EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
Health, Consumers and Reference Materials
Chemical Safety and Alternative Methods Unit
EU Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM)

Summary Record

PARERE Meeting 27-28th November 2017, Ispra, Italy

The meeting of PARERE was held on 27-28th November 2017 (the agenda is included in Annex I).

Welcome and Introductory Session

The JRC's EURL ECVAM welcomed all members and invited all to give updates on the state of the PARERE network in the respective Member States. The establishment of such a network takes time, but progress is being made in most countries. The feedback is valuable and it was noted that the process is formative and educational.

AU mentioned the establishment of an Austrian *in silico-in vitro* safety science platform to support the PARERE and OECD work and improve funding.

SE stated that they started the work of their 3Rs Centre. They will collect the input on consultations of the different Competent Authorities in Sweden. The 3Rs Centre will also be the executive body for the National Committee in Sweden to be established under Directive 2010/63/EU on the protection of animals used for scientific purposes.

SE also asked if EURL ECVAM's Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) was still open for new membership. EURL ECVAM replied that there are currently no plans to extend EU-NETVAL with labs from member countries that are already represented in the network. However in case particular experience or competence for specific tasks is missed then ad-hoc requests for participation in particular studies may be published, under the condition that interested test facilities comply with the EU-NETVAL eligibility criteria.

NO mentioned that it was waiting for their National Committee to be established and that currently the National Institute of Public Health was involved in the PARERE network.

FIN informed that they have started education courses on the 3Rs at the University and that the internal feedback on PARERE consultations was not so much.

ES said that in Spain, the PARERE contact point was REMA, the Spanish Network for the Development of Alternative Methods, which has been nominated by the Spanish Ministry of Agriculture. Currently there are no plans to create a 3Rs Centre.

The UK expressed a strong wish to continue membership of PARERE and very much hoped that the Brexit would not impact on UK's PARERE membership.

PARERE members have been consulted on several occasions over the past year, either on the regulatory relevance of individual methods that had been submitted to EURL ECVAM or on case studies being developed within the EU-ToxRisk project funded by the EU Framework Programme for Research and Innovation, Horizon2020.

Updates on recent consultations with PARERE

Bioelution

The presentation made by EURL ECVAM summarised the main principles of the Bioelution test method, submitted by Eurometaux in July 2016. The bioelution method measures the degree to which metals ions dissolve in a simulated gastric fluid and are potentially available for absorption into systemic circulation (bioaccessible concentration). It is proposed to generate relative bioaccessible metal ion data by comparing the target compound versus the pure metal (reference material) for the oral route of exposure (see section 3.1 in Zuang et al., 2017). PARERE members were asked to give feedback on the interpretation of the CLP legal text, the regulatory applicability of the bioelution test method, its limitations and scientific relevance, the potential further validation of the test method, and the impact on animal testing. A summary of the responses received from PARERE was presented, along with the final decision by EURL ECVAM to ask the test submitter to proceed with a full submission. A number of issues were highlighted by the PARERE members that would need further clarification, such as the correlation between *in vitro* bioaccessibility and *in vivo* bioavailability, kinetic processes that occur *in vivo* are ignored (e.g. continue uptake of soluble ions, absorption, transport, and metabolism), the impact for alloys of potential changes of the matrix during life cycle, and the real contribution to the 3Rs. The need for an independent peer review of all available data was also stressed. The submitter was also asked to address in the full submission all remaining concerns coming from the evaluation of the test method pre-submission and the feedback received from the PARERE consultation. Many of the issues listed were also raised by the ECHA Bioelution Expert Group, which provided advice on the use of the bioelution test data in the context of the CLP Regulation as a refinement for the classification of alloys.

Discussion

It was commented that if you produce an alloy, the composition does not depend on metals only but also on the technological process and how well you can characterise such an alloy. It was agreed that some late input on the consultation could still be taken into consideration.

Some representatives were supportive of the method but they could see issues around ADME and taking this method in isolation. Other assays could complement the bioelution assay. On the question whether the test submitter had been recommended to use the data of the bioelution assay in an integrated way, i.e. with other data from other information sources, EURL ECVAM explained that the data were meant to be typically used in a weight of evidence approach but this depends on the context.

Data from the bioelution assay had been submitted in some EU Member States and they had been used in a read-across context. However there is a difference between human (pH 2) and rat (pH 4) gastric fluids. In a more basic medium there will probably be more release of metal ions. The assay assumes that there is only passive diffusion but there could also be absorption in the GI tract (e.g. duodenum).

EURL ECVAM explained that the test tries to simulate the human situation but that many factors need to be considered.

It was asked if there had been a request to compare the data of the bioelution assay with *in vivo* data. This question had indeed been posed to the test submitter in the assessment report and the submitter was expected to reply.

It was mentioned that in the case of cobalt, this type of data had not been accepted. It had been possible to build trends but for a single case the data were not descriptive enough.

It was mentioned that unless there was a specific use case it would be difficult to find what you should be validating.

EDITOX

This presentation summarised the main principles of the EDITOX test method, submitted by Alcediag on May 31st 2016. EDITOX is an *in vitro* cell-based method suitable to assess the risk for chemical compounds to induce psychiatric adverse side effects (depression, suicide). It quantitatively analyses in the human neuroblastoma cell line SH-SY5Y the RNA editing modifications of the serotonin receptor 2C (5-HT2cR) induced by pharmaceutical compounds. PARERE members were asked to comment on the appropriateness of the method, its regulatory applicability and its possible impact on animal welfare. A summary of the feedback received from PARERE was presented, along with the final decision by EURL ECVAM to ask the test submitter to proceed with a full submission. However, since EDITOX is entirely based upon one MoA and, as a standalone, it is not expected to fully replace *in vivo* testing for depression, EURL ECVAM also asked the submitter to provide with their full Test Submission a review of other possible modes of action of drugs with the risk of causing depression and/or pro-suicidal side effects and of *in vitro* test methods (i.e., battery of tests) that may be available to potentially cover these other MoAs.

