Chronos and Kairos: Understanding time in biology – Time for next generation risk assessment

Workshop Report No. 40

EUROPEAN CENTRE FOR ECOTOXICOLOGY AND TOXICOLOGY OF CHEMICALS

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European Centre for Ecotoxicology and Toxicology of Chemicals
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DISCLAIMER:

Representatives of the European Commission expressed no official view or position on the scientific matters discussed during the breakout sessions and their participation cannot be taken as agreement/disagreement to the views and/or positions expressed during the breakout sessions.

This workshop report is intended to accurately reflect the workshop discussions and conclusions, but it is noted that not all workshop participants provided written input to the workshop write-up.

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1. INTRODUCTION

This workshop aimed to explore the need and approaches to study the influence of time and level of biological organisation (population, organism, tissues, cells etc.) in toxicity testing in next generation risk assessment (NGRA) based on new approach methodologies (NAMs). Notably, the aim was to discuss how to integrate the influence of exposure time window, exposure duration, exposure frequency, and damage accrual rate in developing and interpreting *in vitro* assays, quantitative adverse outcome pathways (qAOP) and quantitative *in vitro* to *in vivo* extrapolation (QIVIVE).

The workshop brought together toxicologists, biologists, bioinformaticians, modelers and risk assessors from different sectors for a two-day workshop (7-8 November 2023; Brussels, with some online participation) to discuss the concept of time in human toxicology. This interdisciplinary setting provided a forum for experimentalists to meet with modellers and map out how the future of chemical safety assessment can utilise knowledge of the effect of time on toxicity, as well as to receive feedback from risk assessors. In so doing, the aim was to develop a strategy for including time variables in NGRA.

This report summarises the outcome of the workshop discussions.
The workshop programme, organising committee, participants, contributors to this report and a list of speakers' abstracts and reflections can be found in Appendices A-F.

2. BACKGROUND TO TOPIC

The factor time is an intrinsic element in toxicology. Firstly, this holds true for the way in which biological systems are exposed to potentially toxic substances: it takes time for compounds to reach the site of toxic action (where the molecular initiating event (MIE) takes place), and it takes time for processes to remove compounds via distribution, excretion or metabolism. These kinetic processes therefore determine the changes over time of the concentration of a toxically active compound at the sites of the MIE(s).

Moreover, the way in which exposure takes place, e.g. one single dose verses prolonged exposure over a longer period (i.e. repeated dosing), will also have an influence on the dynamics. Prolonged or repeated exposure may or may not lead to an accumulation of the compounds in the biological system, thus influencing the course of the exposure at the MIE sites. This is further complicated by the possibility that compounds may influence the elimination processes, e.g. by inducing enzyme or transporter activities. Time is additionally a critical factor in the transition of events though the adverse outcome pathway from MIE to first key biological event (KBE) and thereafter to the adverse outcome. Thus, the dynamics of the toxic reactions will also be influenced by changes in time. Here too, differences may occur with the length of the exposure or after repeated dosing, for example the biological system might be able to counteract the effects with cellular or organismal defence mechanisms. Over time, these mechanisms can be exhausted for example depletion of a key metabolite, resulting in an increased toxicity after prolonged exposure. Similarly a biological system may be able to upregulate its defence, resulting in a decreased toxic sensitivity.

Finally, sensitivity towards toxicity of a compound may be related to certain time frames. This especially holds true for the period of the developing organism. Another example could be the variability of processes during the circadian rhythm.

Hazard results from rate of damage being higher than the rate of recovery. The rate of damage results from the specific mechanism of toxicity of a chemical, its potency, and its concentration in time. The rate of recovery depends on the elimination/excretion of the chemicals and recovery mechanism of the cells. Depending on which is the rate-limiting step, either an internal peak concentration (Cmax) or area under the curve (AUC) can be a better approximation of the biologically effective dose. Examples of this are direct acting mutagens, where there is little recovery and so AUC is a better dosimetric for biologically effective dose (BED), and mitochondrial inhibitors, which tend to have quick recovery and there an internal concentration can be a better approximation (Escher et al., 2011).

The above factors will also play a role in the interpretation of studies performed in *in vitro* systems. An essential element in these studies is a precise insight into the concentrations at different sites of the *in vitro* system (medium, culture plastic, cell surface, intracellular sites), and how these concentrations will be related to an MIE. The time course (length, Cmax, AUC) should ideally reflect the relevant processes in an intact organism, to support relevant *in vitro* to *in vivo* extrapolations (IVIVE). If this cannot be achieved, a more complicated interpretation can possibly be made by modelling approaches such as physiologically based toxicokinetic (PBTK) systems. In all cases, kinetic modelling is an essential element in a QIVIVE process.

Likewise, the adaptive reactivity of the *in vitro* system also plays a role in interpreting the outcomes of an *in vitro* study.

In addition to the above-mentioned kinetics and associated differences that need to be considered when comparing *in vivo* and *in vitro* study, the general transition from *in vivo* to *in vitro* testing will also need to consider how the adverse effect under consideration could progress over time. For example, various processes may lead to increased cell proliferation in chemically exposed laboratory animals. These events may also trigger other pathophysiological events that may take time to develop such as Inflammation. Thus the long-term consequences thereof may result in cancer in some organs, while others are more resistant. Such potential changes in the nature of the adverse effect over time will somehow need to be part of the evaluation of the results of *in vitro* studies.

In view of the advancing field of *in vitro* studies and incorporation of these into NGRAs, it was considered opportune to gather experts in the field to discuss and develop strategies for how to incorporate the influence of time in *in vitro* models, that typically do not allow the same time windows for exposure that are permitted in *in vivo* studies, and how these are developed and interpreted.

3. WORKSHOP PRESENTATIONS

Day 1 of the workshop comprised a series of presentations on topics related to consideration of time in human toxicology, to set the scene and provide inspiration for the Day 2 breakout group discussions.

Nynke Kramer (Wageningen University and Research) shared perspectives on role of time in *in vitro* and *in vivo* toxicity tests, highlighting the importance of understanding exposures in *in vitro* and *in vivo* tests and how PBTK, qAOP and QIVIVE approaches can be utilised to appropriately relate molecular effects *in vitro* to toxic effect *in vivo*.

Huan Yang (EAQLabs) presented the merits of biological systems modelling of qAOPs, integrating exposure, temporal, recovery and PBTK considerations into AOPs, to better understand toxicological data and mechanisms both *in vitro* and *in vivo* testing.

Peter Macko (JRC) highlighted the challenge that current *in vitro* assays may result in potential cumulative chronic effects over time being disregarded, and how incorporation of the time dimension into experimental design, by modelling concentration-time responses using ordinary differential equations (ODEs), can address this.

Ben van Ravenzwaay (Wageningen University and Research) presented an *in vitro* metabolomics case study investigating dose and time dependent responses of intracellular metabolites to nitrofurantoin, and how analysis of the dynamics of such responses may help clarify if a time related change in the quality of the toxicity response (i.e. which organs are affected and to which extent) may occur for a particular compound.

Gladys Ouédraogo (L'Oreal Research & Innovation) continued the possibilities for predicting chronic effects, covering cell transformation assays, carcinogenesis and repeated dose systemic toxicity. Challenges, such as the large number of MoA with may still to be define, human relevance etc. were highlighted.

Cecilia Tan (US EPA) highlighted that current chemical risk assessments use a pragmatic approach to derive reasonable estimates of safe doses and exposure, and uncertainty factors are incorporated to account for variability and uncertainty of various factors, including time. PBTK models were highlighted as a powerful tool to bridge the gap between the dose of interest from *in vitro* assays, the dose within the target tissue, and the exposure dose. The question was raised whether to convert *in vitro* POD into external concentrations, or convert exposure estimates to internal concentrations via PBTK. It was noted that *in vitro* studies integrate some conservatism, e.g. they do not account for the self-repair ability of cells.

Harvey Clewell (Ramboll) brought in a transcriptomic angle to the discussions, noting that gene expression is the most fundamental change at the cell level that can result in a change in biological function or a change in the development of a cell. The opportunity to predict chronic toxicity from transcriptomic dose-responses in short-term *in vivo* studies was highlighted, citing the US EPA EPA Transcriptomic Assessment Product (ETAP) that employs a 5-day rodent transcriptomics dose-response assay to predict the PODs in 2-year bioassays for chemicals lacking useful chronic toxicity information. The data from the ETAP studies, which will be publicly available, can be further analysed to investigate the mechanism of toxicity of the chemical for inclusion in an AOP. The US EPA is currently working to develop an *in vitro* version of the ETAP assay using cells from multiple tissues.

Aaron Redman (ExxonMobil) provided an environmental angle to the discussions, presenting on application of TK and TD as tools to support read across between chemicals and species. Whilst the assumption that internal dose within an organism being in equilibrium with the external exposures is reasonable for many aquatic tests, due to small sizes of the test organisms, this is not the case for e.g. rodent tests which require PBTK models. The importance, for example, of correcting for the fraction unbound and checking for volatilisation were highlighted.

4. BREAKOUT GROUP DISCUSSIONS

The scope of the breakout discussions was to understand how to account for the role of time when performing next generation risk assessment (NGRA) considering new approach methodologies (NAMs). The aim was to discuss how to integrate the influence of exposure time window, exposure duration, exposure frequency and damage accrual rate in developing and interpreting *in vitro* models, quantitative adverse outcome pathways (qAOP) and quantitative *in vitro* to *in vivo* extrapolation (QIVIVE).

