hub for Development and Validation of Integrated Approaches
GLP	Good Laboratory Practice
HRIPT	Human Repeat Insult Patch Test
IA	Integrated Approaches
IATA	Integrated Approaches to Testing and Assessment
KI	Karolinska Institute
LLNA	Local Lymph Node Assay
NAM	New Approach Methods
NGRA	Next Generation Risk Assessment
NKE	Nordic Working Group for Chemicals, Environment, and Health
NZ	Northern Zone
PPP	Plant Protection Product
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
SARA	Skin Allergy Risk Assessment
SciRAP	Science in Risk Assessment and Policy
WoE	Weight of Evidence

Preface

In line with the current European legislation, the NZ member states encourage the reduction of vertebrate testing for the authorization of plant protection product to the necessary minimum required to maintain and improve the human health and safety standards in the region. For the authorization and classification of PPPs, Regulation (EC) No. 1107/2009 (on PPPs) in accordance with Regulation (EU) No. 284/2013 (on data requirements for PPPs) and Regulation (EC) No. 1272/2008 (CLP) need to be considered.

The Regulation (EC) No. 1272/2008 administers the hazard-based classification and labelling of chemicals and chemical mixtures in the EU and thus also applies to agrochemical mixtures. According to the Article 8 of this Regulation which specifically states that animal testing shall be conducted only, if all other means of generating information have been exhausted. Those other means include the use of alternative approach i.e., application of 'bridging principles' (Annex I, Section 1.1.3), the calculation method (Annex I, Section 3.1.3.6), and the validated *in vitro/in silico* methods. Although the evaluation of all this data may be complex, it is encouraged to use a weight of evidence approach involving expert judgement (Paragraph 33)[1].

Such requirements are also formulated and supported in the Regulation (EU) No. 284/2013, i.e., a sufficient information must be provided to allow for the evaluation of foreseeable risks of PPPs for humans, including any information on potentially harmful or unacceptable effects as well as expected cumulative and synergistic effects (Annex, Introduction, Paragraph 1). This information should be generated but not limited to only *in vivo* data, instead, justified alternative approach shall be given preference (Annex, Introduction, Paragraph 5). Hence, for the evaluation of PPPs, the conduction of acute in vivo toxicity studies is not required, if an alternative approach can be justified, which considers possible mixture effects.

[1] A guidance document on the use of the weight of evidence approach in scientific assessments was recently published by EFSA (EFSA Journal 2017;15(8):4971)

However, the challenge is that in the absence of bridging data and the presence of unknown toxicity, no international guidelines currently exist, for the in vitro testing of acute oral, dermal, and inhalation toxicity.

In addition to the acute toxicity, the information on irritation, and sensitization potential of PPPs is also required for a complete hazard assessment to determine the classification and labelling according to the Regulation (EC) No. 1272/2008. For these toxicity endpoints, although validated *in vitro* test methods exist, the data generated needs to be evaluated carefully due to the limitations in their applicability domain i.e., developed for single substance evaluation and not for the mixtures such as PPPs.

The target audience of this workshop was regulatory risk assessors in the NZ working with REACH, PPP and CLP regulations. The purpose of this workshop was to increase the knowledge and understanding of the development, validation and evaluation process of in vitro test methods, the pros and cons of the test methods e.g., in mixture evaluation such as PPPs, as well as to facilitate the harmonization of the practices regarding the acceptance and evaluation of toxicity data in the NZ and in the EU.

Partners and collaborators

The steering group of the workshop constituted Marika Päällysaho, Hanna Lindberg (Finnish Safety and Chemicals Agency), Laura Voitina (State Plant Protection Service of the Republic of Latvia), Pia Hunter, Louise Lundberg (Environmental Protection Agency of Denmark), Diana Lilienblatt, Priit Kersen (Agriculture and Food Board, Republic of Estonia), Dalia Janušauskaitė, Audra Paltanavičienė (State Plant Service under the Ministry of Agriculture, Lithuania), Annike Totlandsdal, Maren Bakkebø, Irene Beate Sørvik, Tirill Medin (Norwegian Food Safety Authority), Imran Ali, Ingrid Berg, Jonas Tägt (Swedish Chemicals Agency).

The workshop was funded by the NKE.

The workshop was organized on 26-27th October 2022 (face-to-face) in Helsinki-Finland. Marika Päällysaho, Hanna Lindberg (Finnish Safety and Chemicals Agency) were organizers of the workshop.

Workshop summary and outcome

Background

Toxicity testing of chemicals is moving steadily toward human cell-based *in vitro* approaches for reasons including scientific relevancy, efficiency, cost, and ethical rightfulness[2,3].

The purpose of this workshop was to increase the knowledge and understanding of the development, validation and evaluation process of *in vitro* methods, the limitations of *in vitro* test methods in mixture evaluation such as PPPs, as well as to facilitate the harmonization of the practices regarding the acceptance and evaluation of *in vitro* toxicity data in the NZ member states and in the EU.