Discussion

To the question if the method was relevant to developmental neurotoxicity (DNT) it was replied that it was for neurotoxicity testing in general but that it could also be applied to DNT.

It was emphasised again that serotonin is only one of the possible mechanisms that can induce depression/suicide and that this mechanism is not fully understood yet.

It was also mentioned that assessment of psychiatric side effects is currently not required by regulation.

It was felt that such a method could be used for prioritisation and screening purposes to further test the very nasty chemicals and those for which there is considerable human exposure.

ToxTracker®

A summary on the progress of the assessment of the ToxTracker® test method, including principles, design of the method, timeline and outcome of the PARERE consultation, was provided in this presentation.

The assay was submitted late November 2016 by Toxys B.V. as an *in vitro* method intended for testing genotoxicity that also include a number of non-genotoxic endpoints associated with human carcinogenicity hazards (Zuang *et al.*, 2017, chapter 3.2[1]). It was proposed to enter the current genotoxicity strategy as an additional *in vitro* test capable "to allowing accurate genotoxicity assessment, providing insight into the mechanisms of (geno)toxicity and reducing the frequency of misleading positive *in vitro* test results that would trigger *in vivo* follow-up testing." The test consists in a microplate format capable of detecting in a set of six different mouse Embryonic Stem (mESC) GFP reporter Cell lines four distinct biological responses: DNA damage, cellular stress, oxidative stress and protein damage as unfolded protein response. The six different cell lines can be simultaneously treated in a 96-well-plate and GFP reporter genes induction and cytotoxicity are detected by flow cytometry.

Based on the information received, EURL ECVAM has considered that the possibility to provide insight into the mechanism of genotoxicity by the combination of various endpoints could make the ToxTracker® a promising method potentially suited to cover human genotoxicity effect and considered the test as being biologically and mechanistically relevant. The above consideration has been confirmed by the positive feedback from OECD members, further to the submission of the project to the OECD test Guideline Programme, and positive comments received during the consultation with PARERE in March 2017. In June 2017, EURL ECVAM asked the test submitter for a full submission.

Discussion

To the question if any type of damage to the cells would be picked up as positives, it was replied that six different mouse Embryonic Stem (mESC) cell lines can be used together. Each one is capable to show the induction, if any, of a specific GFP reporter coupled to a specific signalling pathway/mechanism.

A PARERE representative asked if the final goal was to predict mouse or human carcinogenicity. It was replied that there had been some concerns over the use of mouse embryonic stem cells, however the six different genes had been chosen because they were preferentially activated upon exposure to different classes of known human carcinogens (40 chemicals).

[1] Zuang et al. (2017) EURL ECVAM Status Report on the development, validation and regulatory acceptance of alternative methods and approaches. Available at: https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-status-reports

Another representative mentioned that this test was a good example for applying omics to derive human relevant endpoints. It was also the first example that incorporates non-genotoxic mechanisms.

Toxicokinetic data in regulatory frameworks

The aim of this session on toxicokinetics was to exchange information and views concerning the use of toxicokinetic data in regulatory assessments. European Agency representatives (EFSA, ECHA, EMA) had been invited to provide their perspectives on regulatory requirements and opportunities for using toxicokinetic data, while EURL ECVAM gave updates on current activities. During the Q&A session, EURL ECVAM raised a number of questions for discussion, with a focus on:
a) the OECD Guidance Document for characterisation and description of *in vitro* human hepatic metabolic clearance methods; b) the OECD Guidance Document on the characterisation, validation and reporting of physiologically based models for regulatory applications; c) the OECD Test guidelines and Guidance Document on the determination of *in vitro* intrinsic clearance and extrapolation to fish bioaccumulation; and d) the next steps in the progression of CYP induction methods.

Perspectives of European Agencies

European Food and Safety Authority: Toxicokinetics and Metabolism Data in Chemical Risk Assessment: Background, Open Source Tools and Future Guidance Development at EFSA

In the food safety area, sound hazard identification and hazard characterisation require an understanding of both toxicokinetic (TK) and toxicodynamic (TD) processes for compounds entering an organism via the oral route. This enables the translation of external dose (exposure) into internal dose incorporating absorption, distribution, metabolism, excretion (ADME) of chemicals and toxicity for sound dose response modelling.

In 2014, the EFSA scientific report on “modern methods for human hazard risk assessment” reviewed the use of TK data and models in the food safety area, and identified the need for 1) basic TK data for chemicals in relevant test species and humans, 2) physiologically-based-models (TK/TD) for test species and humans and 3) improving *in vitro* methods to investigate ADME processes. Consequently, EFSA has been involved in collaborative research projects to develop generic open source TK tools including PB-TK models in humans, farm animals and fish as well as Dynamic Energy Budget models for species of ecological relevance. The open source platform ”TK plate” is under construction and should be available to the risk assessment community as a first prototype during 2018.

Recently, new data requirements from pesticide regulation 283-284/2013 request *in vitro* metabolism comparison between rat and humans and *in vivo* TK data for pre-market authorisation of pesticides. These developments highlight the need for the development of a guidance document supporting the use of TK and metabolism data as well as TK models. Such guidance development is planned for the near future together with case studies illustrating application in food safety, particularly for regulated products and contaminants.