Each of the five breakout groups considered a specific AOP:

	AOP
Breakout group 1	Skin Sensitisation AOP
Breakout group 2	Neurodegenerative diseases AOP
Breakout group 3	Carcinogenicity AOP
Breakout group 4	Liver toxicity cholestasis AOP
Breakout group 5	ED-mediated DART AOP

And addressed the following elements and questions:

Elements to address	**Questions**
External and internal scenarios	How should we assess and interpret the influence of time window, duration and frequency of exposure and effect development in *in vitro* assay battery design? Including internal Biokinetic consideration and influence of time in absorption, distribution, metabolism, excretion (ADME) processes.
In vitro assays (biokinetics and dynamics)	Which time points to test in *in vitro* assay relevant? Do we have the relevant methodologies to interpret prolonged/repeated dose toxicity with non-animal methods? If not how to fill this gap?
AOP/qAOP- Bradford Hill (B/H) criteria	Biological - time-scale of a transition from one key event to the next (AOP/qAOP) When is a change related to an adverse effect, and when should a change be interpreted as falling within the boundary of the physiologically 'normal' adaptive range? How should we visualise the influence of time on toxic outcomes in qAOPs?
QIVIVE	*In vitro* versus *in vivo* - influence of time on toxic outcomes in QIVIVE . How do we make appropriate and relevant *in vitro-in vivo* extrapolations in this area? How do we account for the influence of time on toxic outcomes in QIVIVE for human risk assessment? Can the B/H criteria (dose response – time response reversibility) be a means and a solution
NGRA	Inform next-generation risk assessment. Bottlenecks in the application of NAMs with respect to time variable in risk assessment? How should we incorporate exposure duration, frequency and time window and time frame of toxic outcome development in next-generation risk assessment based on NAMs?

Elements to address	Questions
AOB	Are there any other elements missing worth of including and discussing in the context of the workshop? (e.g. integrated approaches to testing and assessment (IATA), read across and quantitative structure-activity relationship (QSAR))

The below subsections summarise the breakout group discussions.

4.1. Skin sensitisation

AOP 40 OECD (2012a), (OECD, 2012b) describes the induction of skin sensitisation initiated by covalent protein binding. Different *in chemico* and *in vitro* assays have been developed to measure the first three key events (KE) leading to the induction of skin sensitisation. The local lymph node assay (LLNA, OECD test guideline 429) in mice measures increased lymph node proliferation compared to control treated animals which is the result at organ level. *In vitro* methods addressing the first 3 KE only, have been validated and implemented into OECD test guidelines (TGs) as well as defined approaches (DAs) which describe their application for hazard identification and classification and labelling. At present, a point of departure for quantitative risk assessment cannot be derived for skin sensitisation based on new approach methods (NAM) such as *in chemico/in vitro* data or DA as a stand-alone. To date, a weight of evidence (WoE) next generation risk assessment approach (NGRA) is applied using, among others, read across, NAM (including in chemico, *in vitro* and in silico), DA, historic animal data and human test data (Gilmour et al., 2020, Gilmour et al., 2023, Lee et al., 2022, Api et al., 2020).

The *in chemico/in vitro* methods have been developed to maximise sensitivity and reduce the number of false-negative results. Their exposure regimes are therefore not developed to describe or reflect the *in vivo* situation. Also, while the cascade of events leading to skin sensitisation induction is well known, it has not been expressed quantitatively. Human patch tests and *in vivo* tests in experimental animals apply external doses expressed as dose per unit/area. *In vitro* methods have been validated against these external doses and apply nominal concentrations. Looking at internal doses could help establishing assay-to-assay comparisons and potentially address some of the time-related uncertainty in testing. However, since validation studies have been completed based on external doses, the prediction models might need to be adapted when shifting to internal doses. Physiologically based pharmacokinetic (PBPK) models for the skin and local exposure in the skin are limited, as far as the breakout group participants were aware, and not employed in skin sensitisation assessment today. There might be interest in taking a closer look at modelled internal doses to explain unexpected findings in humans. For example, Parmasivan and coworkers found that repeated exposure to low doses of contact sensitisers may increase sensitising potency (Paramasivan et al., 2010). In their study, healthy adult volunteers who received a single patch of 60 µg/cm^2 2,4-dinitrochlorobenzene (DNCB) showed the same degree of sensitisation response as those who received three once-weekly applications of 10 µg/cm^2 (Robinson et al., 2000, Kimber et al., 2008, Rees et al., 1990, White et al., 1986, Friedmann et al., 1983).

Another aspect is the time needed for the activation of the different key events. It was discussed if a quantitative AOP for skin sensitisation would be needed and if a better understanding of the time course of events in humans would help to support the endpoint assessment. There was agreement that while interesting this would likely be more of an academic exercise and not help to address the current challenges in skin

sensitisation assessment. The time component in the protein binding step is being investigated and a kinetic variant of the direct peptide reactivity assay (kDPRA) was already developed and accepted as an OECD test guideline (OECD, 2024, Wareing et al., 2020). The DPRA looks at protein depletion at a single time point (24 h) and a single, fixed concentration and does not provide information on potency of a sensitiser as the final protein depletion at 24 h hours may be the same for a potent (fast reacting) or less potent sensitiser. The kinetic DPRA also takes the kinetics of the protein binding reaction into account and measures protein depletion after different exposure times and for different test substance concentrations. The rate constant is calculated and the maximal kinetic rate of the test substance binding with the test peptide can be established to assess skin sensitisation hazard and (to a limited extent) potency. This assay refines some of the missing time aspects in the current NGRA approach.

Another important challenge in test method refinement or development is to broaden the applicability domain of current non-animal test methods and allow to adopt them for difficult to test substances. In this regard, the internal dose or freely available dose in an *in vitro* system may be worth pursuing as external/nominal doses might lead to an underestimate of hazard. Simulating *in vitro* dosimetry might be a useful pre-screening to establish if an *in vitro* model is fit-for-purpose for a specific substance class. This holds true across endpoints and for different *in vitro* test systems..

4.2. Neurodevelopmental toxicity

The AOP considered for DNT was derived from a more complex network of AOPs , comprising AOP 3, 12, 13, 17, 42, 48, 54, 134, 260, as reported by Spinu et al. (2019). The simplified AOP starts with a key event, reduced levels of brain-derived neurotrophic factor (BDNF), followed by a decrease of synaptogenesis, a decrease of neuronal network formation, and finally, the adverse outcome, which is any *in vivo* indication of neurodevelopment adverse effect (e.g. impaired learning, memory, or cognitive function), Figure 1. The reasoning behind the simplification of this AOP network into the linear AOP here evaluated is summarised in Paini et al. (2022). A simplification of the AOPs might miss some aspects of the AOPs that could be important for risk assessment. However, presented with the complexity of the network and lack of standardised data for all the key events, the authors took the empirical decision of simplicity to define a short sequence of well recognised physiological markers leading to DNT.

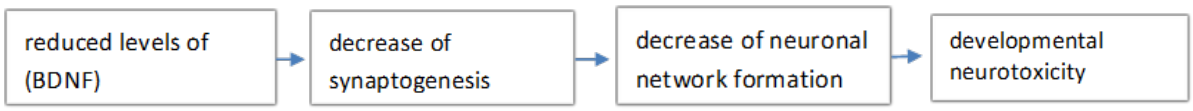

Figure 1. Simplified AOP for NGRA DNT (Adapted from Paini et al.2022)

A good knowledge of the molecular and cellular events depicted in this AOP is needed to understand how time can affect them. BDNF is a neurotrophic factor essential to several neurophysiological processes, some essential for neurodevelopment but not exclusively. The variety of functions of BDNF is dictated by the phase of development but also through the amount and ratio of two BDNF isoforms (m- and pro-BDNF). BDNF synthesis, processing, and extracellular release are controlled by a multitude of transcription factors. Briefly, the m-BDNF isoforms activate molecular pathways that modulate synaptic plasticity and enhances dendritic growth and branching while pro-BDNF activates cascade pathways that modulate neurons survival/death and

the growth cone of neuron (Kowiański et al., 2018). Hence, disruption of the released BDNF isoforms, either increased or decreased can lead to adverse outcomes. A recent human biomonitoring study suggested the use of BDNF as a biomarker of neurodevelopment/cognitive issues and DNT chemicals exposure (e.g., pesticides and heavy metals) after finding epidemiological associations (Rodríguez-Carrillo et al., 2022). The specific manner BDNF is used as a biomarker is the concentration of the isoforms in serum and epigenetic changes in the BDNF gene (Rodríguez-Carrillo et al., 2023). While released BDNF isoforms can transiently change in response to specific chemical insults, epigenetic changes will persist long after the chemical insult is gone, possibly increasing the severity of effects.

Facing the complexity of BDNF regulation, scientists have not focussed on this endpoint for risk assessment but rather more on upstream and functional endpoints. One example is the developmental neurotoxicity *in vitro* test battery (DNT-IVB) which assesses proliferation, differentiation, apoptosis, migration, neurite formation, synaptogenesis, and neural network formation (OECD, 2023, Blum et al., 2023). Usually, this type of assay uses neural stem cells (from embryonic stem cells or induced pluripotent stem cells) or neural progenitor cells derived directly from foetal tissue, usually rodents. The characteristics of these *in vitro* assays (e.g., number of cells, type of cells and endpoint measured) are relevant for the discussion of how to consider differences between the external and internal exposure. Following the questions raised during the workshop, the summary discussion was divided to focus on the sensitive time window of exposure, duration of the exposure, and challenges in modelling aspects with respect to time. In addition, we explored the potential of TK/TD modelling, drawing conclusions and providing gaps and solutions.

Sensitive time window of exposure

What constitutes a sensitive time window depends on a development phase where the embryo or foetus is more sensitive to changes on the key events depicted in the AOP, but also the phase of development where the foetus and embryo can be more exposed to the DNT xenobiotic. While neurodevelopment occurs throughout most of the embryo and foetus development (and after), the first trimester is more sensitive due to the quick onset and intricacies of the neurodevelopment phase and considering that any changes might result in more severe outcomes (the later the stage of development the more fine functions are impacted) (Adams et al., 2000). It is more challenging to define the most sensitive time window from the exposure point of view. It depends on chemicals (e.g., if a chemical is very bioaccumulative), but also not enough information is available on the exposure before the placenta and the foetal blood-brain barrier development and maturation (e.g., whether the yolk sac can be a route of exposure for the embryo). Considering this uncertainty, it is pragmatic to take the first trimester as the most sensitive time window in general. Hopefully, in the future, physiologically based models coupled with *in vitro* ADME and biomonitoring, epidemiologic or clinical studies can confirm if this is the right approach.