Objective

There are still various data gaps for *in vitro* toxicity testing involving the assessment of human health risk from chemical exposure in particularly for PPPs. This workshop addressed both knowledge gaps e.g., reliable extrapolation from *in vitro* to *in vivo*, and delved into the issues of validation and

[2] Kolle, S. N., van Ravenzwaay, B., & Landsiedel, R. (2017). Regulatory accepted but out of domain: In vitro skin irritation tests for agrochemical formulations. *Regulatory toxicology and pharmacology : RTP*, *89*, 125–130.

[3] Roberts, D. W., & Patlewicz, G. (2018). Non-animal assessment of skin sensitization hazard: Is an integrated testing strategy needed, and if so what should be integrated?. *Journal of applied toxicology : JAT*, *38*(1), 41–50.

acceptance for different *in vitro* methods and increased awareness of their usefulness in prediction of health hazard in a regulatory setting.

The future harmonization of practices, for the use of *in vitro* toxicity test methods for health risk assessment of chemicals, in the Northern Zone will contribute in particularly to decrease the cost and time required for animal testing which ultimately benefit not only the authorization holders but also ~~to~~ the farmers, and citizens of all NZ countries. Moreover, it will also promote the collaboration through knowledge transfer with other EU toxicological colleagues. The NZ guidance document will be updated based on discussions and knowledge acquired during this workshop.

Summary

Existing OECD test guidelines developed for the testing of specific endpoints such as inhalation, oral and dermal toxicity rely on the measurement of adverse effect. It is suggested that focusing on the molecular mechanism following chemical exposure instead will help identify grouping of chemicals with similar mode of action. This can further develop and utilize the AOP network to give better understanding of the chemical's behavior and therein cumulative exposure effects. However, existing tools to interpret -omics data yet relies on non-GLP studies which complicates the process of validation for regulatory usage.

In vitro assessment of eyes and skin irritation often demands multiple studies with defined approaches i.e., Top-Down or Bottom-Up, for testing strategies being utilized. Moreover, further development of regulatory validated testing methods is being made for NGRA such as SARA-model for skin allergy although currently only applicable to cosmetic products but can also be developed to PPPs provided that adequate data is generated. In contrast to most *in vivo* studies, validation of new *in vitro* is often based on mixtures rather than single substances although cumulative exposure is still not addressed enough by the available OECD testing methods.

Existing regulatory testing methods still has issues with hazard identification where study results point in different directions. Conclusions drawn seldom integrates both human, *in vivo* and also mechanistic data where developed WoE strategies might fill that gap through combination of data. The central issue of evaluating reliability and relevance of data for WoE assessment is deemed crucial which the tools such as SciRAP might ease, but not eliminate.

Conclusions

The workshop represented good collaboration between academia and regulatory authorities in the NZ member states as well as ECHA. All participants from the regulatory authorities in the NZ member states, considered this workshop very useful and educating. Based on our discussion during the workshop, it was concluded that more efforts are required concerning the development of new alternative *in vitro* methods as stand-alone NAM's for specific endpoints. The development and acceptance of new omics and *in vitro* methods, for assessing and managing the risks relating to chemical mixtures such as PPPs, will help in minimizing the risks to human health. However, one of the main issues, in developing such methods, is the lack of validation for the regulatory use, as many proposed strategies are based on data which does not satisfy the regulatory demands or is non-GLP compliant.

Specific recommendations from the workshop

- ✓ Since, the *in vitro* test methods can provide deeper understanding of the MoA for a substance, this information can be utilized for the risk assessment.
- ✓ Due to the limitations (applicability domain) of available *in vitro* methods, evaluation of the data should be made carefully by using the WoE approach.

- ✓ Further development and validation of suitable *in vitro* methods, especially for mixtures, would be required for use in the regulatory risk assessment of PPPs.

Ongoing and follow-up NAMs activities

Next Generation Risk Assessment (NGRA) work, started by the cosmetics sector, includes non-animal approaches with promising development for use to predict safe levels for human exposure[4]. NGRA may be developed further for use in the RA of agrochemicals such as PPPs in near future, provided that the required data is generated and available. A feasibility study using such approach i.e., SARA-model, has been included under the OECD work program. SARA model uses database of public *in vitro* data from *in-chemico* sensitization, key events, historic LLNA and HRIPT data and may lead to inclusion of a quantitative risk assessment for skin sensitization, under the Defined Approach test guideline, although new *in vitro* data may need to be generated. If an international agreement is obtained, potential application in setting specific concentration limits, assessment of safety for biocidal, pesticides and PPPs might be obtained. Personnel within the regulatory sector of PPPs are prompted to stay contact with their national OECD coordinator for updates.

Biographies of the speakers

Prof. Dario Greco is an expert in the field of toxicogenomic and the director of FHAIVE and Dr. Jack Morikka is a postdoctoral researcher at FHAIVE with expertise in molecular biology. FHAIVE is a research hub in the Faculty of Medicine and Health Technology, Tampere University. It is the GLP national reference laboratory in Finland for validation of alternative methods (ECVAM) and the coordination of the Finnish 3R centre. In FHAIVE, IATA are developed by integrating advanced in vitro models with toxicogenomics an AI-enabled advanced data modelling. The research hub offers GLP IATA (including OECD methods) and validation services regarding chemical safety, drug safety and efficacy, biologics, nano/biomaterials and medical device safety.