European Chemicals Agency (ECHA): Use of toxicokinetic data in the context of REACH

Generation of new toxicokinetic data is not required by REACH Regulation. Annex VIII (10-100 tpa and above) requires "Assessment of toxicokinetic behaviour of the substances to the extent that can be derived from the relevant available data".

In the REACH dossiers, the toxicokinetic data is usually not experimental, but rather estimates or assumptions based on e.g. the physico-chemical properties of the substances. Reference is often made to lipophilicity, hydrolysis, QSAR results, vapour pressure, particle size, metabolism via oxidation etc.

ECHA Guidance advises the registrants to provide toxicokinetic data as "*Supportive evidence*" within the read-across justification. For example, bioelution data has been provided for metal read-across cases.

Another example would be a case where the registrant justified that "*aldehydes are rapidly oxidised to the corresponding carboxylic acids*". ECHA accepted this read-across, provided that "*the metabolism is rapid enough to prevent significant systemic exposure to the aldehyde* (target substance) *and that metabolism to carboxylic acid* (source substance) *is the predominant metabolic pathway*."

Other uses of ADME information are e.g. dose selection for *in vivo* studies, and compensation of missing metabolic competence of *in vitro* methods.

The regulatory relevance of *in vitro* toxicokinetic data depends e.g. on:

- foreseen uses under specific legislation(s)
- validation status and biological relevance
- metabolic competence of the *in vitro* assays
- substance type - under REACH, substance composition is often unknown and variable, and *in vitro* tests can rarely be applied.

ECHA will follow and support projects that aim to develop *in vitro* methods for ADME.

European Medicines Agency (EMA): Use of toxicokinetic data in the context of the development of medicinal products

The need and requirements for toxicokinetic data for development of human medicinal products is described in current guidance documents from ICH (The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use). 3Rs initiatives related to toxicokinetic data, include microsampling, as described below.

The over-arching guidance ICH M3 (R2) describes the need for exposure measurements (AUC), as well as information from *in vitro* metabolic and plasma protein binding data from animals and humans, for calculating safe starting doses in initial clinical trials and also the calculation of safety margins to clinical doses (comparing nonclinical and clinical exposure) as part of risk and safety assessment in humans. Overall, *in vitro* metabolism and protein binding in human and animals, drug interactions and metabolites (resulting in exposures greater than 10%) of parent compound are generally characterized as well as area under curve (AUC), maximum exposure (Cmax), time of maximum exposure (Tmax) and half-life (t½) for the parent compound.

ICH S3A offers more specific guidance for assessing systemic exposure in toxicology studies. The primary objective of toxicokinetics is to describe the systemic exposure in animals and its relationship to dose level and the time-course of the toxicity study. Toxicokinetics is an integral part of non-clinical testing program, to understand the toxicity testing results (e.g. delayed toxicity could be related to peak in toxic metabolite).

Toxicokinetic measurements should be included in single and repeat-dose studies, reproductive studies, genotoxicity and carcinogenicity studies, as well as in studies supporting a change in route-of-administration.

The ICH S3A Question and Answers document introduce the concept of microsampling, which allows for kinetic sampling to be performed in rodent studies in the main study animals. Additional animals are not required due to the smaller blood volumes needed for the sampling and analysis.

Finally, the EU guideline on strategies to identify and mitigate risks for first-in-human and early clinical trials with investigational medicinal products (EMEA/CHMP/SWP/28367/07 Rev. 1) includes also a section on toxicokinetics and states “These data should adequately support the interpretation of data from *in vivo* pharmacodynamic (PD) models and safety/toxicological studies before starting first-in-human (FIH)/early phase clinical trials (CTs)”.

EURL ECVAM's current activities in the area of toxicokinetics

Human hepatic metabolic clearance

The presentation first introduced the EURL ECVAM Strategy for achieving 3Rs impact in the assessment of toxicokinetics and systemic toxicity (Bessems et al., 2015) which describes opportunities to generate and integrate toxicokinetic (TK) data in chemical risk assessment while ultimately avoiding the need for animal studies. The TK strategy comprises four aims and the first one target the development of *in vitro* methods which can generate information on Absorption, Distribution, Metabolism and Excretion processes.

EURL ECVAM decided to advance *in vitro* methods that can provide information on hepatic metabolic clearance since metabolism is frequently the main driving ADME process within TK and also because there are already several non-guideline *in vitro* methods which can significantly vary for the experimental-settings, stage of development, intended use, reliability, relevance, etc.

Therefore, EURL ECVAM has decided to develop an OECD Guidance Document (GD) to characterise and describe this specific class of *in vitro* methods for measuring hepatic metabolic clearance as a first practical step to promote the use of TK information.

With a view to enhancing the use of *in vitro* methods for hepatic metabolic clearance in chemical hazard and risk assessment, the objective of this GD is to establish a consistent and transparent framework focused on identifying the relevant elements to be considered when characterising and describing *in vitro* hepatic metabolic clearance methods in order to facilitate the assessment of their performance, method comparison and increase confidence in their use to support chemical risk assessment.

CYP Induction

CYP enzymes play a key role in human metabolism, including biosynthesis of steroid hormones, prostaglandins and bile acids and are the first-line defence in the oxidative biotransformation and detoxification of xenobiotics. CYPs can either biotransform a xenobiotic into a harmless and

excretable metabolite or transform a non-toxic parent compound into a toxic metabolite. Xenobiotics can induce or inhibit these enzymes, affecting the metabolism of endogenous substrates, of the xenobiotic itself or other xenobiotics (e.g. mixtures) to which we are co-exposed. The overall result is a deregulation of the normal metabolism and homeostasis with potential toxicological effects. The Molecular Initiating Event (MIE) of CYP induction is the binding of endogenous and exogenous ligands to the nuclear receptors/transcription factors Aryl Hydrocarbon Receptor (AhR), Constitutive Androstane Receptor (CAR) and Pregnane X Receptor (PXR). PXR primarily induces the transcription of CYP3A family, CAR of CYP2B family and AhR of CYP1A; however, cross-talk between CAR and PXR pathways is possible.