Duration of exposure

Chemicals associated to this AOP can be drugs or environmental pollutants for which *in vivo* exposure scenarios are different; for drugs there are more defined dosing schematics whilst for environmental pollutants a daily intake needs to be assumed. Physiologically-based toxicokinetic (PBTK) models can be used to simulate the target site peak and area under the curve concentrations following the different *in vivo* exposure scenarios. In silico models can also be applied to simulate the kinetics of an *in vitro* repeated exposure scenario which can be especially useful for accumulative. Considering that the majority of DNT chemicals are stable (do not get metabolised or degraded) in the *in vitro* system, the only difference between a longer duration of exposure and a repeated exposure with the same total duration is the potential accumulation of the chemical. This

accumulation can be modelled with *in silico* models and in fact has been used to disentangle the different apparent sensitivities of brainspheres models to a pharmaceutical amiodarone following different exposure scenarios chemicals (Nunes et al., 2023).

Dynamic challenges in the modelling systems

The modelling the kinetics of the chemical enables the calculation of target sites concentration from both *in vitro* and *in vivo*. However, there is still a question as to how time is reflected in this dosimetry. Cmax and AUC are extremes of this dimension, where Cmax does not account for time and AUC gives as much importance to time and concentration in the target site. Ideally, there is a seamless integration of toxicodynamics-toxicokinetics (TK-TD), with the use of frameworks such as qAOP, and this informs how the chemical, in time, causes the different effects. However, this proves to be a challenge for this selected DNT AOP, where a lack of mechanistic knowledge and experimental data merges with a 'moving target', which is the developing brain. The challenge of the 'moving target' is that it complicates the definition of reversibility or irreversibility, and thus complicates the definition of adaptability and adverse effect.

TKTD model to predict BDNF levels in serum and neuron migration

Inspired by Peter Macko's presentation on 'Time variables and exposure *in vitro* testing strategies' we developed a theoretical and simplified example of the TKTD model for BDNF and decrease of neurons migration (Figure 2). The model was developed on R, and the rates are fictitious just so they represent the curves that can help illustrate the case study. Figure 1 exemplifies how, without chemical exposure, the BDNF has a steady state that enables the migration of neuronal stem cells, which is a continuous process in time that reaches a maximum at 1500 µm. As part of the exercise, we set that a threshold for normal development is to have neurons migrated 1500 µm by 300 h. Once the chemical is added to the system, it reaches a steady level in time, it is decreasing BDNF production in serum which gets a lower steady-state, consequently decreasing the migration of neurons. If the assay is 120 hours, it looks like all concentrations affect migration, but in fact, with the lowest concentration of chemicals, the cells still reach the threshold for normal development at 300 h. Adding a washout period, where the chemical is removed from the cells' culture medium can provide some information on how quickly BDNF and migration recovers, which is crucial to understand how to extrapolate the exposure-effect relationship to *in vivo* scenarios.

An *in vivo* exposure of a chemical with relatively high clearance shows the daily peaks of the chemical but also shows the lack of considerable accumulation. Although the peaks of the chemical cause decrease of BDNF in serum, as soon as the chemical is cleared, BDNF recovers to basal levels. Consequently, the effects on migration are much decreased comparing to the same nominal concentrations as *in vitro*. In the case of a more accumulative chemical, the BDNF does not recover and continues decreasing in serum, causing a much higher impact on migration of the neuron progenitor cells.

This theoretical example that needs to be validated has very simplistic assumptions allowing to highlight the importance of recovery and resilience of the system. In this example, without knowing how quickly BDNF returns to baseline after the chemical insult, we cannot understand what the effect would be in a different exposure scenario, such as *in vivo*. It should be noted that the possibility of BDNF recovering quickly to baseline levels, cells might also change their epigenetics to overcome the insult and themselves overexpress BDNF to maintain a normal migration rate. Harris et al. (2018) for example showed how rotenone added to a 3D dopaminergic system affects mitochondria and energy parameters as well as neurite outgrowth. Once the chemicals are washed from the system the cells functionally recover but upon a second episode of exposure cells show increased resilience comparing to cells that are being exposed for the first time (Harris et al., 2018).

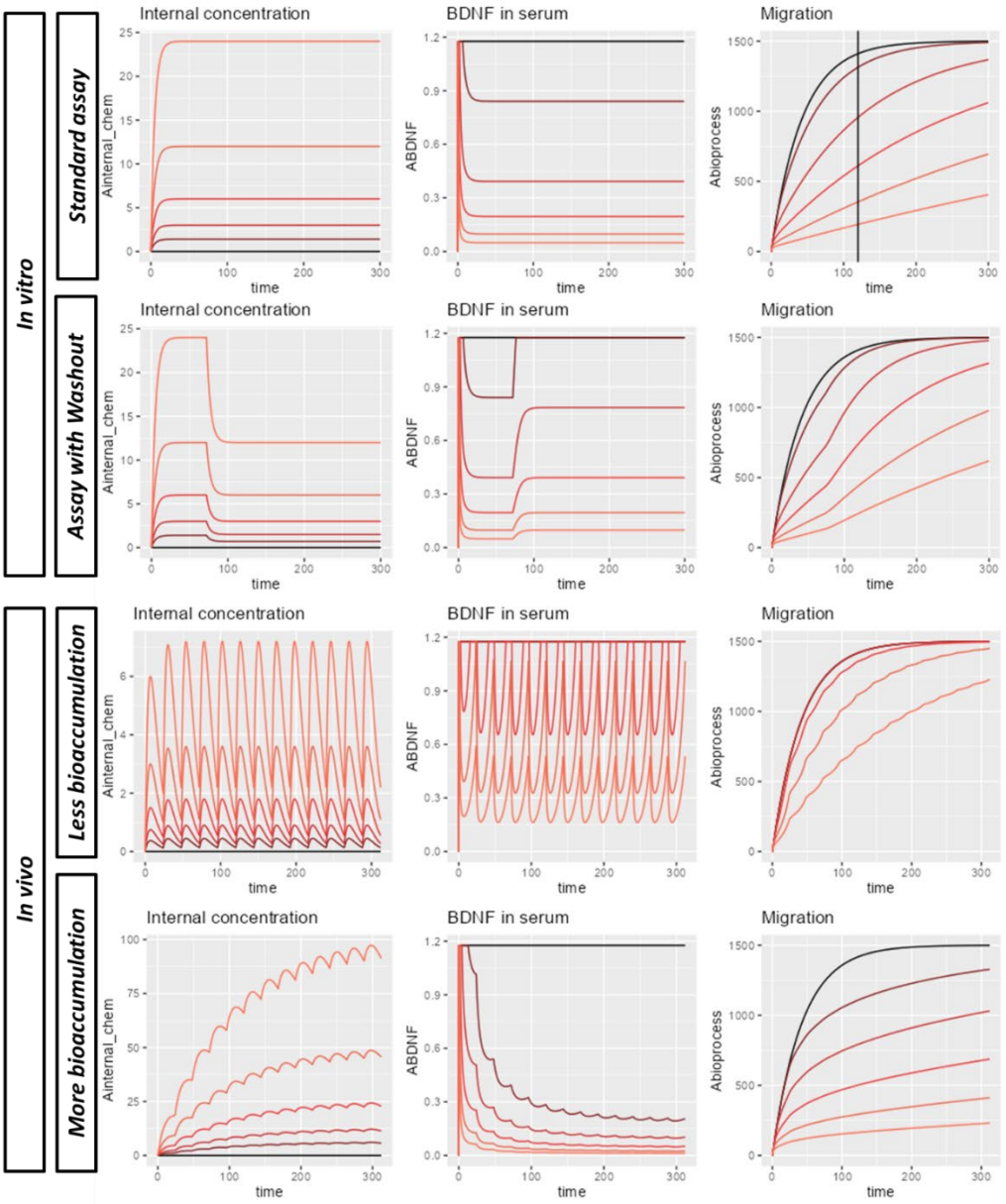

Figure 2. Theoretical model of the effects of a virtual chemical on BDNF synthesis/release and consequently migration, depending on different exposure scenarios. Black lines symbol no chemical exposure, so basal cells activities and with increasing lighter orange colours it symbols increasing concentrations of the virtual chemicals. The concentrations simulated are the same for all exposure scenarios.

Based on the breakout group discussions and modelling and simulations the conclusions were:

1. Measurements of *in vitro* endpoints should be minimum at two timepoints: first after a duration deemed suitable to measure the specific process being observed (e.g., changes in gene expression might be faster than migration of differentiation of the cells) and secondly after a washout period (Figure 1 - Assay with Washout). This last timepoint will give information on how quickly the *in vitro* system recovers to baseline levels which is important to predict the effect caused by different exposure scenarios.

2. It is important to consider *in vitro* kinetics since for some chemicals, the release of the chemical after the washout period might not be as efficient as for less bioaccumulative chemicals (Nunes et al., 2023). Hence, a model similar to the Virtual cell based model (Paini et al., 2017) which considers both *in vitro* kinetics but also effect in time (cell growth) might be suitable to integrate the knowledge of such an *in vitro* assay and use it in QIVIVE.

The current framework proposed here, although pragmatic, is still complex and time-consuming to do in a high-throughput way. Hence, it should only be applied to chemicals that are flagged through more high-throughput techniques. Still, we have to highlight that *in vivo* kinetics and systemic bioavailability, should be considered early on.

Gaps of DNT AOP and possible solutions

Even with the integration of *in vitro* assays data and TKTD modelling and simulations there is still gaps that needs to be addressed and are required for prediction of the adverse effect.

