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TERUG NAAR INHOUD

Editorial

After a well-deserved summer break, we hereby present you the new edition of the TCDD. I hope that you all enjoyed the summer and its extension to the autumn.

During brainstorming sessions of the editorial team, we always try to think about what is essential for toxicologists, what we are struggling with, or even what is new and people would like to learn about it. For this edition, we decided to dedicate the special theme to the complicated task of choosing the relevant dose for toxicity studies. A lot of questions are raised about the proper choice of dosing *in vitro* but also *in vivo*. Are all studies relevant with their doses? Could we use certain approaches like Benchmark Approach? This question will be answered by Guangchao Chen. Another issue raised by Susana Proença is about the misunderstandings around dosage that would also be a cause for the lack of reproducibility and translatability. Additionally, the importance of the solvent used will be approached by Marcha Verheijen.

The months before the summer and September have been very busy for a lot of members with the NVT annual meeting, the workshop from the PREMIER/TransPharm projects, as well as symposia from sections Pharmaceutical Toxicology and Teratology & Reproduction Toxicology. These events provide opportunities for discussions and networking that we are all glad to be allowed to do!

I hope you all enjoy reading this edition,

Sincerely,

Héloïse

Photo by Jess Bailey on Unsplash

NVT Annual Meeting 2023

The PhD day of the NVT meeting kicked-off with the keynote lecture of Prof. Dr. Ivonne Rietjens (WUR) titled ‘PBK Modeling and QIVIVE in New Approach Methodologies (NAMs): Does One Size Fit All?’. The keynote lecture was followed by a break, which was combined with a poster session. The big salon of the Reehorst was filled with a variety of posters displaying all sorts of interesting and important research. The posters could be viewed during all breaks and participants were scheduled to present their poster to the jury during their allocated time slots. These sessions gave young scientists the chance to present their latest findings.

After the break we continued with the PhD/MSc speed presentation session, which were 3-4 minute presentations in which students highlighted their most interesting and remarkable findings. They were given by Job Berkhout (RIVM/UU), Lennart van Melis (UU), Maria Kloukinioti (Maastricht University), Jiayi Yang (UU), Danaëlla Lachat (Radboudumc), Kirsten Lassing (TNO/Radboudumc), Elise van der Koogh (UU), Jurriaan Varekamp (UMCG) and Meike Verheul (UU).

In the next session the participants attended one of the three workshops. During the ‘Graphs in R’ workshop, participants learned how to correctly format their data to use in ggplot2 and why it’s so important. They also gained a basic understanding of the Grammar of Graphics and how that is implemented in ggplot2. In the ‘Grant writing’ workshop, participants were introduced to the concept of grant planning and were provided with information on national and international funding possibilities, especially for early researchers. Next, participants learned how to master the necessary skills to write effective and compelling research stories, through the use of two writing templates and examples.

The third workshop was the ‘meet the expert’ workshop, where participants were divided into small groups and could have discussions with and ask questions to professionals in the toxicology field.

The last session of the day was the ‘inclusivity’ session. During this session, we discussed several questions, including ‘Why does inclusion oftentimes evoke such strong and different emotions in people?’ And ‘Why does inclusion oftentimes receive verbal support, yet very little action?’.

Afterwards, it was time for a reception with drinks and a dinner at the Reehorst. During the evening program a bowling competition was held at Bowlingcentrum Groeneveld.

The second day, the ‘members’ day, was opened with a keynote lecture by John Colbourne (Bham) titled ‘Toxicity by Descent: Using Phylogenetic Relationships to Predict Inter-Species Differences in Toxicity Pathways’. During this lecture, we got to know more about Precisiontox and how it employs six model species to uncover, as well as molecular toxicity pathways shared across the animal kingdom. After the break, it was up to four PhD candidates, Fabian Wagenaars (VU), Victor Amstutz (Maastricht

University), Lora-Sophie Gerber (UU), Deniz Bozdag (UU, in their third or final year to present their research during the PhD platform session.

Next, we had the first themed session about ‘Toxicology for all’. Jorke Kamstra (UU) started off with discussing IATA workflows as a basis for future chemical assessment, followed by Robert Landsiedel (BASF) addressing inhalation toxicity and choosing the right fit for different particle sizes and different assessment methods. Angela Maas (RUMC) ended the session with a talk on ‘how women make the difference in cardiology’.

The second themed session covered ‘new Approach Methodologies’ with Ad Ragas (RU) discussing modeling of risk diversity and the option to capture extremes into the models we currently have. Nick Beijer (RIVM) addressed the blind spots of implant safety: can *in vitro* or *in silico* models be used for the biological evaluation of implantable medical devices. Lastly, Christine Mummery (LUMC) showed the models based on hPSCs for cardiovascular disease and their use for understanding disease mechanisms and cardiotoxic effects of drugs.

The second and last day ended with an award ceremony. Hanna Marta Durza was awarded the 2023 Joep van den Bercken PhD. award for her dissertation ‘Contaminants of emerging concern in the fetal environment: unravelling the exposure and effects of endocrine disrupting compounds and micro(nano)plastics *in utero’*. She will present her research during the NVT of next year.

All posters and presentations were thoroughly judged by the NVT jury, which consisted of experts from academia, industry and regulatory agencies. As expected, the competition was high since a lot of valuable and novel research was presented. The people that were awarded for their presentations were: Deniz Bozdag (UU, PhD platform award), Joyce van der Heijden (Radboudumc, PhD poster prize), Kirsten Lassing (TNO/Radboudumc, MSc poster prize), Lennart van Melis (UU, PhD speed presentation) and Elise van der Koogh (UU, MSc speed presentation).

We look back on a great event with many great lectures, presentations, posters and social interactions. Once again, we would like to thank everyone who was, directly and indirectly, involved in participating and organizing the annual meeting of 2023. We’re looking forward to seeing you next year!

2023 organising committee - *Nathalie Dierichs, Irene Gosselink, Joyce van der Heijden, Viënna van de Laarschot, Julia Meerman, Kiri Romano Olmedo, Hans Bouwmeester, Laura Hondebrink, Juliette Legler, Yvonne Staal, Peter Theunissen.*

Spring symposium from NVT Sections Pharmaceutical Toxicology and Teratology & Reproduction Toxicology