CYP induction requires intact hepatic cells retaining transporter and drug/chemical metabolic functions over the experimental period and relatively stable in the expression of relevant functions; therefore it is an ideal marker for assessing the functional machinery of longer-term metabolic competent test systems. Two CYP induction *in vitro* methods using cryopreserved primary human hepatocytes and cryopreserved HepaRG cells as hepatic metabolic competent test systems underwent an EURL ECVAM-led validation study involving five laboratories i.e. two different technically proficient laboratories for each *in vitro* method plus EURL ECVAM as the only naïve laboratory for both *in vitro* methods. Test compounds from the pharmaceutical sector with well documented *in vivo* induction properties have been tested. The study showed that HepaRG cells correctly classified the CYP1A2, CYP2B6 and CYP3A4 *in vivo* induction properties of all 10 tested compounds. PHHs classified the induction properties of 12 compounds correctly for CYP2B6 and 3A4 but miss-classified three as to their *in vivo* classification of CYP1A2 induction potential.

In parallel, a cocktail approach for *in vitro* CYP induction and an analytical method for the quantification of the CYP specific metabolites of interest in the same run have been successfully validated.

The validation study indicates that both CYP induction *in vitro* methods are mostly similar in their ability to detect and classify correctly xenobiotics in terms of CYP1A2, CYP2B6, CYP3A4 induction. As such, the CYP induction *in vitro* methods respond to the demand of standardised *in vitro* methods for informing ADME processes and facilitating the generation and use of non-animal data to assess human risk. It opens the possibility for further *in vitro* method development for other processes (e.g. metabolic transformation by other biotransformation enzymes, metabolite identification, and clearance) and integration into batteries of methods for systemic toxicity. EURL ECVAM continues to engage with the relevant expert groups at OECD level to place the methods in an appropriate regulatory context for their use in chemical safety assessment.

Physiologically Based Kinetic models

Physiologically Based Kinetic (PBK) modelling is a scientifically-sound approach to predict internal dose metrics for chemical risk assessment applications. A PBK model involves a set of differential equations to describe the critical physiological, physicochemical, and biochemical processes that determines the fate of a chemical in an organism by means of Absorption, Distribution, Metabolism and Excretion (ADME). A PBK model is built using chemical-independent (anatomical and physiological) parameters as well as chemical-dependent parameters (physicochemical and ADME properties). Traditionally, the calibration of model parameters of a PBK model and the validation of its predictive capacity rely heavily on comparing model simulations with relevant *in vivo* data. However, such data (in particular *in vivo* TK data) are available only for a limited amount of chemicals and species of interest, which is a limiting factor in the validation and uptake of these types of models. There is therefore a need for validation efforts to shift the emphasis from the ability to directly predict the *in vivo* toxicokinetics for the chemical of interest to other lines of evidence supporting the credibility of PBK models. Although aspects of existing guidance documents are applicable to PBK modelling in general, they are all based on the premise that model

performance must ultimately be assessed by direct comparison between predicted kinetic profiles with equivalent in vivo data. In a recent international PBK modelling workshop held at the Joint Research Centre (JRC) in November 2016, experts unanimously agreed that there is a critical need for a GD for characterising and reporting a new generation of PBK models that increasingly integrate non-animal ADME data but for which animal TK data may not be available for calibration purposes[2]. This critical need was further affirmed by respondents to an international survey, launched by EURL ECVAM, on scientific and regulatory applications of PBK models[3]. The survey results showed that, within the PBK modelling community, there is a need to harmonise the characterisation, validation, and reporting of PBK models that are based on data derived from non-animal methods. Thus, the aim of the proposed guidance document is to address this need so that the credibility to promote the acceptance and use of PBK models in a regulatory context.

Aquatic bioaccumulation

In vitro methods to assess fish metabolism have the potential to provide information on bioaccumulation and may be used in testing strategies, for screening purposes, in a weight-of-evidence approach or read across. However, these methods lack standardisation. In the light of this, the OECD project "Fish *in vitro* metabolism" was launched in 2014, which is co-led by US and EC.

During 2014-2016, a ring trial was carried out to assess the reproducibility of two *in vitro* methods using either cryopreserved rainbow trout hepatocytes or rainbow trout subcellular S9 fraction to determine in vitro intrinsic clearance rates. Based on the protocols used in the ring trial, two draft OECD test guidelines (TG) have been developed. They are accompanied by a draft GD, which describes critical steps and limitations. It further includes an example model for *in vitro-to-in vivo* extrapolation to estimate whole-body clearance rate and its possible use in *in silico* fish bioconcentration models. By including information on possible biotransformation their reliability may be increased.

The two draft TGs, the draft GD and the ring trial report underwent two OECD public commenting rounds in 2017.

Q&A Session on ADME

Following the presentations on EU agency perspectives and current EURL ECVAM activities in the area of toxicokinetics, a Q&A session was chaired by EURL ECVAM. The aim of this session was to discuss any points arising from the presentations and to start discuss specific questions that had been circulated in advance to PARERE members.

The regulatory value of all of these projects was acknowledged, as were the commonalities in their applications (e.g. providing ways of performing interspecies extrapolation and assessing human relevance of animal data).