How do we define thresholds for the higher level key events (that are measured *in vitro*), below which we are confident there will be no adverse effects *in vivo*? Connecting this *in vitro* key events to higher level key events that can only be observed *in vivo* is challenging; human foetus development is partially a black box due to the ethical considerations of invasively investigate this phase of life. Although there are animal studies on neurodevelopment there are not fully relevant for a discussion on the time onset of neurodevelopment events (Zhao and Bhattacharyya, 2018). We conclude that the best model for humans are humans and possibly human epidemiological data that connects gestational exposure to childhood cognitive and ideally even some biomarkers (BDNF and imageology of the brain) can be useful to develop a more empirical model (e.g. Bayesian models) that can fill the gaps between the key events observed *in vitro* and the adverse outcome. Such a model would be supported by chemical-specific epidemiological data but being constructed based on an AOP it should be chemical agnostic once kinetic are considered.

Such a simplistic AOP for risk assessment (especially one where we ignore the MIE and earliest KE) has as core assumptions that the MIE and key events happen similarly in an *in vitro* system as in an *in vivo* system. For this it is important not only to evaluate the *in vivo* time window of brain development that is comparable to the *in vitro* models but also if there is sufficient similarity between the *in vitro* models and *in vivo* embryo brain. A specific aspect is the epigenetic changes of BDNF. Depending on the background epigenetics of the BDNF gene and of molecules that control its processing and release, epigenetic changes caused by chemicals *in vivo* might be different between *in vitro* and *in vivo*. Uncertainties like this can be cleared with validation of the *in vitro* models as it is being done for the DNT-IVB (Blum et al., 2023) and organised by OECD guidelines (OECD, 2023). Related to this is also the fact that some effects can be sensitive to population variability, and so this variability of key events needs to be characterised. Some of the *in vitro* models for DNT are derived from stem cells which can open the door to testing different donors. However also integration of the knowledge of the AOP and knowledge on human variability can help target these studies (e.g., BDNF serum levels have been indicated to be 30 % explained by heritability (Li et al., 2020).

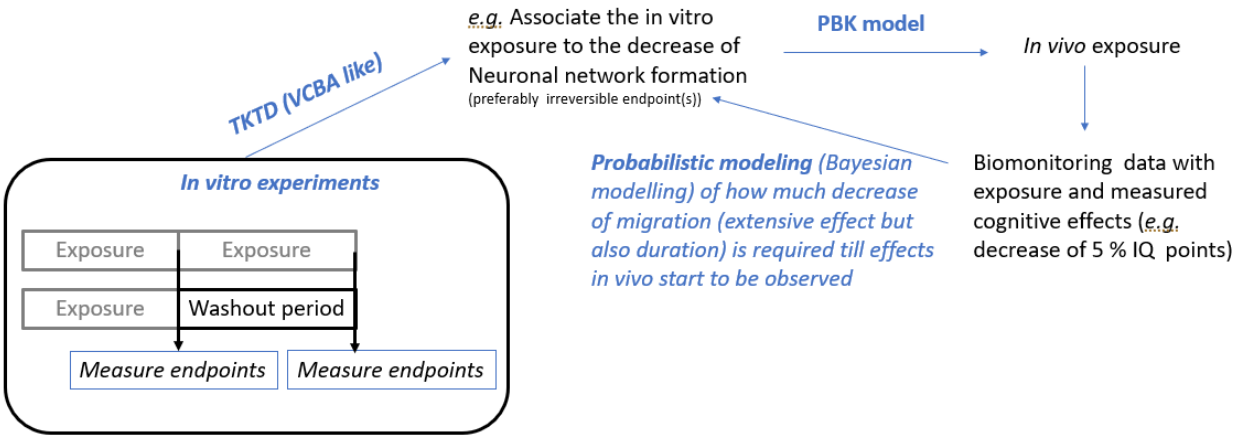

Figure 3. Proposed IATA for assessing BDNF-mediated DNT.

An Integrated Approaches to Testing and Assessment (IATA) has been proposed for DNT testing, but the focus has been on hazard identification and not necessarily on characterisation and exposure. We propose in Figure 3 an IATA that summarises the different models and assays herein discussed and so it considers time effect in the different aspects of risk assessment. While standard operation protocol has been developing for the *in vitro* models and assay for DNT, we suggest here they should also include the exposure scenarios (e.g., adding the washout period instead of repeated exposure) but also characterisation of aspects important for assessing the *in vitro* kinetics. SOPs for the in silico tools (DNT TKTD model and PBK models for DNT) should also be developed to ensure the tools are suitable for such an integration. This IATA is based on simplifications; uncertainties should be accounted for by using as much as possible probabilistic risk assessment and, when possible, making more conservative estimations. Fundamental research on evaluating the core assumptions of these IATA should proceed parallelly and be incorporated as scientific and regulators consensus is reached.

There is a pressing need for NGRA to produce neurodevelopmental toxicity (DNT) data (Smirnova et al. (2024) and [1]) and the time needed to disclose all details related to the pathway will significantly delay this type of risk assessment.

4.3. Carcinogenicity

As reflected by the existence of lifetime rodent bioassays, carcinogenicity is the endpoint with the longest time component in toxicology, introducing unique needs for the implementation of NAMs.

This breakout group was presented with a specific case study on a well-investigated non-genotoxic, liver-mediated thyroid carcinogenicity AOP: Sustained perturbation of the hypothalamus-pituitary-thyroid (HPT) feedback loop caused by increased hepatic T4 clearance, resulting in thyroid hyperplasia and follicular cell tumours.

[1] https://www.efsa.europa.eu/en/events/event/oecdefsa-workshop-developmental-neurotoxicity-dnt-use-non-animal-test

In this specific example, the chemical of interest activates the hepatic transcription factors constitutive androstane receptor (CAR), pregnane X receptor (PXR) (*Molecular Initiating Event, MIE*). CAR and PXR, known to impact xeno- and endobiotic metabolism in various ways, induce Phase II enzymes such as hepatic T4-uridine diphosphate glucuronosyl transferase (UDPGT), resulting in increased clearance and reduced plasma levels of T4 (*Key Event (KE) 2*). In turn, the hypothalamus responds with an increased release of thyrotrophin-reducing hormone (TRH), stimulating the pituitary gland to release thyroid stimulating hormone (TSH) (*KE 3*). *KE 4* constitutes increased follicular cell proliferation, resulting hyperplasia and/or tumour formation (*Adverse Outcome*) (Figure 4).

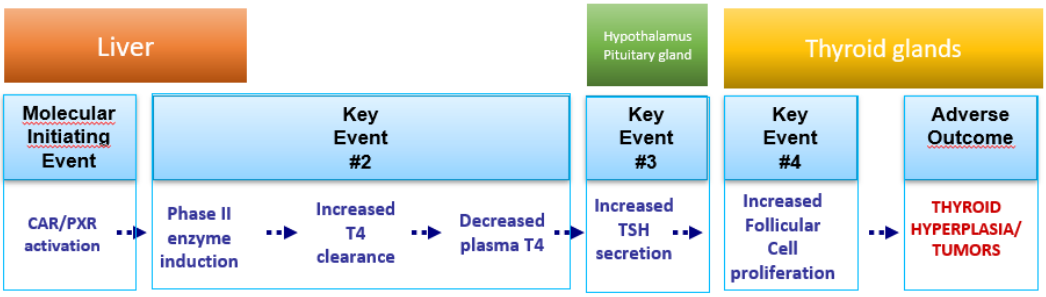

Figure 4. Non-genotoxic, liver-mediated thyroid carcinogenicity AOP

Besides the time component, the group identified various interesting challenges for NAM-based testing and assessment of this carcinogenicity AOP, including species-specific sensitivity and the involvement of multiple organs within the body.

Due to the necessary sustained nature of the processes in all four KEs to induce the adverse outcome (AO), one-time exposure is not believed to suffice to cause it; repeated exposure is deemed necessary for this AOP. However, as outlined above, activation of CAR and PXR may also induce other hepatic Phase I and/or Phase II enzymes involved in xenobiotic metabolism. The potential of certain chemicals to autoinduce their own hepatic clearance after repeated but not single exposure is well described in public literature. Therefore, for this particular AOP, the determination of the internal exposure to the target chemical would need to move beyond default assessment and include an investigation of the potential impact of the MIE and KE 1 on its biokinetic profile. Current *in vivo* testing necessitates repeated short-term exposure of a few days to identify such a mechanism. The limited lifespan of primary hepatocytes does not allow prolonged repeated exposure *in vitro*. Therefore, physiologically-based toxicokinetic (PBTK) modelling is considered a necessary element in the kinetic assessment to include the time dependency of internal exposure.

The group discussed various possibilities to assess this AOP in a NGRA context. For a full implementation *in vitro*, the following challenges were identified:

- Although it might be technically feasible to address KEs 1-3 by several single-exposure *in vitro* assays, the considerations above, along with the time required for T4 plasma concentrations to drop sufficiently to impact the HPT feedback loop, would nevertheless necessitate a time-/dose-response characterisation. Also, in order to reflect the long latency of this toxicological endpoint, a robust assessment of the impact of repeated exposure would be required to identify the presumably far lower exposure concentrations needed to cause an adverse outcome (as opposed to single exposure).

- Full implementation of the AOP *in vitro* would in addition require the combination of different assays/organ systems or even multi-organ approaches like organ-on-chip models due to the involvement of endocrine processes.

- Late KEs like hyperplasia/tumour formation were deemed challenging to be addressed directly *in vitro*. Due to the different target tissues involved in this AOP, direct *in vitro* testing of the target chemical in a Cell Transformation Assay would not be meaningful. Also, with carcinogenicity being a stochastic process, no *in vitro* assay would suffice to provide enough cell divisions to allow upscaling and establishing a robust quantitative link between chemical exposure and tumour formation. This difficulty is not unique to this particular AOP, but in a more general view indicates that NGRA will has to develop new assessment concepts e.g. to infer the AO based on an IATA, instead of to directly test the AO.