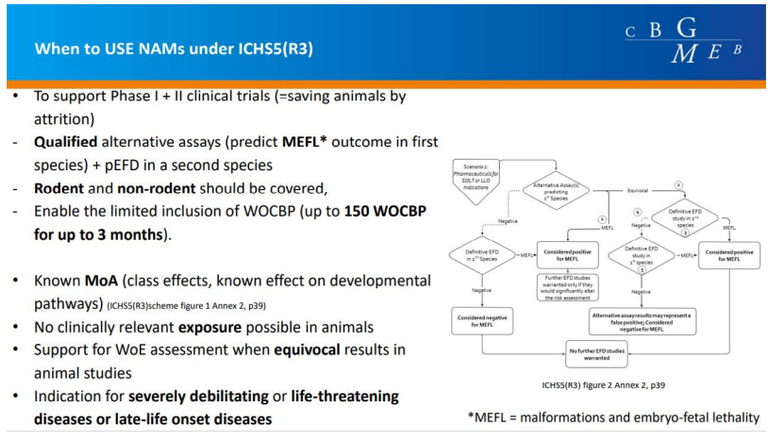
The NVT Sections Pharmaceutical Toxicology and Teratology & Reproduction Toxicology held on the 18th of April 18 2023 at the Leiden University a Spring co-symposium with the topic: "*Advances in reproductive & developmental toxicology testing With the emergence of a new and updated guideline (ICH S5 (R3)) on reproductive toxicity testing of pharmaceuticals*". The door has thereby been opened for alternative testing methods, to reduce animal use and improved scientifically based risk assessment. An overview of the opportunities, advances and challenges in this field, from a regulatory, academic and industry point of view were given during this symposium. The following presentations were given and most of these can be found on the member-login part of the NVT website:

- New regulatory opportunities in DART for pharmaceutical development Peter Theunissen, CBG (a written summary given below)
- ReproTracker and animal free developmental toxicity predictions Amer Jamalpoor, Toxys
- Development and application of *ex vivo* models for estimation of foetal exposure Rick Greupink, Radboud UMC (a written summary given below)
- Challenges from an industry perspective Nicola Powles-Glover, AstraZeneca

New regulatory opportunities in DART for pharmaceutical development; NAMs in DART testing under ICHS5(R3)

Peter Theunissen, CBG

New approach methods (NAMs) for embryo-foetal developmental (EFD) toxicity testing have been in development for more than three decades. For most of these NAMs the basis lies in whole embryo culture, larvae culture (zebrafish, C. elegans) or use of (induced) pluripotent animal/human derived stem cell differentiation. Pharmaceutical companies have been using NAMs mainly for in-house screening purposes and drug candidate selection. However, since 2020, the ICHS5(R3) harmonized guideline on detection of reproductive and developmental toxicity for human pharmaceuticals provides guidance on how to implement NAMs in a testing strategy to support clinical phase I and II trials and marketing authorization applications (MAA), including compounds with a known developmental toxic mode of action, and certain indications, such severely debilitating and life threating diseases or geriatric patients. Rather than endorsing or discussing specific assays or methodologies, the ICHS5(R3) guideline describes characteristics which NAMs designed to replace EFD toxicity testing should possess and identifies criteria that should be used to qualify the (battery of) assay(s) within a specific context of use. To assist the qualification, the guidance provides a reference list of compounds which are known to induce malformations and/or embryo foetal lethality (MEFL) in test animals and/or human. Furthermore, the guidance

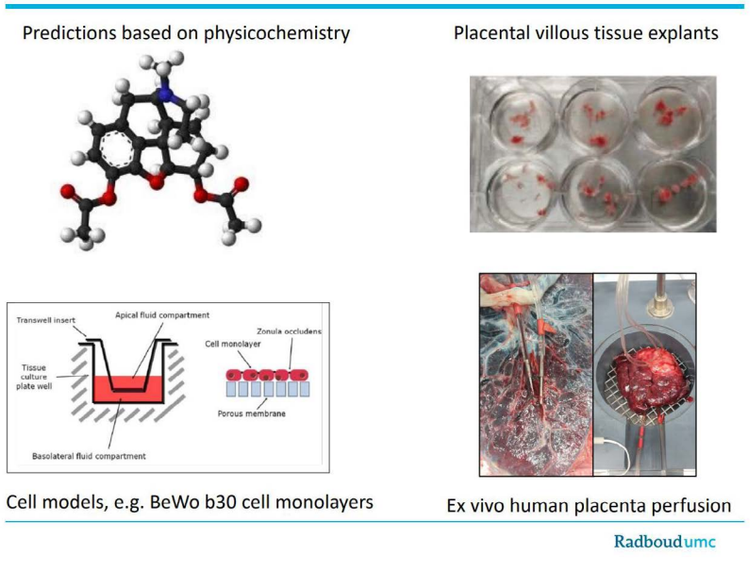


describes the circumstances under which alternative methods can be used. Yet, currently pharmaceutical companies remain reluctant to share use data from NAMs in a regulatory context for various reasons. However, regulatory agencies have a need to become familiar with NAMs data, to facilitate the incorporation of regulatory assessment of NAMs in the marketing authorization application process. Breaking this impasse should enable the use of NAMs to fulfil regulatory requirements of EFD toxicity testing. To facilitate this, at EMA there are a number of regulatory routes available. The first route is through the Innovative task force, which aims to increase the knowledge and experience in use of NAMs for regulatory purposes. In this process, assay developers can have free of charge informal talks with EMA experts and ESEC members to discuss proof of concept and possibilities for qualification. Qualification of NAMs through a qualification advice can then be a next step to establish qualification of NAMs for DART testing within a specific context of use.

Development and application of ex vivo models for estimation of foetal exposure

Rick Greupink, PharmD PhD, Division of Pharmacology and Toxicology, Department of Pharmacy, Radboud university medical centre, Nijmegen. Contact: rick.greupink@radboudumc.nl

The placenta plays a key role in maintaining a healthy pregnancy. In order to improve drug safety during pregnancy, it is therefore relevant to understand to which extent and at which rate drugs are transferred across the placenta and how pharmaceuticals may affect placental function. Pregnant women are not readily enrolled in clinical pharmacology trials, therefore data on drug efficacy and safety during pregnancy are scarce and/or become available only at a late stage after market introduction of a pharmaceutical. At Radboud university medical centre, a multidisciplinary research program dedicated to obstetric and paediatric pharmacology has been established. Embedded within this program, our research group conducts non-clinical and clinical studies which aim to improve drug safety and outcomes of pharmacotherapy during pregnancy by translating molecular-based knowledge of drug exposure and action to a clinical or risk assessment setting. Particularly the *ex vivo* dual-side human placenta perfusion technique and trans-well studies employing placental syncytiotrophoblast cell lines, are instrumental in establishing rate and extent of placental transfer in the laboratory. Next to conducting laboratory investigations, we explore how data from *in vitro* experiments may be combined with physiology-based pharmacokinetic (PBPK) modelling approaches to predict foetal exposure of drugs *in vivo*. This includes verifying model predictions with clinical data (e.g. via umbilical cord blood sampling in observational clinical studies or on a case-by-case basis). Next to pharmacokinetics, human placenta tissue can be used to study adverse effects of drugs on the level of the placenta. In this respect, placental villous explants can be cultured in the lab for up to several days, to allow relative long-term exposure with xenobiotics and study their effects on key aspects of placental function. In the presentation (slide deck available on NVT website member area), a few examples were highlighted that centred around the work we did on anti-retroviral agents and tyrosine kinase inhibitors. For future work, funding was secured to further develop *in vitro* and *in silico* placenta models that are relevant for human reproductive toxicology. This aims to contribute to New Approach Methodologies for reproductive toxicity testing, in order to help replace, reduce or refine animal experiments. An alternative and ongoing line of research in our group focusses on employing pregnancy-PBPK modelling to improve clinical drug dosing during pregnancy. Here we use a PBPK modelling approach to predict how pregnancy-induced changes in ADME may affect clinical pharmacokinetics and whether this warrants dose adjustments to optimize pharmacotherapy. If you are interested in the work presented, or have questions, please do not hesitate to contact us for further discussion.