In relation to the proposed OECD Guidance for characterisation and description of *in vitro* human hepatic metabolic clearance methods, NL informed the participants of a new and complementary SPSF submitted to the OECD WNT by the Netherlands entitled “A feasibility study for establishing TGs for *in vitro* human hepatic metabolic clearance and metabolite formation”. Whereas the JRC-led guidance document will develop a framework for characterising a family of TK (hepatic clearance) methods, the NL-led project would explore the application of this framework in developing specific

[2] Paini et al., 2017 - https://ec.europa.eu/jrc/en/publication/eurl-ecvam-workshop-new-generation-physiologically-based-kinetic-models-risk-assessment
[3] Paini et al., 2017 - https://www.ncbi.nlm.nih.gov/pubmed/28866268

protocols and guidelines. If adopted, this project would also entail the establishment of a standing expert group under the Working Group of National Coordinators of the OECD Test Guidelines programme (WNT) addressing multiple aspects of toxicokinetics.

It was acknowledged that the OECD work on determination of intrinsic clearance using rainbow trout hepatocytes and S9 subcellular fractions, and its extrapolation to bioaccumulation, is at an advanced stage. It was also noted that the "package" of test guidelines and explicit guidance on how to interpret the *in vitro* data provides a useful approach at WNT. This could be extended to the mammalian TK area, where there are many methods, often providing similar information, but which is challenging to interpret since it is only indirectly related to toxicity (hence the need for PBK models and other approaches for extrapolating TK data).

In relation to the CYP induction methods, the possibility to apply the methods to chemicals other than pharmaceuticals, and possibly with a broader "cocktail" of enzymes, was discussed as a means of contributing to the screening of chemicals.

Since there was insufficient time to address all questions, it was agreed that these would also be the subject of a written consultation.

Summary outcome of the PARERE consultation on some of the EU-ToxRisk case studies

In the following text, the EU-ToxRisk core team has briefly summarised the case studies that were presented at the PARERE meeting at the JRC on 28 November 2017. In addition, a summary of the consultation process and the discussions is provided. The presentations (without unpublished data) are available to PARERE members on CIRCABC.

1. EU-ToxRisk - PARERE consultation process

The EU-ToxRisk team circulated several documents ahead of the PARERE meeting; a summary of the EU-ToxRisk case study strategy, summary overviews of four representative case studies and a set of questions on the regulatory relevance of the EU-ToxRisk case studies. In total EU-ToxRisk received answers from nine PARERE members. Some partners chose only to answer certain questions due to their specific expertise and each question received answers from five PARERE members on average. In general the answers were diverse in that the interpretation of the questions differed between PARERE members and then of course the answers also differed. On the whole there was a general consensus that supported the EU-ToxRisk approaches and some valuable feedback was received from the PARERE members. The presentation of the answers to the questionnaires triggered very interesting and fruitful discussions at the PARERE meeting in Ispra on the 28th of November 2017. This discussion is further summarised below. In addition, a brief summary of the case studies that were presented is shown below.

1.1 Case study 1: Prediction of microvesicular liver steatosis - a read-across case study with (un-)branched carboxylic acids

Short branched carboxylic acids, like valproic acid (VPA), induce microvesicular liver steatosis in animal studies. It is known, that microvesicular liver steatosis can be induced via several different AOPs. In total 19 structurally very similar (un)branched carboxylic acid have been selected that are either *in vivo* positive or *in vivo* negative. We selected different *in vitro* models to learn how to predict the adverse outcome, microvesicular liver steatosis, in a quantitative and qualitative way. Reverse dosimetry based on a PBPK model was used to determine a relevant concentration range for the *in vitro* studies. Also, an "In Vitro Distribution (IVD) model" will be used to predict the intracellular concentrations in the different *in vitro* systems. This information is needed to perform quantitative *in vitro* to *in vivo* extrapolation (QIVIVE).

The testing of 30 reporter genes with the CALUX assays discriminated well between *in vivo* positive and negative compounds based on the amount of activated reporter genes. In addition, lipid accumulation following a single exposure in HepG2 cells was predominately seen for *in vivo* positive compounds. Next, repeated dose testing will be carried out in primary human hepatocytes and 3D-HepaRG cells. The combination of different *in vitro* models and complementary as well as conflicting data will be supported by statistical tools, e.g. based on Dempster Shafer theory. Using these new tools, we will integrate data into an analogue and category approach to elaborate their usefulness in regulatory risk assessment.

1.2 Case study 2: Valproic acid analogues and DART liabilities

Developmental and Reproductive Toxicology (DART) is a complex toxicological endpoint for which new approach methodologies (NAM) need to be developed: i.e. *in vitro* models, and toxicokinetic models that together replace animal tests. The current *in vitro* DART battery is composed of: Zebrafish Embryo Test (ZET), mouse Embryonic Stem cell test (mEST; cardiac differentiation), human pluripotent stem cells (iPSC; neuronal differentiation), ZET reporter assay (bone malformation), and the CALUX assays (among others various receptor assays). For toxicokinetic analysis static and dynamic *in vitro* distribution models, and PBPK models including a placental compartment were developed for human, rat and mouse. In this case study we will develop, test and improve these NAM to identify and characterise one specific DART effect: Neural Tube Defects (NTD). Valproic acid (VPA), a human anticonvulsant drug, is known to induce NTD in humans and animals. A number of structural VPA analogues (either *in vivo* positive or negative) were chosen for this case study. The exact mechanism underlying this teratogenicity is unclear and likely multifaceted. The concrete objectives of this case study are to determine if it is possible to: 1) correctly predict the teratogenic properties and potency of VPA analogues by using NAM in a read across approach, 2) demonstrate with these NAM that a no or lowest observed effect concentration identified in this test battery represents a suitable point of departure. Testing of all VPA analogues in all models has resulted in *nominal* IC10, and EC10 concentration values for each VPA analogue; from this data a preliminary conclusion is that the DART battery reasonably well predicts the VPA analogues as being 'positive' or 'negative' for NTD properties.