- While many NAM-based assessments of AOPs would focus on the MIE, this was considered insufficient in this particular case. As outlined above CAR and PXR play a prominent role in various metabolic processes, and are known to be activated by many exogenous and endogenous substances. The inclusion of additional KEs was therefore deemed necessary.

Therefore, the group agreed that a qAOP-based assessment necessitates the inclusion of systems (TK/TD) modelling to both reflect the time element and increase the robustness of quantitative assessments.

With the HPT feedback loop being a normal endogenous physiological process to maintain homeostasis of thyroid hormones, an in-depth quantitative assessment of Key Event Relationships (KERs) and quantifiable concentration- and duration-related thresholds for physiological processes was considered key in the identification of the adverse outcome, with the temporality component of the Bradford-Hill criteria being kept in mind. The primary site of action for the presented AOP being the liver, one can ask if once the in-depth assessment would be achieved it would be possible to focus on the key events taking place in the liver. Many overarching questions remains to be answered concerning the concordance of the dose responses of the different key events as a function of time. This has been demonstrated between 28-d and 2-yr in the use case presented. However, how conserved the phenomenon is across different AOPs, different species remain to be seen. Ultimately, can those early key events even be transposed in an *in vitro* test system in a physiological relevant manner?

Lastly, for successful implementation of an NGRA-based approach, it would be critical to benchmark NAMs results for a series of already characterised compounds including true positive carcinogens, CAR and PXR activating compounds not being carcinogens and true negative compounds to build qAOP modelling (TK/TD). This would allow a characterisation of the uncertainties and limitations of the developed IATA, but also the use of the final assessment concept for e.g. within-read-across to assess new chemical entities for this mode of action. This approach could then later be expanded to cover a greater chemical space.

4.4. Cholestasis

This breakout group discussed the relevance of time within the context of AOP 27 which is titled ‘Cholestatic Liver Injury induced by Inhibition of the Bile Salt Export Pump (ABCB11).’ The AOP relates the inhibition of the bile salt export pump (BSEP) to cholestatic liver injury through direct and indirect mechanisms. It has been shown that BSEP is cis-inhibited competitively by various drugs known to induce cholestasis. The most direct

effect of this inhibition is the accumulation of bile acids (BAs) in hepatocytes since the role of BSEP is to export BAs from hepatocytes into bile canaliculi. The accumulation of bile acids itself is already a central feature of cholestasis and contributes to its pathogenesis and clinical manifestations. Further, the accumulation of BAs triggers processes at the cellular level which are aimed to counteract BA accumulation, like gene expression changes in bile acid transport and metabolism related genes, which then ultimately also lead to deteriorative responses, such as inflammation, oxidative stress, and even cell death. Despite being equally important for the triggering of cholestasis, these secondary adaptive cellular responses are less direct, and therefore more difficult to quantify, since they are strongly regulated processes bounded by various homeostatic feedback mechanisms. In the current OECD test guidelines (OECD TG 407 and OECD TG 422) these may be captured, however, without capturing the specific underlying mechanisms.

Furthermore, it is well-know that time plays a big role in the manifestation of cholestasis and that there is considerable variability in the velocity of its manifestation. It is known that cholestasis can occur rapidly (hours or days), but it may also take much longer (weeks to months) and in certain situations it has been described to occur after a certain lag time. Reversely, even after withdrawal of the triggering agent, it can still worsen for some time. And in certain life stages like pregnancy, subjects are more prone to it. All these effects support the fact that the manifestation of the *in vivo* observed cholestasis adversity is much more complex and time-dependent than the simple underlying inhibition of BSEP itself could explain. This dependence on complex regulatory cellular mechanisms and the effect of time creates a special challenge for capturing such cholestasis-inducing effects with *in vitro* methods and NAMs.

The breakout group further discussed the relevance of time-dependent effects on the various levels outlined in the introductory text to Section 4 of this report. Despite the complicating factors mentioned before, the fact that BSEP inhibition itself is a relatively straightforward mechanism makes it at least conceptually easy to test for it *in vitro*. Kinetic considerations may play an important role *in vitro* and *in vivo* and related effects, such as accumulation or metabolization, need to be considered to yield relevant testing outcomes. But apart from those considerations the measurement of the molecular interaction of compounds with BSEP is relatively immediate and direct. However, at the next step the difficulty of time-dependency then comes into play. As outlined before, there are many adaptive mechanisms to bring back elevated levels of BA, to repair cell damage and adaptations to apoptosis. Therefore, it is difficult to determine which quantity of change is outside the normal adaptive range that an organism can compensate for. To deal with these complexities, either requires the establishment of heuristic thresholds that would allow to judge which molecular perturbations would actually lead to cholestasis, or ideally full quantitative modelling like quantitative adverse outcome pathways (qAOPs). And integrated approach of using kinetic modelling of *in vitro* and *in vivo* exposure, together with quantitative adverse effect models, such as qAOPs, would then allow for full consideration of the effect of time on all biological levels.

4.5. ED-mediated DART: Teratogenesis

AOP 19 describes how androgen receptor (AR) antagonism can lead to adverse effects in the male foetus of mammals. The molecular initiating event (MIE) is the binding of a compound to the androgen receptor resulting in a displacement of the natural ligand testosterone (T) or the more active dihydro-testosterone (DHT) and because of that an antagonising effect. It should be noted that the binding of a compound to the receptor can be reversible (i.e. a completive binding) or irreversible. At a cellular level the antagonistic effect

leads to a decrease of gene transcription associated with the androgen receptor and an alteration of the Wnt signalling pathway. At an organ level this then results in a feminisation of the male foetus ultimately leading to reduced fertility and impairment of reproductive capacity (see figure 5).

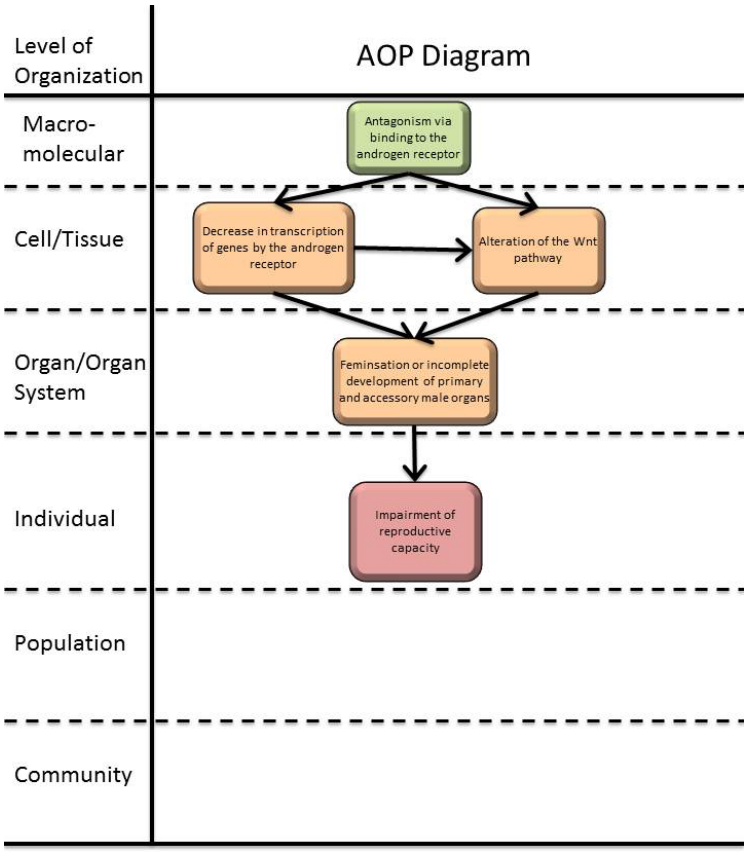

Figure 5. AOP 19 Androgen receptor antagonism leading to adverse effects in the male foetus (mammals)

There are various *in vivo* studies that are able to identify such an effect, particularly the prenatal developmental toxicity studies (OECD TG 414) and the reproduction toxicity guidelines (OECD TG 416 and TG 443, but also OECD TG 421 and TG 422). An *in vivo* study specifically designed to assess such effects is the Herberger Assay. Although there are many *in vitro* studies for the detection of teratogenicity none of these have been accepted as a full replacement of the above-mentioned *in vivo* studies. The MIE, however, can be readily determined by various *in vitro* studies which provide quantitative information on the strength of the bindings to the AR and which can be compared to the natural ligand. With respect to the induction of an adverse effect, it should be noted that there is a window of sensitivity during foetal development, which is in rats from gestational day 15 – 19 and in humans from week 11 – 20 (for details see Sharpe (2020)). Consequently, *in vitro* studies addressing the full AOP should be able to function within this window of sensitivity.

In developmental toxicity studies the external and internal scenarios are more complex than for most other forms of toxicity. The external exposure is to the mother while the effects are observed in the foetus. Therefore toxicokinetics (TK) need to take into account the concentrations in the mother vs foetus. This includes considering the placenta as a barrier. Without factual knowledge the concentration in the foetus may be the considered same as in the mother, however significant differences may exist. Measuring placental barrier

function (which can be done *in vitro* using the BeWo system) may help to improve the estimate of foetal concentrations relative the maternal ones. In addition, there are IVIVE/in silico methods already available to measure some of these parameters.

Consideration in the TK needs to be given to the type of exposure – acute verses chronic. Both need to be considered in the model. For chronic exposure time may be less important for TK than for acute or intermittent exposure because for most types of developmental toxicity windows of sensitivity exist. For the androgen receptor mediated effects, the window of sensitivity is relatively late. i.e. during foetal development, rather than during organogenesis. Therefore, *in vitro* assays should take into account both biokinetics and perhaps even more important the toxico-dynamics, ensuring that the window of sensitivity is taken into account in the assay.