Use of the benchmark dose approach for risk assessment

Author: Guangchao Chen, Afdeling Chemische Voedselveiligheid, Centrum Preventie, Leefstijl en Gezondheid, Rijksinstituut voor Volksgezondheid en Milieu (RIVM)

What is Benchmark dose (BMD) modelling?

In human health risk assessment of chemicals, the process of hazard characterization often involves analyzing data from toxicity studies (or in some cases epidemiological studies). The overall aim of this analysis is to derive a dose that can be used as a starting point for the risk assessment. This dose is referred to as the Reference Point [1,2] or Point of Departure [3]. The derived Reference Point forms a basis to establish a safe level of human intake of chemicals, i.e. the health-based guidance values such as the acceptable daily intakes for food additives and pesticide residues, and the tolerable daily intakes or tolerable weekly intakes for contaminants[1].

More than 30 years ago, the benchmark dose (BMD) approach was proposed to refine the estimate of the Reference Point [4]. This is due to some major shortcomings of the no-observed-adverse-effect level (NOAEL) method [1,2,5], which has been long used to derive Reference Points for risk assessment. As acknowledged by international agencies, including WHO, US-EPA, ECHA and EFSA, the BMD approach is a scientifically more advanced methodology as compared to the NOAEL [6-8,1].

In a BMD analysis, the overall dose-response relationship for a particular endpoint is analyzed using all the dose-response data at hand. The analysis yields a dose-response curve and an estimate of the BMD, together with its one-sided 95% confidence interval, whose lower bound (BMDL) serves as the Reference Point for risk assessment. The BMD is the dose associated with a predetermined change in response, usually 1-10%, termed the benchmark response (BMR). As an example, a 10% change in response would be termed BMD10 while its lower 95% confidence interval would be termed the BMDL10. See Figure 1 for the illustration of these key concepts.

A BMD analysis can be performed in available software tools[3,9], which have been made user friendly over the years, making computational skills in the user unnecessary. Nevertheless, some conceptual understanding will help the toxicologists and risk assessors in deriving the BMD(L), and these will be given below. The discussion here will focus on continuous toxicological endpoints, however, some of the issues discussed are also relevant for quantal endpoints.

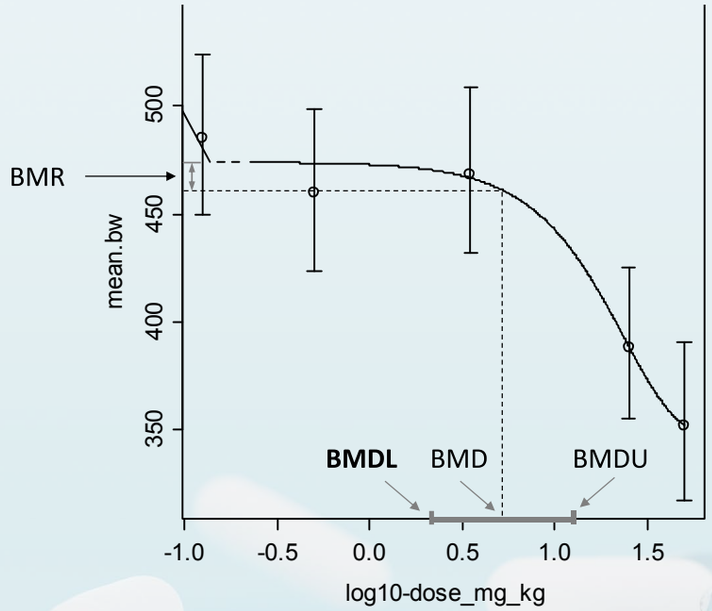


Figure 1. Illustration of key concepts of the benchmark dose (BMD) approach, taking the continuous endpoint body weight decrease as an example. The benchmark response (BMR) is defined as the change in response relative to the background response. The 95% confidence lower and upper confidence intervals termed BMDL and BMDU, respectively.

Why should we not use the NOAEL as the Reference Point for risk assessment?

The use of the NOAEL in deriving Reference Points comes with major disadvantages, such as:

- A NOAEL is often mistaken for a dose without effects. Nevertheless, a dose at which an effect was not observed in an experiment should not lead to the conclusion that there is no effect at that dose (not seen does not mean non-existent). The smaller the effect, the more experimental units (e.g., test animals) are needed to observe that effect. Therefore, zero effects are beyond (experimental) science;
- The NOAEL strongly depends on experimental setup, e.g. choice of doses, dose spacing, group size. For example, with fewer animals it is less likely to detect an existing effect;
- The uncertainty in a NOAEL is not made visible, and is simply ignored in practice;

The change of biological effect (relative to the background) at a NOAEL remains unknown (is it protective?). And thus, NOAELs cannot be compared among studies or study groups.

The NOAEL approach does not use all the dose-response information available. Both very rich datasets and very poor datasets result in one single value, without acknowledging the quality of the data. This hinders the refinement of study designs.

However, a recurring point of discussion is what to do if a BMD analysis results in a very wide BMD confidence interval, so that using the associated BMDL as an Reference Point appears unwarranted. One popular argument is to fall back to the NOAEL approach [6,2], stating that a large BMD confidence interval means that the BMD is highly uncertain, making the BMDL unsuitable as a Reference Point. However, this argument is not valid. When

the BMD is found to be highly uncertain, the uncertainty in the NOAEL will be even larger. This is because a large uncertainty in the BMD is caused by the limitations of the specific dataset (and not by the BMD approach). The BMD approach can make those limitations in the data visible (by the BMD confidence interval) while the NOAEL approach cannot. Thus, if the data are so poor that the derived BMD confidence interval is very uncertain, then the data itself are just not of sufficient quality to serve as a basis for risk assessment. The NOAEL approach ignores that, and simply results in a value, without indicating that this value is unreliable.

Instead of resorting to the NOAEL, which hides the problem, the answer to the problem of poor data could be to repeat the study with a better study design, which is in practice often not realistic. In that case, one option is to simply use the BMDL, even if it is unrealistically low. Possibly even with that low Reference Point, the exposure might be known to be even lower, so that there is no reason for concern. The other solution is to extend the BMD analysis by using information from historical dose-response data. There are two options for doing this. One is to combine the poor dataset with other datasets on the same endpoint, available for other chemicals tested in the past. The BMD approach allows for taking differences among chemicals into account by the so-called covariate approach[1]. This method usually results in considerably smaller BMD confidence intervals [10]. Another approach relies on Bayesian methods, which aims to comprise historical information of dose-response shapes in so-called prior distributions. The latter methodology is being developed in parallel by EFSA and US-EPA [2,3]. However, it may take some time before reliable prior distributions are available to make the Bayesian method a useful approach.