1.3 Case study 4: Prediction of Parkinsonian like liabilities based on AOP aligned testing linked to mitochondrial toxicity

The overall aim for this CS is to determine whether evaluation of key events (KEs) of an AOP in a quantitative model could contribute to the assessment of the safety of pesticides with respiratory chain inhibitor function. An AOP named "Inhibition of the mitochondrial complex I of nigra striatal neurons leads to parkinsonian motor deficits" has been developed (AOP-wiki #3). This AOP assumes that the Parkinsonian motor deficits are directly related to mitochondrial respiratory chain (MRC) complex 1 inhibition. Various insecticides, pesticides and fungicides are targeting MRC complexes with unknown neuronal effects. A range of pesticides, insecticides and fungicides were selected that target the mitochondrial respiratory chain complex I, II or III. The toxicity of the different target chemicals will be predicted based on NAM data generated. The NAMs are aligned to the KEs of the AOP for a number of different test systems (liver cells, kidney cells and neuronal cells). Briefly, effects on mitochondrial respiration will be measured through the seahorse assay and by measuring mitochondrial membrane potential. Also, effects on proteostasis and neurite outgrowth as well as cell survival are being assessed. Our results will define if the AOP is fit-for-purpose to predict Parkinson-like defects caused by complex 1 inhibition. Based on the different model systems we will be able to define the test systems that can be integrated in a testing strategy. This will be of relevance since current standard animal testing strategies do not allow identification of Parkinson-like adverse events and can therefore not assess this important adverse outcome.

1.4 Case study 8: Repeated-dose toxicity: Popcorn Lung – read-across on diketones

Alpha-diketones such as diacetyl (2,3- butadiene) are known to induce an obstructive pulmonary disease also known as "popcorn lung". Observed effects include inflammation and/or fibrosis of the

bronchioles. Alpha-diketones are known to have a high electron affinity and are able to transfer electrons which lead to ROS production and oxidative stress. Case study compounds include four α-diketones but also one beta and one gamma diketone, which have a different mode of action (MoA) to α-diketones. Furthermore, Acetone and Butanone were included to serve as *in vivo* negative contols. Test systems including neuronal, primary bronchiolar epithelial cells and precision cut lung slices will be exposed via Air-liquid exposures. Also, High-Throughput-Screening methods in liver HepG2 cells will be used to study cellular stress pathway activation. The aim of this case study is to learn, how far the selected NAMs are able to predict the toxicity of α-diketones and differentiate β and γ-diketones, which share a high structural similarity but differ with regard to their specific MoAs. We will further explore the applicability of EU-ToxRisk models and tools to predict toxikokinetics with the aim to predict a point of departure. Finally, we aim to explore to what extent chemical similarity can be enriched by biological data, e.g. derived from omic investigations or cellular read outs. The majority of *in vitro* tests will start in January 2018.

2. Summary of the discussion round

It was appreciated by the members of the PARERE network, that EU-ToxRisk did take up the comments from the first meeting in 2016 and that the case study descriptions improved significantly. Furthermore, it was recognised that EU-ToxRisk had made huge progress over the last year. For this summary, the outcome of the discussion was structured in five main topics: the definition of the regulatory question addressed, the application of AOPs in read across, the read across case study approach, the ab initio case study approach as well as the understanding and consideration of uncertainty. The discussion on these main topics is summarised further below.

It was discussed that the case studies (CS) should have a focus to be pragmatic and precise examples for the development of read across approaches based on NAMs and to have direct integration into risk assessment. “A lot of read across cases today are solely based on chemical similarity in which physico-chemical properties are taken as surrogate for kinetic data. Additional toxicodynamics and ADME data could improve these cases”, some of the members mentioned. An important aspect would be to know and explain the uncertainty and limitations connected to the above-mentioned approaches. Nevertheless, it was agreed by everyone, that we also need a good scientific basis for these approaches. Therefore, it is obvious, that some scientific questions per case study will not directly contribute to the IATA but to a better understanding of the robustness and relevance of the NAMs.

During the discussion, it was generally agreed that NAM-based defined approaches for testing/IATAs could well be used in regulatory toxicology. However, there was no consensus as to what extent this would be possible at the short-term. It was also stressed, that currently it is not foreseeable in how far NAMs could be used in the mid-term future to assess the hazards for compounds besides read across (so called ab initio testing). EU-ToxRisk is perceived as having the great opportunity to generate answers to these questions by showing the potential as well as limitations based on the current case-study work.

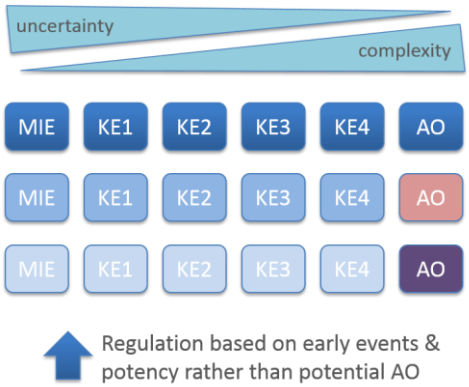
The members of the PARERE network encouraged EU-ToxRisk to proceed with its work and to update the PARERE network, soon. All participants in the discussion highlighted the value of such interaction and are now looking forward to the next discussion in 2018.