This leads to the question whether we have the relevant methodologies to interpret prolonged/repeated dose toxicity with non-animal methods, and if not how to fill this gap?

With respect to exposure and internal dose, i.e. TK, absorption can be determined / modelled in Caco2 (worst case is 100%). Plasma binding and metabolism (addition of S9, or microsome, consideration of the induction of P450) can also be determined with appropriate in silico and *in vitro* models. In addition, BEWO cells, as a placental cells line that can be added to the *in vitro* intestinal absorption determination (in silico or using the CaCo2 cell line) to model the placental barrier. Active transport processes may need to be taken into account; however, this is an area where there is far less knowledge and both in silico as well as *in vitro* methods are not yet well developed.

The time or duration of exposure has a TK as well as a toxico-dynamic component. The duration of exposure may change the internal (maternal and foetal) concentrations and as mentioned above as windows of sensitivity exist, this also needs to be considered in terms of the length of the exposure.

With respect to an effect on the androgen receptor (AR), ToxCast assays for the AR can detect agonism and antagonism but do not consider the critical time period of exposure and effect. However, having identified an interaction with the receptor then the time component of the exposure can be considered. Therefore, this assay this is a useful first molecular initiation event (MIE) stage.

Considerations of the type of this MIE on the adverse outcome several aspects need to be considered.

(1) How long does a receptor need to be blocked to have a downstream effect ?

(2) What is the pharmacological nature of the interaction of the antagonist with the receptor? Is the interaction reversible or irreversible and how competitive is the antagonist for the receptor. This has a time component that will need to be considered in the assay.

In addition to an interaction with the AR, similar adverse effects can be induced by a reduction of the production of the hormone (e.g., testosterone) and here too the question needs to be addressed how long the exposure needs to be, to reduce the hormone levels to such an extent that adverse effects can occur. Therefore, for *in vitro* models to be accurate such an assay should be able to be tracked through the foetal stages in order to determine effect or cover the critical time points of exposure.

The first step of understanding time is to understand its effects on the MIE and AOP. In terms of understanding the effects of time on the various steps in an AOP (the key biological events (KBEs)), it is essential that the species needs to be considered as the windows of sensitivity are different for the rat to the human. This is also important for the risk assessment, when considering time related exposure.

Considering the KBEs in the biological - timescale of a transition from one key event to the next is it possible to determine when is a change related to an adverse effect and would it be possible to interpret a change as falling within the boundary of the physiologically 'normal' adaptive range? To address this question, it was

considered necessary to move from the effects in the foetus alone to subsequent affects in the whole organism and in particular effects on fertility and reproduction.

There might need to consider adaptation or reversibility. If exposure ceases, is there a resumption of 'normal' and how does time of exposure affect reversibility. There is a balance to be considered between damage and repair. Malformations are irreversible, however effects on fertility can be reversible, which was demonstrated for the antiandrogenic drug flutamide, a prostate cancer drug. During the period of exposure infertility occurs but there is recovery after the cessation of exposure to the substance. The duration of exposure itself may also lead an adaptive or reversible change to become irreversible. For example, if there is an epigenetic adaptation that leads to a continued alteration of gene expression, this may result in non-reversible adverse effects. Thus, there is a relationship to the effect of dose as well as time. Time also needs to be considered in the period of exposures e.g.: adolescent verses older male for example and similarly for females. Time scale of KBEs in the AOP is not considered to be the rate limiting effect because the time between the KBEs is fast therefore the rate limiting steps are the kinetics of the exposure.

Considering these aspects of duration of exposure and (time related) windows of sensitivity how do we make appropriate and relevant *in vitro-in vivo* extrapolations in this area? How do we account for the influence of time on toxic outcomes in QIVIVE for human risk assessment? The TK part of this question can be reasonable addressed by adapting existing models to take into account placental transfer and perhaps some additional foetus specific factors such as metabolism and plasma protein binding. The major challenge is the lack of good representative *in vitro* systems. Also, there is a need to understand the dynamics of interaction for example the affinity constant of the substance verses the endogenous hormone. Therefore, to advance next generation risk assessment approaches it is necessary to develop appropriate NAMs that take into account the dynamic development of the foetus, foetal-maternal interactions and specific knowledge related to species dependent windows of sensitivity.

5. WORKSHOP RECOMMENDATIONS

Time matters in toxicology and therefore the toxicokinetic profile and the toxicodynamic activity of a compound in an experimental system, be it *in vivo* or *in vitro*, need to be assessed and characterised for its influence on the test outcome and the extrapolation to human. These elements need to be captured in the problem formulation. This can then be used to determine if the chosen assay or assay battery is fit-for-purpose for capturing the time element and event that are dependent on time. This should all form part of the consideration of the applicability domain of the assay(s) for the substance and adverse outcome under consideration. While time is an important factor to consider, discussions in the workshop confirmed that there are additional limitations of assay applicability domains that remain significant hurdles for the use of non-animal methods (e.g. for difficult-to-test substances, UVCB substances, particles testing).

A tiered approach is recommended, moving from external exposure considerations to toxicokinetic and toxicodynamic considerations. If a conclusion can be reached at any stage with the time aspect sufficiently addressed the assessment can be concluded.

When moving from the traditional risk assessment paradigm to next-generation risk assessment, the following recommendations are made about time and toxicokinetics:

- Assess the time, frequency, and duration of real-life exposures to estimate the worst-case external exposure.
- Assess the exposure window and the criticality of the exposure time point, e.g. for exposures during different stages of development, and ensure the *in vitro* system can mimic this sensitivity.
- Use IVIVE models to derive the internal exposure at the target site with specific attention to the kinetics and use of peak concentration (C_{max}), for substances eliminated fast and efficiently vs. area under the Curve (AUC), for substances with slower ADME kinetics and mimic as far as possible *in vivo* exposure directly measured or calculated from PBPK.
- The potential for cumulative exposure and mimicking these *in vitro*.

Identify time-sensitive toxicodynamic aspects using target Adverse Outcome Pathway analysis where appropriate:

- Assess damage accrual rates and potential for repair with specific attention to the reversibility of the Molecular Initiating Event (MIE) and each of the Key Events (KE) to understand if any build-up over time is expected.
- Where possible defining a quantitative AOP will express KE-KE relationships quantitatively. This allows to assess the time critical component of the AOP and ensure any compensatory and recovery behaviours can be modelled. Specifically assess any rate limiting steps within this such as rate limiting conversions.

It is likely that there will be no one-to-one replacement for animal studies and therefore integration of results from different toxicologic assays with different exposure metrics will be necessary. Internal exposures at sensitive tissues and site of action can be used as basis for extrapolation, and benchmark modelling from acute and chronic data, and should be further explored. There will be no one perfect answer and the problem formulation leading to a careful consideration of the applicability domain, which will include time, for the substance and exposure window under consideration will lead to improved prediction for hazard from *in vitro* systems.

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APPENDIX A: WORKSHOP PROGRAMME

Day 1: 7 November		
10.30 – 11.00	*Arrival and registration for in-person participants*	
11.00 – 11.20	Welcome, introduction and workshop objectives	**Blanca Serrano** (ECETOC), **Alicia Paini** (esqLABS, DE)
11.20 – 11.40	Studying and comparing the role of time in in vitro and in vivo toxicity tests	**Nynke Kramer** (Wageningen University and Research, NL)
11.40 – 11.50	Systems modelling of quantitative adverse outcome pathways: progress on temporal integration of toxicokinetics and beyond	**Huan Yang** (esqLABS, DE)
11.50 – 12.00	General discussion/Q&A	
11.50 – 12.10	Time variables and exposure in in vitro testing strategies	**Peter Macko** (JRC, IT)
12.10 – 12.20	General discussion/Q&A	
12.20 –12.40	Dose and Time Responses using in vitro Metabolomics	**Ben van Ravenzwaay** (Wageningen University and Research, NL)
12.40 – 12.50	General discussion/Q&A	
12.50 – 13.50	**Lunch**	
13.50 – 14.10	Bringing the pieces of the puzzle together: considering time and biological scale with new approach methodologies	**Gladys Ouedraogo** (L'Oréal, FR) *(online)*
14.10 – 14.20	General discussion/Q&A	
14.20 –14.40	Integration of time-related factors in dose-response analysis and exposure assessment	**Cecilia Tan** (US EPA, US)
14.40 – 14.50	General discussion/Q&A	
14.50 - 15.20	**Coffee break**	
15.20 – 15.40	Preparing the way for short-term in vitro assay prediction of in vivo chronic toxicity	**Harvey Clewell** (Ramboll, US) *(online)*
15.40 – 15.50	General discussion/Q&A	
15.50 – 16.10	TK and TD as tools to support read across between chemicals and species	**Aaron Redman** (ExxonMobil, US) *(online)*
16.10 – 16.20	General discussion/Q&A	
16.20 – 16.40	Brief summary/overview of presentations, discussion & last remarks/notes	**Nynke Kramer**
16.40 – 17.00	Introduction to day 2	**Alicia Paini**
17.00 – 18.30	**Aperitivo**	

Day 2: 8 November		
09.30 – 09.45	Welcome and introduction to Day 2	
09.45 – 12.30	Breakout groups on case studies, guided by charge questions and a matrix	