BMD modeling allows for including general toxicological assumptions

One of the strengths of BMD modeling is that it can be designed such that it is in line with key toxicological assumptions and requirements. One key assumption in risk assessment is that, when extrapolating from the Reference Point to an equipotent human dose, the ratio of BMDs among groups (e.g. animals vs. humans, average human vs. sensitive human) does not change when changing the BMR. This means for example that whether the BMR for the reduction in body weight is 5 % or is 10%, the ratio of the BMDs stays the same (e.g. the interspecies assessment factor does not change with the BMR). This key assumption is true if dose-response relationships are parallel on log-dose scale. In general, toxicological data do indeed show that property, at least when considering the same endpoint [10]. To be sure of parallel dose-response relationships on log-dose scale, the BMD modelling requires models such that changing the potency/ sensitivity parameter (the BMD) only shifts the dose-response relationship horizontally (see more explanation below, and Figure 2 upper right panel). In that case, the BMD ratio does not depend on the BMR. To further illustrate how toxicological assumptions should dictate the development of suitable dose-response modeling, we briefly discuss three principles relevant for the dose-response modeling of continuous endpoints.

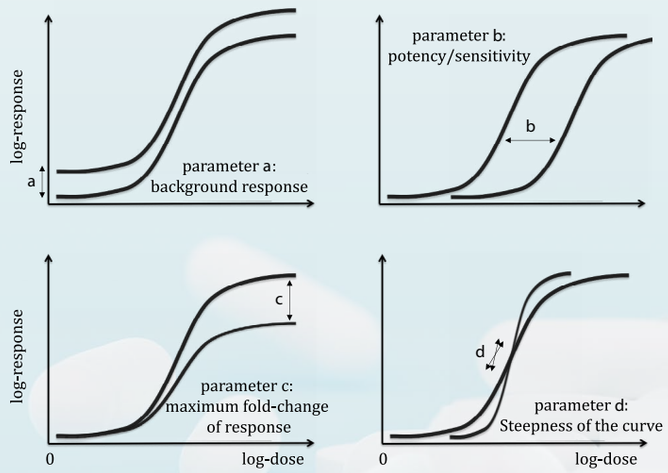


Figure 2. The four canonical parameters a, b (or equivalently the BMD), c, and d of the dose-response models and their interpretation for continuous endpoints. The arrows indicate how the curve would change when changing the respective parameter.

Principle I - canonical parameters

A dose-response model should reflect the essential properties of a dose-response relationship [1]: the response at dose zero (control response), the dose corresponding to a given response level (potency of the chemical or sensitivity of the test population), the steepness of the curve, and the maximum fold-change of response (i.e. the ratio between the maximum response and the background response). Therefore, the first principle is that a dose-response model should contain the following four "canonical" parameters:

- the background response parameter (parameter a);
- the potency parameter (parameter b, or equivalently BMD);
- the parameter for the maximum fold-change of response (parameter c);
- the steepness parameter, where steepness relates to log-dose (parameter d).

Figure 2 illustrates how these canonical parameters can change the curve.

Principle II - canonical models

On top of the parameters, another aspect is the model expression. The second principle of BMD modelling is based on the biological assumption that biological effects work multiplicatively [10].This is to say that, the biological response induced by dose (and by other factors in the experiments) is proportional to the background response, rather than additive to the background response. This assumption implies that a dose-response model should have the expression:

$$y = a \cdot c^{\mathrm{F}(x)} \qquad (1)$$

where *y* stands for the continuous biological response, *x* the dose, and F(x) is a function of *b* (or *BMD*) and *d* that increases from 0 to 1 (with dose *x* from 0 to infinity). Models that have the expression (1) and the four canonical parameters are called

canonical models. The canonical models currently in use in the PROAST software[9] are shown in Table 1.

Table 1. Canonical models currently used in BMD modelling (PROAST software)

Model	Formula
Exponential model	$y = a \cdot c^{1-e^{-(\frac{x}{b})^d}}$
Hill model	$y = a \cdot c^{\frac{x^d}{b^d+x^d}}$
Inverse Exponential model	$y = a \cdot c^{e^{-(\frac{x}{b})^{-d}}}$
Log-Normal model	$y = a \cdot c^{\Phi(\ln b + d \cdot \ln x)}$

Principle III - Internal consistency in modeling

Generally, in good modeling practice the assumptions made should be internally consistent and not conflicting with each other. The general assumption of biological effects working multiplicatively implies that the within-dose group standard deviations will be proportional to the associated means. This assumption also indicates that, for each dose group, the scatter around the mean will be lognormally distributed. Because the scatter is caused by other unintended factors in the experiments which also act multiplicatively on the effect, just like the dose. Therefore, using model expression (1) is internally consistent with an assumed lognormal distribution for the within-dose groups scatter of continuous endpoints. Similarly, the definition of the BMR should be consistent with the choice of expression (1) and the lognormal distribution, i.e., it should be defined as a percent change relative to background, and not as an absolute difference. While internal consistency in the assumptions underlying a scientific theory or methodology is an evident requirement, it is often overlooked in practice.

A recent review showed that the approaches in the current BMD guidances of US-EPA [7,3] and the latest EFSA BMD guidance (2022) deviate from one or more of these three principles [2]. This is a rather unfortunate development, that may lead to anomalous Reference Points in some cases. For this reason, we advocate applying the BMD methodology that is in line with the three aforementioned principles. These principles are essential for the toxicological soundness and transparency of the methodology, and help avoiding misleading or incorrect results, while toxicologists will (better) understand their BMD analyses. Readers are welcome to contact the author for further discussions.

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Good methodology reporting is essential to understand the dose *in vitro*

By *Susana Proença*

Research, particularly in psychology and biomedicine, faces growing concerns over reproducibility and the challenge of translating *in vitro* and animal findings to real-life scenarios. These issues are highlighted in mainstream media, including the book “Rigor Mortis: How Sloppy Science Creates Worthless Cures, Crushes Hope, and Wastes Billions”. While lack of reproducibility and translatability is hardly an issue that is specific to *in vitro* methods, the push to use *in vitro* models to replace animal testing has the most to lose since these issues sow seeds of skepticism. Data fabrication, statistical inconsistencies, and cell line errors are among several causes. Here, I argue that misunderstandings around dosage are also a cause for the lack of reproducibility and translatability.

In 2015, I embarked on my master’s thesis. My initial assignment involved reviewing literature to assess the distinctions between 3D and 2D hepatic models. There was, and still is, competition to develop the most realistic *in vitro* hepatic model. Cultivating cells within a 3D structure is a technique to enhance the hepatic phenotype. Typically, these emerging models are gauged against 2D counterparts by subjecting them to specific hepatotoxicants and comparing the resultant dose-response curves. As part of my review, I collated dose-response curves from various research papers.