1) Definition of regulatory question

- EU-ToxRisk should be quite pragmatic! The CS should be targeted to much more specific regulatory purposes: Which hazard endpoint is a CS focusing on and which regulatory target/ which regulations is a CS heading for.
- CS should not be too complicated and too theoretical.
- One (still) very important topic and an aspect often forgotten in the current discussions/ research projects is to prioritise chemicals for further testing (and therewith potentially a "low-hanging-fruit" for EU-ToxRisk). It will remain on the question: How can we identify those compounds, for which animal testing is really needed? How can we identify the potency of the compounds for which animal testing is really needed?

2) Application of AOPs

- It was not clear what biological/ mechanistic similarity would be. The EU ToxRisk team shared a picture on MIEs leading to AOs and the associated uncertainty and complexity. This was further discussed using the example of the steatosis AOP. The discussion on uncertainty is summarized under 3).



- One general question was how to select good KE to build a read across on. It was mentioned that the basis is a good data base with all the KE/key event relationship (KER). Based on this data base a good justification why to select a certain KE should be documented. Then, other close KE, which belong to other AOPs should be excluded. The tests on the resulting KE should be designed to be cheap, understandable, easy to measure, etc.
- It was felt that EU-ToxRisk is on the right way concerning the application of AOP-based read across, even though it is clear that the current case studies do have limitations. Some members of the PARERE-network mentioned that "we just have to start and gain experience and learn from it with groups of chemicals and defined questions." One clear limitation of EU-ToxRisk is that only a part of what has to be done in terms of regulatory testing can be covered within the project. In this context, it was suggested to be as close as possible to the adverse outcome to get highest certainty that a compound really does exert that particular AO. Other participants suggested to look more into the early events because these were assumed to be better understood mechanistically. Furthermore, they might be easier to be tested *in vitro* (an example would be preneoplastic lesions that cannot be measured *in vitro*).
  Finally, to be more pragmatic and applied, rather than only conceptual, the project partners were encouraged to start with limited approaches (not covering all endpoints/ toxicities/

AOPs), since this could be of significant help already. Within EU-ToxRisk it would have to be accepted that not everything could be covered and that the approaches developed might not be 100% accurate. Important, though, is to obtain knowledge about coverage and limitations.

- Furthermore, it was discussed that there are inherent limitations for a DA/IATA concept based on certain AOPs. As an example, within the ongoing work at the OECD developing an IATA for non-genotoxic carcinogenicity, it was highlighted that for such examples of complex networks of relevant AOPs, it is problematic to ascertain how the downstream key events, closer to the AO can be examined with test chemicals, when these KEs and KERs are far removed from the MIE interactions with the test chemical, and it is the signaling consequent to the MIE that one can examine in the upstream KEs which generally have mechanistic *in vitro* test methods. How can this be adequately addressed with our NAM-based approaches? How can we make the connection between the initial chemical interaction with subsequent effects that happen more indirectly? An assumption raised in this context was that testing a more downstream KE would automatically mean that the applied methods can cover the upstream KE/KER, too. These methods needed to be more complex (covering the complexity of the AOP at that stage). There is a need to find a way to reasonably explain the process to the AO even though the chemical might only play a role in early KEs. This is currently being explored by the OECD expert group developing an IATA for non-genotoxic carcinogenicity, where there is also an important regulatory need. This issue should perhaps also be considered in EU-ToxRisk case studies and models.
- It was raised that the regulatory community could also discuss that when using NAMs we may cover certain pathways of toxicities with agreed testing approaches (e.g., DA, IATAs), which are not fully congruent with current animal testing based endpoints and classifications (e.g. they do not include all potential pathways leading to liver organ toxicity, but also have relevance for carcinogenicity and developmental toxicity). However for those cases, the regulatory community might nevertheless use the outcome in a regulatory context, since classifications and limit values are also currently usually based on positive evidence for adversity. In this way regulatory toxicology may evolve towards becoming less and less dependent on animal test data.

3) Uncertainty

- Referring to the above-mentioned figure on MIEs leading to different AOs, uncertainty and complexity were not clear to everybody and needed definition: the EU-ToxRisk team highlighted the example of steatosis. PPARs activation is described as a MIEs in the AOP, but is also involved in many other processes and might not ultimately lead to steatosis. The closer one comes to the AO with the DA used, the lower the uncertainty becomes that the AO really occurs. However in relation to the figure above, it was stressed that complexity may be considered as just another type of uncertainty. Testing at higher levels of complexity means that more elements are combined (e.g. organ systems in the whole animal) and many factors may influence the outcome (e.g. (epi-)genetic background, pre-existing disease-stage, (co-)exposure, diet, environment, stress), but the combination of these potentially variable elements and factors is fixed in the experiment. One such complex system is not necessarily representative of another complex system and without deep mechanistic understanding, extrapolation of results from one complex laboratory model to real world effects is quite uncertain. However, at the moment, the mechanistic understanding has matured. Assessment based on testing at lower levels of complexity may be even less

uncertain than assessment based on testing at higher levels of complexity (Paparella *et al*., 2017[4]).