	Breakout group 1	*Skin Sensitisation AOP (e.g. AOP 40)*	Moderator: **Daniela Holland** (ExxonMobil, BE) Rapporteur: **Petra Kern** (Procter & Gamble, BE)
	Breakout group 2	*Neurodegenerative diseases AOP (e.g AOP 3)*	Moderator: **Alicia Paini** (EsqLABS, DE) Rapporteur: **Susana Proenca** (Wageningen University and Research, NL)
	Breakout group 3	*Carcinogenicity AOP*	Moderator: **David Rouquie** *(online)* (Bayer, FR) Rapporteur: **Barbara Schmitt** *(online)* (Evonik, DE)
	Breakout group 4	*Liver toxicity cholestasis AOP (e.g. AOP 27)*	Moderator: **Nynke Kramer** (Wageningen University and Research, NL) Rapporteur: **René Geci** (esqLabs GmbH/ University Hospital Aachen, DE)
	Breakout group 5	*ED-mediated DART AOP (e.g. AOPs 19, 23)*	Moderator: **Ben van Ravenzwaay** (Wageningen University and Research, NL) Rapporteur: **Tim Gant** (Imperial College London, UK)
12.30 – 13.30	**Lunch**		
13.30 – 15.30	Plenary feedback from breakout groups		**Rapporteurs from breakout groups**
15.30 – 16.00	Summarise and close		**Ben van Ravenzwaay**

APPENDIX B: WORKSHOP ORGANISING COMMITTEE

Paolo Boffetta, Bologna University
Tim Gant, Imperial College London
Daniela Holland, ExxonMobil
Nynke Kramer, Wageningen University and Research
Philippe Lemaire, TotalEnergies
Alastair Middleton, Unilever
Alicia Paini, esqLABS
David Rouquie, Bayer
Kees van Leeuwen, Utrecht University
Ben van Ravenzwaay, Wageningen University and Research

Blanca Serrano, ECETOC
Andrea Salvadori, ECETOC
Lucy Wilmot, ECETOC

APPENDIX D: WORKSHOP PARTICIPANTS

Name	Surname	Affiliation	Attendance Day 1	Attendance Day 2
Bas	Blaauboer	Utrecht University	F2F	F2F
Phil	Botham	Syngenta	online	
Laure-Alix	Clerbaux	UCLouvain	F2F	F2F
Harvey	Clewell	Ramboll	online	online (in part and later in day for breakout group 4 follow-up call)
Rebecca	Clewell	21st Century Tox Consulting	online	online (in part and later in day for breakout group 4 follow-up call)
Richard	Currie	Syngenta	online	
Philipp	Demuth	BASF	online	online
Sylvia	Escher	Fraunhofer ITEM	online	online
Tim	Gant	Imperial College London	F2F	F2F
René	Geci	esqLabs GmbH/University Hospital Aachen	F2F	F2F
Na	Guan	Dow	online	
Valerie	Herno	Solvay	online	
Daniela	Holland	ExxonMobil	F2F	F2F
Heli	Hollnagel	Dow	online	online
Charles	Humfrey	Lubrizol Ltd	online	
Petra	Kern	Procter & Gamble	F2F	online
Susanne	Kolle	BASF SE	online	online
Nynke	Kramer	Wageningen University	F2F	F2F
Peter	Macko	European Commission Joint Research Centre	F2F	F2F
Sue	Marty	Dow	online	
Bette	Meek	University of Ottawa	online	online later in day for breakout group 4 follow-up call
Gladys	Ouédraogo	L'Oréal Research & Innovation	online	
Alicia	Paini	esqLABS GmbH	F2F	F2F
Susana	Proenca	esqLABS GmbH	F2F	F2F
Aaron	Redman	ExxonMobil Biomedical Science, Inc	online	online
David	Rouquie	Bayer	online	online
Asta	Ruzgyte Frère	DSM-Firmenich	online	online
Barbara	Schmitt	Evonik	online	online
Katrin	Schutte	DG ENV	online	online
Blanca	Serrano Ramòn	ECETOC	F2F	F2F
Cecilia	Tan	US Environmental Protection Agency	F2F	F2F
Ben	van Ravenzwaay	Wageningen University	F2F	F2F
Sanjeeva	Wijeyesakere	Dow	online	

Name	Surname	Affiliation	Attendance Day 1	Attendance Day 2
Lucy	Wilmot	ECETOC	F2F	F2F
Andrew	Worth	European Commission Joint Research Centre	online	online
Huan	Yang	esqLABS GmbH	F2F	F2F

APPENDIX E: WORKSHOP REPORT CONTRIBUTORS

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Daniela Holland, ExxonMobil
Petra Kern, Procter & Gamble
Nynke Kramer, Wageningen University and Research
Alicia Paini, esqLABS GmbH
Susana Proenca, esqLABS GmbH
Ben van Ravenzwaay, Wageningen University and Research
David Rouquié, Bayer
Asta Ruzgyte Frère, DSM
Barbara Schmitt, Evonik

APPENDIX F: SPEAKER ABSTRACTS AND BIOGRAPHIES

Studying and comparing the role of time in in vitro and in vivo toxicity tests

Nynke Kramer, Wageningen University and Research, NL

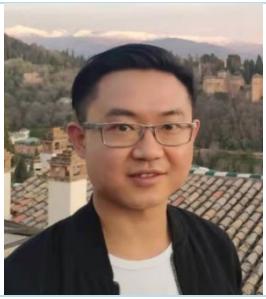
In risk assessment, the role of time on the toxic potential of a chemical is generally assessed using a suite of toxicity assays on animals which are exposed and observed for a defined period of time. These different tests result in different toxic endpoints and potencies. With the shift in paradigm towards the use of non-animal testing methods for toxicity testing and risk assessment, new challenges arise aligning in vitro-derived toxicity data to the different in vivo toxicity tests with defined exposure durations. Toxicokinetic-toxicodynamic (TK-TD) modelling from ecotoxicity studies may be able to help overcome these challenges and provide a mechanistic approach to understanding the role that time plays in toxicology. In this presentation, studies illustrating the application of TK-TD modelling in in vitro toxicology and quantitative in vitro-in vivo extrapolation (QIVIVE) will be highlighted. These include two repeat-dose in vitro studies integrating TK-TD modelling to assess the neurotoxic and hepatotoxic potential of amiodarone, a highly lipophilic drug to treat arrythmia.

Nynke Kramer is associate professor in toxicology in the Toxicology Division of Wageningen University and Research. Her research focusses on enhancing the uptake of in vitro models in toxicological risk assessment by developing models extrapolating effect concentrations obtained from in vitro cell assays to toxic doses relevant to humans and animals. She teaches pharmacokinetics and (eco)toxicological risk assessment at undergraduate, graduate, and postgraduate level. Her teaching and research neatly integrate the skills she acquired as an assistant professor and post-doctoral fellow at the Institute for Risk Assessment Sciences of Utrecht University, as well as during her PhD in toxicology at Utrecht University, her MSc in Environmental Change and Management at Oxford University, and her BSc in Life Sciences at University College Utrecht.

Systems modelling of quantitative adverse outcome pathways: progress on temporal integration of toxicokinetics and beyond

Huan Yang, esqLABs, DE

Biological systems modelling aims to predict and understand input-output relationships through computational modelling and simulation of relevant biological mechanisms. Quantitative adverse outcome pathways (qAOPs) take the activation of molecular initiating events (MIEs) as systems inputs. Along with quantitative AOPs, activation of MIEs will be modelled further to activation of key events (KEs) and adverse outcomes (AOs). Time scales vary at different biological levels across MIEs/KEs/AOs, and intrinsic feedback loops in biological levels could make time aspects more complicated. Besides qAOP, further integrated quantitative systems modelling with physiologically-based pharmacokinetic (PBPK) and qAOPs could offer integrated risk assessment tools to predict exposure-response relationships. We will demonstrate the merits of integrated systems modelling in NGRA through not only visualization of (temporal) response-response relationship but also advanced computational analysis to better understand toxicology data and mechanisms.



Huan Yang is a principal scientist in systems toxicology and qAOP platform lead at esqLABS GmbH, Germany. He obtained his PhD in applied mathematics and systems neuroscience at the University of Twente, the Netherlands in 2015. 2016-2021, he worked as a postdoc at Leiden University. His research topics focused on quantitative systems toxicology by applying mathematical modelling of adverse outcome pathways.

Time variables and exposure in in vitro testing strategies

Peter Macko, Joint Research Center (JRC), European Commission

In vitro methodologies serve as valuable alternatives to animal testing, forming integral components of novel approach methodologies for toxicological hazard and risk assessments. However, in vitro experiments often have limitations in terms of their duration, measurements of responses, and rarely consider more time points, which may result in the disregard of potential cumulative chronic effects over time. To address this issue, we propose an experimental design that not only characterizes the toxicodynamics of a response in relation to concentration but also incorporates the dimension of time. The concentration-time responses are modelled using a set of ordinary differential equations (ODEs). This approach enables the characterization of the dynamics of key events and their relationships, thus facilitating the development of quantitative adverse outcome pathways.

Peter Macko received a degree in physics from Comenius University in Bratislava before obtaining his PhD in laser spectroscopy from Joseph Fourier University in Grenoble. During the early years of his career, he focused on experimental and computational physics, primarily utilizing highly sensitive spectroscopic techniques to investigate atmospheric, interstellar, and plasma physics and chemistry. He possesses a wealth of experience in laser detection techniques, optical systems, microscopy, and computational skills, including the modelling of optical systems, and the dynamics and kinetics of chemical, transport, and diffusion processes. Later on, his research shifted towards biomolecular imaging. He has spent over a decade working at EURL ECVAM with high-throughput and high-content imaging platforms for in vitro methods, and with computational toxicology.