Deciphering these dose-response curves turned out to be more challenging than I thought. Often, the differences in dose-response curves for identical chemicals were more pronounced across different studies than between the 2D and 3D hepatic models themselves. For one particular chemical, the IC_{50}s—whether derived from 2D or 3D studies—varied drastically. Upon querying the authors of one study, they mentioned their use of 10% FBS in their cell culture medium, speculating that other researchers might not have incorporated FBS. This revelation triggered my interest, leading me to delve into toxicokinetics. I was taken aback by how significantly serum could alter the chemical availability and by the realization that the quantity of cells used could also influence results, especially with lipophilic chemicals like amiodarone.

Armed with this newfound insight, I endeavored to decipher the tables of IC_{50}s and IC_{20}s, taking into account the specifics of the *in vitro* conditions. Regrettably, much of this pivotal information was absent in the published methodologies—a frustration familiar to anyone who has attempted to replicate an experiment from existing literature. Whether due to concerns over competition or a lack of awareness about the importance of transparent methodologies, many crucial details necessary for experiment replication are omitted. Key aspects, such as the medium volume, cell count, and the presence of serum or albumin in the culture medium, are essential to retroactively deduce differences in test chemical availability. This journey made me reflect: if drawing comparisons between *in vitro* methods proved this challenging, how can we confidently use these models for predicting *in vivo* scenarios?

Throughout my career, I’ve been driven by the quest to better understand dosing in various systems and to determine the effective dose. My journey has taken me from a traineeship at ECVAM’s Joint Research Center in Italy, where I learned to model *in vitro* kinetics, to my PhD work at IRAS, Utrecht University, where I sought to measure these kinetics. Later, during my brief stint with the ONTOX project, I collaborated with a team focused on PBK models, aiming to devise strategies for quantitative *in vitro* to *in vivo* extrapolation. This growing passion for mastering PBK models and QIVIVE has now led me to my next venture at esqLABS, where I’m excited to begin work next month.

Throughout my career, I’ve dedicated significant time to advocating for sound methodologies and robust experiment design. Yet, I’m optimistic, seeing advancements in the field. Within ONTOX, there’s a commendable emphasis on detailed method reporting, especially for *in vitro* methods, exemplified by the ToxTemp template[1]. I highlight this as a positive example and appeal to journal editors and all involved in the peer review process: ensure research papers detail complete methodologies to allow for study reproduction and always provide raw data. Clear, rigorous methodologies guarantee research reliability, replicability, and validity, thereby laying a dependable foundation for future explorations and insights.

Author note: To lead by example, this commentary, although personal, was revised by chatGPT4.

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Solvent Selection in Experimental Design: A Critical Factor

In most toxicological experiments, subjects or samples are exposed to a compound of interest. For some fields of research, such as environmental or dietary exposure, the exposure occurs naturally. However, many compounds are tested using specified experimental designs in which one or more specified doses are investigated and compared to a control condition. In these cases, compounds are usually dissolved in a stock solution and/or diluted before use. The solution used for this purpose is known as a solvent or vehicle and should be carefully selected based on the properties of the compound (polarity, solubility, stability), the route of administration, the final intended dose and possible confounding effects of the solvent. Especially for *in vitro* exposures, where ethical considerations play a lesser role, the possible confounding solvent effects are often overlooked because researchers tend to stick to conventional procedures.

By *Marcha Verheijen*

In the old days, high compound doses were used to be able to see toxic effects with the techniques that were available back then. Present day, techniques have become very sensitive and capable of identifying minor changes even at therapeutic doses or lower. That is also why the consideration of the applied solvent (type and concentration) should be carefully considered.
Dimethyl sulfoxide (DMSO) is a commonly used solvent in toxicological *in vitro* studies and a great example for this story[1]. DMSO is widely applicable because it can be used for poorly soluble polar or non-polar molecules. A commonly used “low” dose of DMSO is 0.1%, but do researchers realize that this is actually 14.1 mM[2]? Often this means that the DMSO dose is higher than the dose of the tested compound, which is usually in µM range or even lower.

It has been reported that DMSO is nontoxic below 10% (v/v) and, in practice, the impact of DMSO is typically regarded as negligible[3-5]. But nontoxic is not the same as negligible. Interestingly, DMSO was actively researched for medical applications (anti-inflammatory, analgesia, diuretic, vasodilation and muscle relaxation[6]), but this was halted in 1965 due to adverse effects. While DMSO effects are dose dependent, this clearly demonstrates that DMSO is not inert. Furthermore, one may argue that data can be corrected for vehicle effects by using a vehicle control. That is absolutely true, if it is done properly. This correction becomes an issue when the vehicle has an effect on processes that are involved in compound effects. For example, inflammation induced by a compound may not be detectable if DMSO has anti-inflammatory responses in the same system. It has also been found that DMSO may impact epigenetic regulation in some cell systems[1]. Then correction for vehicle effects becomes more complex.

While DMSO was used as an example in this story, the same reasoning also applies to other solvents. While the use of solvents cannot always be avoided, researchers can pay attention to the type of solvent that is used and make sure the concentrations are as low as possible. Furthermore, to be able to correct for vehicle effects, the researcher needs to know what the vehicle actually does. One way to approach this is by including an untreated control (without compound and solvent) in the experimental design. In that way, one can always verify whether obtained results are influenced by the compound, the vehicle or both.

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Microneedles, the Future of Drug Delivery?

Drug delivery is an important part of drug development that is gaining increasing attention

Microneedles, as the name implies, are micrometer-scale needles that are loaded with an active pharmaceutical ingredient (API) that is then injected at the site of delivery. Due to their size they're less painful[1] than traditional hypodermic needles and due to the smaller pores crated, are less likely to cause infection[2]. Microneedles vary in designs. They can be made of a single needle or an array of microneedles and can be applied as a patch or even as a pill.

By *Barae Jomaa*

The dosage form (e.g. pills, drops, aerosols) and its route of administration (e.g. oral, nasal, intravenous) together form the basis of drug delivery. Drug delivery devices include, among other, syringes, nasal sprays, eyedroppers and more recently microneedles. Another term that is used for microneedles is drug delivery system which is defined as DDS, a "formulation or device that delivers an API in site-directed applications or provides timely (i.e., immediate, delayed, or sustained) release of the API. The system, on its own, is not pharmaceutically active, but improves the efficacy and/or safety of an API that it carries." This more general term therefore covers drug delivery devices and excipients such as adjuvants, preservatives and drug carriers/vehicles. Preferably a DDS would include both the drug delivery device and the relevant excipient aimed at improving safety/efficacy.