Prof. Dario Greco

Dr. Jack Morikka

Laura Rossi, Scientific officer at ECHA holding MSc in biochemistry and with a background in academic research in Treg development in humans. Expertise in in vitro methods covering skin and eye irritation and skin sensitisation, with current focus more on the skin sensitisation methodologies. Laura is involved in the OECD expert groups on irritation and sensitisation, in individual TGs and Defined Approaches and Guidance Documents.

Laura Rossi

[4] Gilmour, N., Reynolds, J., Przybylak, K., Aleksic, M., Aptula, N., Baltazar, M. T., Cubberley, R., Rajagopal, R., Reynolds, G., Spriggs, S., Thorpe, C., Windebank, S., & Maxwell, G. (2022). Next generation risk assessment for skin allergy: Decision making using new approach methodologies. *Regulatory toxicology and pharmacology : RTP*, *131*, 105159

Dr. Anna Beronius, Toxicologist and senior research specialist at the Institute of Environmental Medicine, Karolinska Institutet, Sweden. Dr. Beronius conducts research in the science-to-policy interface, focusing on advancing methodology for hazard and risk assessment of endocrine disruptors and chemical mixtures. This includes optimizing use of mechanistic data in chemical assessments, development and application of Adverse Outcome Pathways (AOP), and development of weight of evidence assessment methodology. Dr. Beronius is also Deputy Programme Director at the Global Master's Programme in Toxicology at Karolinska Institutet and is involved in training of professionals at national and European authorities, such as EFSA and ECHA.


Dr. Anna Beronius

Presentations

Dr. Jack Morikka from FHAIVE at Tampere University spoke about the needs and challenges in developing and validating GLP models for regulatory purposes. Dr. Morikka explained the importance of GLP in the quality and reproducibility of the scientific data and how the GLP validation is organized in Europe and what kind of validation services FHAIVE can offer. Also, the limitations of GLP and the possibilities to extend GLP into IATA and NAM were discussed.

Following the GLP discussion Professor Dario Greco, also from FHAIVE at Tampere University, explained how different toxicogenomic approaches can be used in toxicological risk assessment. During the discussion, Prof. Greco emphasized that a lot of data is already available to learn from and that new tools are being developed for GLP-compliant computational analysis of the data to identify and understand the mechanistic information behind the toxicity of a substance. The development and validation of such tools is proposed to facilitate the inclusion of toxicogenomic data in the regulatory risk assessment. This would also include deeper knowledge about cumulative exposure while also utilizing and developing the AOP network for better future grouping of chemicals.

On the second day of the workshop, Laura Rossi from ECHA spoke about the available validated in vitro toxicity test methods for the assessment and evaluation of skin corrosion/irritation and serious eye damage/eye irritation. She explained how to design the testing strategies e.g., top down or bottom-up approach, the known limitations of the in vitro tests (in particularly the applicability domain, inability to analyze gases/aerosols and the false negative prediction) and how these methods can be used to make reliable predictions. Laura also discussed the validated in vitro skin sensitization approaches in particularly the Defined Approaches, based on key events from AOPs including the in-chemico methods. She also mentioned the development of NGRA of skin sensitization and the newly developed SARA model, with a feasibility study primarily focused on cosmetic products but which can potentially be expanded and used for PPPs. Laura emphasized that is it very important for risk assessors to stay in contact with OECD national coordinators to get updates on the new approaches.

The last speaker. Dr. Anna Beronius from the Institute of Environmental Medicine at Karolinska Institutet, spoke about WoE assessment in chemical hazard and risk assessment. Dr. Beronius explained the basic principles of WoE, when it could be applied in the regulatory settings and the known limitations and challenges of the approach. She also mentioned the SciRAP tool and its use for

evaluating the reliability and relevance of non-GLP toxicity data from scientific literature, where the aim is to bridge the gap between academic research and regulatory assessment of chemicals.

Acknowledgements

We would like to thank the organizers Marika Päällysaho and Hanna Lindberg from Tukes for hosting the workshop in Helsinki, the speakers for sharing their invaluable knowledge and all NZ tox participants for active discussions. We would also like to thank NKE for funding the workshop and providing the opportunity for meeting and discussing points of interest within the NZ tox group.

Conflict of interest

Professor Darion Greco and Dr. jack Morikka are employees of the Tampere University, Finland and perform research at FHAIVE research institute which is part of the Faculty of Medicine and Health Technology (MET). FHAIVE is devoted to the development and validation of integrated approaches (IATA). In FHAIVE, IATA are developed by integrating advanced *in vitro* models with toxicogenomic and AI-enabled advanced data modelling.

Dr. Anna Beronius is an employee at the Institute of Environmental Medicine (IMM), Karolinska Institutet, Sweden. IMM conducts research and provides training in health risk assessment for participants from academia, authorities, and industry. Moreover, at IMM, a secretariat for risk assessment handles requests and assignments from national authorities. The secretariat also coordinates more extensive national and international health risk assessment projects.