Preparing the way for short-term in vitro assay prediction of in vivo chronic toxicity

Harvey Clewell, Ramboll, US

One of the most challenging applications of NAMs to reduce animal testing requirements is in the prediction of chronic toxicity. While a variety of functional tissue cultures can now routinely be maintained in vitro for a period of several weeks, the relationship of dose-responses for toxicity over such short periods in vitro to dose-responses observed after chronic exposure in vivo has not yet been elucidated. A way forward in this area may be provided by the unique temporal characteristics of transcriptomic dose-response data and the remarkable correspondence that has been demonstrated between points of departure (PODs) based on the transcriptomic dose-response in short-term in vivo studies and apical PODs from 2-year chronic toxicity assays. Based on these studies, a new USEPA risk assessment approach, the EPA Transcriptomic Assessment Product (ETAP), is under development that will employ a 5-day rodent transcriptomics assay to predict the PODs in 2-year bioassays for chemicals lacking useful chronic toxicity information. Importantly, the data the USEPA collects will be publicly available and could readily be further analyzed to assess toxic modes of action and relative potencies. Health Canada and NIEHS are currently investigating the use of in vitro transcriptomic assays for these purposes (Rowan-Carroll et al. 2021, Reardon et al. 2021). A possible path forward for an in vitro alternative to the ETAP to predict the outcome of a 2-year bioassay would require: (1) conducting 5-day in vitro assays in one or more tissue cultures using chemicals included in the analyses described in the ETAP documentation, (2) transcriptomic pathway analysis on the same studies to provide evidence to support AOP identification, and (3) development of an agreed battery of in vitro genotoxicity assays that could be used to determine whether the POD from the in vitro transcriptomic study could represent a threshold for nongenotoxic carcinogenicity.

Harvey Joseph Clewell III, PhD, DABT, ATS, is a Principal Consultant with Ramboll US Corporation, located in Research Triangle Park, North Carolina. He has more than 45 years of experience in environmental quality research, toxicology research, chemical risk assessment, and hazardous materials management, and has authored more than 200 peer-reviewed scientific publications and book chapters. Dr. Clewell has gained an international reputation for his work on the incorporation of mechanistic data and mode of action information into chemical risk assessments, having played a seminal role in the first uses of physiologically based pharmacokinetic (PBPK) modeling in cancer and non-cancer assessments by the EPA, FDA, ATSDR, OSHA and Health Canada. He is also an expert in the use of cellular genomic response data to inform the mode of action for chemical toxicity and to determine alternative points of departure for risk assessments. Dr. Clewell has served on the external peer review panels for EPA guidelines on development of reference concentrations, cancer risk assessment, risk characterization, benchmark dose modeling, PBPK modeling and dermal absorption, and has participated in many chemical-specific reviews conducted by the EPA Scientific Advisory Board and the FIFRA Scientific Advisory Panel. He also served as a member of the ECVAM Scientific Advisory Committee from 2012 to 2016. In 2007 the Society of Toxicology recognized Clewell with the Arnold J. Lehman Award for his major contributions to chemical safety and risk assessment.

Bringing the pieces of the puzzle together: considering time and biological scale with new approach methodologies

Gladys Ouedraogo, L'Oréal, FR

There is a growing need of new approach methodologies for addressing hazard and risk assessment. Some regulations like the European Cosmetics' one banned animal testing.

Several initiatives are trying to address this need. For local effects like skin and eye irritation, there are some strategies to addressing them with new approach methodologies -NAMs-. When it comes to complex endpoints like systemic/reproductive toxicity more effort is needed to establish tools and approaches allowing safety assessment and the likelihood of causing adversity which is a requirement for chemical registration. It is now commonly accepted that no one to one replacement nor a 'one size fits all' approaches are suitable.

Characterizing adversity with new approach methodologies is challenging for many reasons:

- There are multiple ways to cause systemic toxicity and most of the underlying mechanisms leading to adversity are unknown.
- The temporality aspect between exposure and when toxicity occurs.
- Scaling from molecular, cellular effects to organs or organisms/populations.

A pragmatic approach allowing proposer biological coverage of key pathways related to human health.

Here, examples of using NAMs to address long term effects like general repeated dose systemic toxicity and carcinogenesis will be presented. They will feed into the discussion of establishing relevant experimental conditions when developing NAMs.

Gladys Ouédraogo has extensive experience in the development of New Approach Methodologies.

She joined L'Oréal R&I in 2003 to establish a unit for predicting cancer without animal testing. During her career, she created and led various research projects on technologies and emerging topics in the field of toxicity assessment. In doing so, she worked on genotoxicity, molecular modeling, systemic toxicity and endocrine modulation. In 2013, after leading teams working on alternative methods for predicting toxicity and efficacy for three years, she has been managing several collaborations and activities in areas such as repeat-dose systematic toxicity – an area that she is also actively developing within L'Oréal R&I.

Dose and time responses using in vitro metabolomics

Ben van Ravenzwaay, Wageningen University and Research, NL

In regulatory toxicity testing the duration of exposure has an influence on both the quality (which organs are affected and to which extent) of the toxicity observed as well as the quantity (dose without an effect). Depending also on the kinetics, time may only have a moderate aggravating effect or can be even more important than dose (complete carcinogens). How can we account for such time dependent properties in in vitro studies? Using in vitro metabolomics dose and time dependent responses of 256 intracellular metabolites were investigated following 3, 6, 24 and 48h exposure to various concentrations of nitrofurantoin. Increasing the dose and exposure duration were observed enhance the metabolic response. For the high concentration a non-linear response was seen for some metabolites, most likely related to the occurrence of cytotoxicity at the later time points. For the low concentrations this was not the case. Analysis of such dynamics may help to clarify if a time related change in the quality of the toxicity response may occur for a particular compound. What might happen beyond 48h would require further investigation.

Bennard van Ravenzwaay is a doctor of Environmental Sciences/Toxicology from Wageningen University, Netherlands in collaboration with the German Cancer Research Centre in Heidelberg, Germany. He worked for 34 years at BASF SE, Ludwigshafen, the last 20 as Senior Vice President of the Department for Experimental Toxicology and Ecology and BASF Metabolome Solutions.

He is an associate professor for Reproduction Toxicity of the University of Wageningen and had a teaching assignment at the University of Kaiserslautern until 2021.

He is Chairman of the Scientific Committee of the European Centre for Ecotoxicology and Toxicology (ECETOC) and a member of editorial boards of ‘Archives of Toxicology’, ‘Chemical Biological Interactions’ and ‘Toxicology Letters’.

He was member of the board of trustees of the Health and Environment Science Institute (HESI) from 2012 – 2018. He is a member of the German Society for Pharmacology and Toxicology, a European registered toxicologist and SOT-Member.

He is an author more than 250 peer reviewed publications.

Since 2022 he is an independent consultant for environmental sciences.

Integration of time-related factors in dose-response analysis and exposure assessment

Cecilia Tan, U.S. Environmental Protection Agency, Durham, NC

In conventional chemical safety testing, animals are exposed to varying durations to simulate scenarios relevant to human exposure. For instance, acute toxicity tests aim to replicate one-time, high-dose accidental exposure, while chronic studies attempt to emulate a lifetime of continuous exposure. Nevertheless, it remains challenging for animal studies to cover the full range of potential human exposure scenarios, encompassing time-related factors such as exposure duration and frequency, and critical exposure windows. Instead, a pragmatic approach is taken, where dose-response analysis estimates a reference dose; exposure assessment predicts potential exposure ranges; and comparing the reference dose with exposure estimates to assess risk. Time-related factors are integrated into both dose-response analysis and exposure assessment, yielding estimates of 'doses'. Such a pragmatic approach also applies to in vitro testing, which can be used to identify doses that trigger molecular initial events within adverse outcome pathways. To bridge the gap between the dose of interest from in vitro assay, the dose within the target tissue, and the dose being exposed, physiologically based kinetic (PBK) models can be a powerful tool. In addition, PBK models possess the capability to integrate time-related factors into exposure-relevant or response-specific doses in risk assessment.

Disclaimer: The views expressed in this abstract are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency

Dr. Cecilia Tan is a senior science advisor at the Health Effects Division at the US. Environmental Protection Agency's Office of Pesticide Programs. Her main role is to review and apply pharmacokinetic data and models to improve the scientific basis for inter- and intra-species extrapolations in pesticide risk assessment. Before joining the Office of Pesticide Programs in 2018, Dr. Tan was a researcher at the EPA's Office of Research & Development. Her research involved using computational modeling to understand the quantitative relationships between external exposure, internal doses, and adverse outcomes. She is actively involved in several physiologically based kinetic (PBK) modeling-related committees to facilitate more applications of PBK modeling in regulatory risk assessment. Dr. Tan has a MS degree in Environmental Health Sciences from the Harvard School of Public Health, Ph.D. in Environmental Engineering and Sciences from the University of North Carolina, Chapel Hill, and MBA from North Carolina State University.

TK and TD as tools to support read across between chemicals and species

Aaron Redman, ExxonMobil, US

We present a framework for comparing different routes of exposure using TK and TD concepts and modeling tools based on the estimation of the concentration of freely dissolved (e.g., fraction unbound) chemicals. This approach provides a technical basis for quantitatively comparing the relative toxicity observed in aquatic test species (e.g., zebrafish), to that observed in rodent tests, and potentially other alternative methods. Typical aquatic tests apply constant exposure methods for acute and chronic endpoints, where the internal dose in the organisms is reasonably in equilibrium with the external exposures due to the small size of the test organisms. This approach has resulted in several hundreds of relatively high-quality toxicity data for more than 100 individual species and provides a basis for probabilistic estimation of acute and chronic thresholds. The exposure situation for rodent tests differs from aquatic test systems and therefore require PBPK models to estimate the internal dose from different routes of exposure characterized by the maximum concentration of the fraction unbound in the venous blood. The result of this approach provides a basis for comparing the relative sensitivity of rodent and aquatic test species and endpoints and provides a basis for describing the relative change in the toxicity thresholds against the exposure durations.

Dr. Redman has been engaged in research on fate and effects of chemicals for about 20 years, including development of toxicity models for UVCBs and application of probabilistic methods to develop risk-based toxicity thresholds. Recent work includes evaluation of TK data to characterize time dependent toxicity of hydrocarbons for aquatic and rodent species. Dr. Redman has worked at ExxonMobil Biomedical Sciences in New Jersey since 2011 and is actively engaged in scientific communications and collaboration efforts to develop data and tools to advance risk assessments https://orcid.org/0000-0002-5933-7906.

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