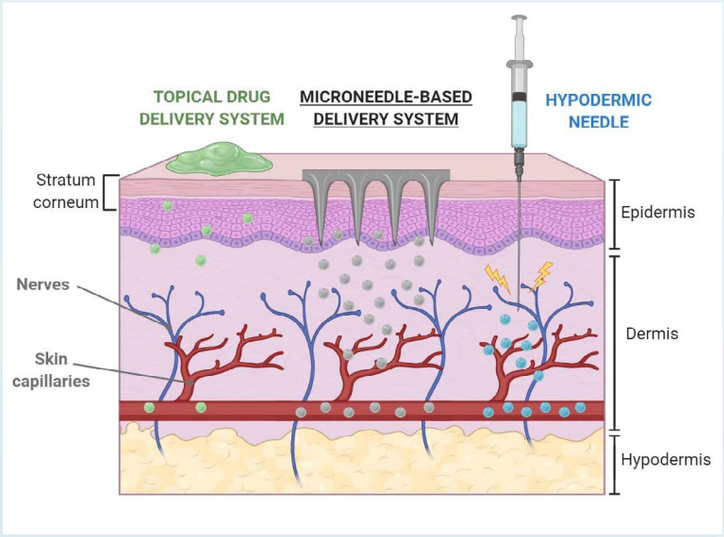

Figure 1: Microneedle delivery system compared to a hypodermic needle and topical drug delivery system. Image attributed to Guillot et al., 2020[3] and reproduced under the creative commons license 4.0.

The two main types of microneedles are solid microneedles and hollow microneedles. Solid microneedles pre-treat the skin by forming pores then a patch coated with the drug is applied. Alternatively, the solid microneedle may be coated with the drug. Hollow microneedles on the other hand, have a core containing the drug that is released into the skin upon penetration, allowing for quick absorption. Hollow microneedles may also be made of biodegradable polymers and would then be named dissolving microneedles[4].

As witnessed during the COVID-19 pandemic, drug delivery systems are key to a vaccine's success. Since mRNAs are large molecules, they cannot enter cells on their own and degrade in biological fluids. This is where lipid nanoparticles (LNPs) played an essential role to make mRNA vaccines a reality[5]. LNPs were found to be the most successful drug delivery system that enables the delivery of mRNAs into cells by endocytosis and eventual release of the mRNA into the cytosol and ribosome. The lower level of expertise needed to deliver a microneedle patch as compared to hypodermic needles means that rolling out vaccines would be quicker in upcoming pandemics[5]. Moving this technology even further, researchers have recently developed an automated microneedle vaccine printer that can manufacture thermostable COVID-19 mRNA vaccines in a microneedle patch[6].

Microneedles are also being explored as part of a drug delivery system for melanoma treatment. A research team from the Wyss Institute, MIT, and Brigham and Women's Hospital has developed a locally applied immunotherapy that combines focused ultrasound (FUS) with nanoparticle-bound activators that stimulate immune responses. Furthermore, the researchers created a minimally invasive microneedle platform, capable of absorbing biomarker-containing fluid from deep skin layers. This platform serves a dual purpose, allowing drug delivery and biomarker detection simultaneously[7].

One of the more creative uses of microneedles has been developed by researchers at MIT and Massachusetts General Hospital (MGH). They have designed a novel drug capsule

coated with microneedles that can inject drugs directly into the stomach lining after being swallowed[8]. This innovation could offer an alternative to traditional injections, especially for large protein-based drugs that cannot be administered orally due to degradation in the stomach.

While the applications seem limited only by the extent of our imagination, there are some potential disadvantages to microneedles. For one, hollow and coated microneedles might not be very effective in delivering the drug and may even leak onto the skin when damaged, potentially during application[9]. Another concern is that microneedles are more fragile than hypodermic needles and may break, leaving fragments in the skin that may cause irritation[10]

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Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE)

By Jochem Louisse

Disclaimer: The views and opinions expressed in this article are those of the authors and do not necessarily reflect the views or positions of any entities they represent.

Hazard and risk assessment of chemicals is typically based on data from toxicity studies performed on laboratory animals, thereby providing information on the dose-dependent adverse effects of chemicals in test species, based on which safe exposure levels in humans are derived. Already for decades, various research initiatives have aimed to make a paradigm shift happen to perform human hazard and risk assessment of chemicals based on non-animal, human-relevant methods (also called novel approach methods (NAMs)), forming the basis for a 'next generation risk assessment' (e.g., Carmichael et al., 2022).

The development of human cell-based (*in vitro*) test systems is rapidly evolving, and *in vitro* models are available with different complexities, from simple 2-D monolayer cell-based test systems to complex (stem) cell-based 3D structures, including microphysiological systems. Irrespective of the complexity of the (*in vitro*) model, if such models are aimed to be used for hazard and risk assessment, the information obtained on concentration-dependent toxicity in the *in vitro* test system needs to be translated to an *in vivo* potency that can be used to derive a point of departure for the risk assessment (e.g., Blaauboer et al., 2012; Yoon et al., 2015; Louisse et al., 2017). For systemic toxicity endpoints, various examples of such translations are available in the literature, in which *in vitro* potency information has been translated to *in vivo* potency information, usually referred to as quantitative *in vitro* to *in vivo* extrapolation (QIVIVE). With QIVIVE, *in vitro* effect concentrations are translated to *in vivo* effect doses by so-called physiologically based kinetic (PBK) modelling-facilitated reverse dosimetry. A PBK model describes the ADME processes in a species of interest and allows to relate information on external exposure to internal exposure, such as concentrations in blood and/or in a target tissue. The external (oral) dose that relates to an internal concentration associated with an effect in an *in vitro* test system of interest is often referred to as (oral) equivalent dose.

Pioneering work on QIVIVE has been done in the Netherlands. The first publication in this regard is from DeJongh and coworkers, who translated concentrations related to neurotoxic effects in cells to oral equivalent doses using PBK modelling (DeJongh et al., 1999). In a next study, Verwei and coworkers translated effect concentrations obtained in an *in vitro* model that assesses chemical-induced disturbance of embryonic stem cell differentiation to oral equivalent doses (Verwei et al., 2006). Both studies compared obtained oral equivalent doses in test species with NOAEL/LOAEL values reported for the test items, showing for most cases a good concordance. After that work, more examples have become available for diverse

toxicity endpoints, providing proofs-of-principle that with QIVIVE, data obtained from *in vitro* test systems have the potential to be used to derive points of departure for the hazard and risk assessment of chemicals (e.g., Louisse et al., 2012; Strikwold et al., 2013). Currently, PBK modelling-based QIVIVE is often applied in research projects that aim to develop next generation risk assessment approaches based on NAMs. Although the science progresses, QIVIVE approaches are so far only limitedly/not yet applied in regulatory risk assessments.