- In line with these thoughts, it was also mentioned that the highest certainty for testing and assessment may be obtained when answering to the question if/how strong a MIE or early KE is likely to be affected.
- It was commented that the OECD non-genotoxic carcinogenicity expert group is working on the question above, but that also EU-ToxRisk may like to consider the different types of uncertainty and how they influence the process (AOP) looked at (e.g. Inflammation → under which circumstances does this lead to cancer?) and what this means for the validation of NAM-based approaches. Complexity should be considered for IATA development, but not necessarily included into the test systems. EU-ToxRisk should also evaluate, in how far one could generalise from the results/case studies.
- Some regulators mentioned that using the NAMs for screening and then following up *in vivo* might not work for all compounds/regulations.
- The EU-ToxRisk representatives asked whether omics data could help us to alert for something that is not expected to be covered by the DA/IATAs developed in EU-ToxRisk in order to reduce uncertainty? It was highlighted that for read across, first it would have to be shown that the CS does what it should in the NAMs applied. Some members of the PARERE-network expected that transcriptomics data will more likely add confusion/complexity rather than yielding relevant information ("Data is generated since decades, but not used in regulatory decision-making. Lovely heatmaps do not help, however, for *ab initio* cases, omics might help to trigger the next steps."). Other members were more positive in terms of using omics data for read across. In order to enable their use, it has to be clearly defined what the context in the problem formulation is and how the data would help to answer the defined regulatory question. The importance of the data base that one compares against was highlighted. Some first examples for the added value have been published in the last years.

4) Read across case study approach

- A general remark on the EU-ToxRisk CS was to include the OECD toolbox for similarity estimates.
- It was questioned why and how animal testing could be replaced. Physiological reactions in humans and animals are so close that one can (and should) use that data for hazard assessment.
- It was highlighted that REACH accepts good and substantiated category approaches. One goal of EU-ToxRisk should be to identify how *in vitro* data can support/ substantiate such category approaches. In this context, it was appreciated that read across justifications as developed within EU-ToxRisk might serve are much better for the formation of a category approach than what has been submitted in reality. The approach to use AOPs could be the way to generalise for the future.
- Furthermore, high accuracy is not needed in supporting read across because it just needs to be confirmed that the source and target compounds have similar effects.
- It was discussed that the prerequisites for read across are
  1. Chemical/ physical similarity

[4] Paparella *et al*., 2017. Uncertainties of testing methods: What do we (want to) know about carcinogenicity? ALTEX 34 (2). https://doi.org/10.14573/altex.1608281

2. Toxicokinetic similarity
3. Toxicodynamic similarity

It was agreed that the work of EU-ToxRisk could specifically benefit the toxicodynamic, but also the toxicokinetc similarity. Currently, read across in many cases is lacking any kind of data. Consequently, every (reasonable) additional data point could support a chemically-based read across. The scenarios generated within EU-ToxRisk are valuable even if it is clear and common understanding that the NAMs cannot tell us everything. Nevertheless, "hundreds/ thousands of animals could be saved."

The EU-ToxRisk representatives appreciated this feedback on the added value generated with the project.

- Suggestions for the next set of case studies:
    - Evaluate case studies within the current regulatory framework (e.g., in other projects) and build on them.
    - Also look into the future: How can we foster a change in regulatory toxicology? Key to this is a really clear problem definition.
    - Stay pragmatic on the short-term: Based on the *in vivo* data make an informed decision on which *in vitro* assays to select. Than base the read across on the in vitro results.

5) *Ab initio* case study approach

- It was felt that it is premature to really put an unknown substance into our systems and predict the effects (this would be the ab initio scenario). It needs some level of pre-existing information to be acceptable by ECHA.
- In other areas, however, the use of NAMs could support hazard assessment already (e.g., food constituents).
- Furthermore, the potential value of omics data was discussed in this context (see summary on uncertainty above)

Annex I – Agenda

Meeting of the Preliminary Assessment of Regulatory Relevance (PARERE) network

1st day: 27th November 2017

Building 58 Auditorium

13:00-14:00 **Welcome and Introductions from EURL ECVAM**

- Approval of draft agenda
- Updates on the state of the network in Member States
(PARERE representatives)
- Updates on recent consultations with PARERE
Bioelution *(Pilar Prieto, EURL ECVAM)*
EDITOX *(Francesca Pistollato, EURL ECVAM)*
ToxTracker *(Federica Madia, EURL ECVAM)*

14:00-16:00 **Toxicokinetic data in regulatory frameworks**

Perspectives of European Agencies

EFSA – EFSA guidance document on integrating toxicokinetics data *(Jean-Lou Dorne, EFSA)*

ECHA – Use of toxicokinetic data in the context of REACH *(Kimmo Louekari, ECHA)*

EMA - Use of toxicokinetic data in the context of medicinal products *(Ellen Vestergaard, EMA)*

EURL ECVAM's current activities

- OECD Guidance for characterisation and description of *in vitro* human hepatic metabolic clearance methods *(Alfonso Lostia, EURL ECVAM)*

- OECD Test Guideline on CYP Induction *(Camilla Bernasconi, EURL ECVAM)*

- OECD Guidance document on the characterisation, validation and reporting of physiologically based models for regulatory applications *(Alicia Paini, EURL ECVAM)*

JRC Ispra, 27-28th November 2017

- OECD Guidance on the "Determination of *in vitro* intrinsic clearance using cryopreserved hepatocytes (RT-HEP) or liver S9 sub-cellular fractions (RT-S9) from rainbow trout and extrapolation to *in vivo* intrinsic clearance" (and two related test guidelines) for fish bioaccumulation testing *(Marlies Halder, EURL ECVAM)*

16:00-16:30 Coffee break

16:30-18:00 **Q&A session on ADME**
(moderated by *Andrew Worth, EURL ECVAM)*

18:30 Departure

2nd day: 28th November 2017

Building 58 Auditorium

09:00-10:00 **Presentation of case studies in EUToxRisk**

10.00-11.00 **Outcome of PARERE consultation on case studies in EUToxRisk**

11:00-11:30 Coffee break

11:30-13:00 **Further discussion on case studies**

13:00-14:00 Buffet lunch

14:00 **Start of joint PARERE-ESTAF Meeting**