There may still be several hurdles to overcome to allow general application of these approaches in risk assessment. These include the selection of relevant *in vitro* test systems and related read-outs for which *in vitro* effect concentrations are to be extrapolated to external doses. From that regard, the developments in the field of (quantitative) adverse outcome pathways may be important to select those test systems with related endpoints/readouts that are considered most relevant for setting points of departure. Another challenge is to determine and select the *in vitro* dose metric to relate the *in vitro* effect concentration to an internal dose (Cmax, AUC (what timeframe?), free or tissue concentration, etc.) to be used for the QIVIVE (Groothuis et al., 2015). Another important aspect to consider is that for the majority of chemicals, availability of kinetic data, let alone *in vivo* kinetic data, required for PBK model parameterization, is limited. In case PBK models are parameterized based on data obtained from *in vitro* and *in silico* methods, more insight into the uncertainty of such PBK model predictions is required to allow for regulatory application (Punt et al. 2022a,b). Furthermore, guidance/guideline documents for *in vitro* kinetic studies are needed to obtain robust *in vitro*-based PBK model input parameter values, for example related to hepatic clearance (Louisse et al., 2020), which is expected to lead to more robust PBK model predictions (Punt et al. 2023).

Regardless of the hurdles, PBK modelling-based QIVIVE is considered to be an important aspect of next generation risk assessment (see e.g., Luijten et al., 2020), allowing a quantitative interpretation of *in vitro* data in the hazard and risk assessment of chemicals.

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The PREMIER and TransPharm workshop:

The transition towards sustainable pharmaceuticals – assessing sustainability, from design & production to prescription & use

Adapted from the newsletter of PREMIER/TransPharm by Héloïse Proquin (RIVM) and Caroline Moermond (RIVM)

Objectives

TransPharm[1] is a Horizon Europe project about transforming into a sustainable European pharmaceutical sector, coordinated by Ghent University in Belgium. It aims to move towards a sustainable pharmaceutical industry and to improve the European readiness for sustainable production of small molecules (active pharmaceutical ingredients, APIs) that have environmental footprint concerns. To reach the envisaged aims, the project will deliver four toolboxes for the development of greener pharmaceutical products and APIs, with the aim of analysing and predicting flow behaviour and environmental biodegradability and ecotoxicity of APIs and their synthesis pathways, identifying greener and more sustainable alternatives to pharmaceutical products / APIs of concern, reducing the footprint in synthetic schemes of APIs, and assessing the sustainability of pharmaceuticals over their entire life cycle.

The PREMIER project[2] is an IHI project, where the non-industry part of the consortium is financed by the European Commission and pharmaceutical industry contributes an equal amount. The PREMIER project aims to design a database and assessment system for identifying and addressing environmental risks of medicines, especially for those with limited data availability. Besides this, options are explored to incorporate environmental considerations earlier in the drug development process to steer the development of medicines in a greener direction.

These projects joined forces into a combined workshop on sustainable pharmaceuticals.

Workshop

What is a sustainable pharmaceutical product? Can sustainability considerations be included in the discovery/design process of new active pharmaceutical ingredients (APIs)? And what information needs does the health sector have when it comes to the sustainability of pharmaceuticals?

These are some of the questions discussed during the PREMIER/TransPharm workshop on 4-6 April in Nijmegen, the Netherlands. The workshop brought together a unique range of experts and professionals representing different stakeholders involved in the discovery, production, prescription, use and regulation of pharmaceuticals with around 100 participants. During interactive small group sessions, the following topics were discussed in depth:

1. *Moving towards the design of APIs with less impact on the environment after use – Needs of actors in drug R&D and the health care sector*

It was first discussed what a green API is. Different answers showed different ways of approaching this (e.g., regarding biodegradability, metabolites/by-products, toxicity in the environment, but also carbon footprint and process intensification). After this, the main needs were identified which need to be met in order to implement/stimulate use of green APIs. The most important aspects were green procurement, education and awareness of prescribers (and others) to prescribe green APIs, criteria to define/assess green APIs and data to do so, R&D and development of green APIs. Incentives and regulation are needed to create push and green market access.
Identifying and elaborating on priority needs showed that e.g. factoring the environmental impact into the price, a cultural/behavioural change, stakeholder engagements, and funding are key to solve the identified needs.

2. *Environmental criteria for APIs to reduce their impact after use*

It was concluded that it is possible to take up environmental considerations in (early) drug R&D, but there are also some challenges. The most important condition is that criteria, tools

and cut-off values are clear. They must be robust and reliable to allow stop/go decisions to be made. Which topic(s) these criteria are about is of lesser importance to R&D specialists. Most of the screening should be performed *in silico*, early in the R&D process.

3. *Implementation of environmental criteria in drug discovery and design of small molecules*

Implementation seems to be possible, but criteria should be well-defined and have a clear relation with actual environmental hazards and risks. R&D needs a toolbox with easy to use, simple, meaningful assays, which are high throughput and fit within the appropriate stages of R&D. The test endpoints should be selective and specific, with clear cut-off values. There also needs to be a system for ranking/weighing of criteria, against each other but also against other R&D criteria. Off-target effects identified in drug R&D may help to identify which environmental organisms may be at risks. The field of bioaccumulation may be the easiest area to start to work together, as this is usually not a good property for patients as well as the environment. Tools to assess bioconcentration in the environment are well developed and may be of use in early pre-clinical development. Successful cooperation between R&D experts and environmental scientists to provide such assays could stimulate further progress.

4. *Criteria for greener production of pharmaceutical products – Is there a business case?*

A Mentimeter quiz was organized on the topic "Is there a business case for green pharmaceuticals?". It consisted of 8 questions. A few responses are summarized below.

- A large majority of the respondents (82-88%) thinks that a greener pharmaceutical product does not have to outperform conventional pharmaceutical products in other areas, e.g. clinical efficacy, safety for the patient (acceptable adverse effects), convenience of use, secure drug supply or shelf life.
- The respondents consider all environmental impacts related to pharmaceutical products more or less equally important (i.e., impacts of production and impacts after use).
- The respondents identified a lack of guidelines on what constitutes a greener API as the most important barrier to replace current APIs by greener ones, followed by (examples of) greener APIs and reducing the uncertainty on the reimbursement of costs/investments.
- The majority of the respondents (22 out of 36) thinks that the production of greener APIs and products should also be possible outside Europe.
- A large majority (67%) of the respondents thinks that a business case should also be possible if not all stakeholders are on board yet.

5. *Sustainability assessment of pharmaceutical products – The need for a sustainability assessment system and the criteria to consider when designing such a system, as well as opportunities for education and awareness raising.*

Participants discussed the question for whom and why **mapping the life cycle of pharmaceuticals and designing an assessment system** is needed. Participants found that sustainability assessment in daily work is complex, with many interconnections between actors and processes. The assessment should preferably be harmonized, resulting in one evaluation result that can be used by all members of the product chain, such as health insurance companies, clinicians, etc. It should lead to appropriate drug use. Unintended consequences should be minimalized (e.g., less access to medicines).

Conclusions

Although there was a consensus on the urgency for sustainable pharmaceuticals, it is generally seen as a complex task. Communication and inclusion of the different disciplines will be key, in order to align the goals and vision, and to share knowledge and experiences. This workshop marked the start of bringing these disciplines together.

"It is a mammoth task to develop a system that measures all aspects of sustainability", concluded host Ad Ragas at the end of the workshop. "But let's not wait until we have perfect criteria on what constitutes a sustainable pharmaceutical. That discussion might take ages. We should move forwards and start experimenting. We will make mistakes, but we will learn from them. We need the courage to experiment and fail on our path to create more sustainable pharmaceuticals."

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Artificial Sweeteners Facing Further Scrutiny

By *Barae Jomaa*

Splenda, a leading artificial sweetener, is under scrutiny due to concerns over potential health risks. Research suggests that sucralose, the key component of Splenda, may transform into a compound called sucralose-6-acetate within the human body. Sucralose-6-acetate has also been found as an impurity in commercial samples at levels up to 0.67%. A study led by Susan Schiffman[1] showed that this compound is genotoxic, could also harm the lining of the intestine and trigger inflammation, oxidative stress, and cancer-related gene expression.

Despite these findings, experts caution against jumping to conclusions, emphasizing that further research is needed to understand the practical health effects of consuming sucralose-sweetened products.

Artificial sweeteners, including Splenda, have faced scrutiny for years, with studies suggesting links to health risks. For instance, combining sucralose with carbohydrates was found to elevate blood sugar levels, and the World Health Organization discouraged using artificial sweeteners for weight control due to concerns about diabetes, cardiovascular diseases, and mortality risks. Health experts recommend considering healthier, low- or no-sugar alternatives, such as seltzer water and fruits with natural sugars, accompanied by fiber and phytonutrients, over highly processed sweet foods and drinks.

According to a news report by Reuters on the 29th of June, the International Agency for Research on Cancer (IARC), part of the World Health Organization (WHO), is considering labeling aspartame as “possibly carcinogenic to humans,” causing a stir in the food industry and regulatory circles. Aspartame, a widely-used artificial sweetener, is found in various products. This potential classification by the IARC is based on an evaluation of evidence related to aspartame’s potential cancer risks, without considering safe consumption levels. This has raised concerns about consumer confusion. The Joint WHO and Food and Agriculture Organization’s Expert Committee on Food Additives (JECFA) has long deemed aspartame safe within accepted daily limits, a stance shared by national regulators.

The IARC’s classification system focuses on evidence strength rather than inherent danger, with four levels: carcinogenic, probably carcinogenic, possibly carcinogenic, and not classifiable. Aspartame’s potential “possibly carcinogenic” label places it in a category with limited but suggestive evidence. Critics, including the International Sweeteners Association (ISA), have criticized the IARC’s review as scientifically inadequate.

Previous studies on aspartame’s safety have produced mixed results, with some suggesting a slightly higher cancer risk but failing to establish causation. Questions have also been raised about the methodology of studies linking aspartame to cancer in rodents. Despite these studies, regulatory bodies worldwide have approved aspartame for use following extensive reviews of available evidence, and major food and beverage companies have defended its use.

The IARC’s consideration of aspartame as a possible carcinogen aims to encourage further research, providing a more comprehensive understanding of its potential risks. However, it is also expected to reignite debates over the IARC’s role and the safety of sweeteners.

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AIO toxafette - Tim Somers

In the toxafette, PhD-students working in the toxicology field get the chance to share their experiences in performing research. Every issue a new candidate answers a series of questions, and then pass the baton to a fellow PhD-student. This time Tim Somers, from Radboud University in Nijmegen tells us about his project.

Can you introduce yourself?
Hello, my name is Tim Somers. I am born and raised in the beautiful city of Nijmegen. I do not have the classical background as many of my precedents in the toxafette. I studied medicine at the Radboud University. After graduation, I started working in the hospital as doctor at the department of Cardiothoracic surgery (heart and lung operations). This was the place where I built and studied several clinical databases during my masters. As research continued to interest me and I like to be challenged, I explored the possibilities of conducting PhD research. Since databases lost my curiosity and I dislike computer work all day, an ideal experimental project at the department of Pharmacology and Toxicology crossed my path. Within this project I study the metabolic effects of statins on the heart. Besides spending lots of time in the hospital and the labs, I like to participate in and watch all kinds of sports. I have been swimming for a long time, but currently more into cycling, running, and walking through the green and hilly surroundings of Nijmegen. In order to do all these sports, I love good food with good company.

How would you explain the subject of your research to a layperson?
Statins are the world's most used cholesterol-lowering drugs. Many people, especially after a heart attack or stroke, use them to prevent new episodes of cardiovascular diseases – the world's number one cause of death. Although statins have shown to be extremely effective, there are some side-effects. Muscle pains are the most reported. For long it was unknown how statins caused these muscle complaints. Our group unraveled that mitochondria play an important role. Mitochondria are the powerhouses of our cells. Their function is depressed by statins, making cells struggle for sufficient energy. The result is an increased production of lactic acid, leading to muscle cramps. This is comparable to what you feel after very heavy/strenuous exercise. We were curious how statins affect the heart, since nobody complains of chest pains and our heart is the most important muscle of your body (at least to our opinion). To study the effects of statins on the metabolism of the heart, we used heart cells grown from stem cells (cells that can grow in any cell type of our body) and tissue from people undergoing heart surgery.

How is your research related to the field of toxicology, and why did you choose this subject?
Statins have shown to be toxic to mitochondria in skeletal muscle cells. We want to explore whether statins would have a cardiotoxic effect as well. I chose this subject because of my interest in the heart. Moreover, I want to contribute to a more personalized medicine, as currently all patients receive statin therapy. In case statins would hamper mitochondrial function, some people with poor cardiac function would benefit less. Finally, I get the chance to acquire new skills and combine preclinical and clinical studies.

What was your motivation for performing PhD-research?
I wanted to start as a PhD candidate as I was looking for a new challenge during my clinical work. I was done with all the databases and lost my interest in spending all days behind a computer. Also gaining lots of knowledge in a particular field so that you get the chance of explaining it to others and teaching students has motivated me. Although only a minor incentive, I also started as a PhD student as earning this title would help me in obtaining a residency (training as a specialist, *red.*).

How do you see the future of your research topic (follow-up research / social impact)? What do you hope for?
I hope my research forms the foundation for future studies on the effects of statins in older cardiomyocytes. This could potentially help in creating a more personalized statin therapy rather than all patients with heart attack or stroke receiving it without contemplation.

Does the project meet your expectations, why or why not?
My PhD-research exceeds my expectations. How cool is it to

see live beating cells (cardiomyocytes) in a dish under the microscope. I never thought I would see this and never thought that I would be able to make them myself. The same applies to making heart tissue from heart surgery patients beat again outside the body. My PhD-research keeps amazing me.

What is the biggest challenge for you in doing PhD research?
The biggest challenge for me also has to do with how I wanted my PhD project to be. And this is the combination with clinical work. I never wanted to do fulltime PhD-research, since the experience gained is easy to maintain. On the other hand, this sometimes means I have to do evening- and nightshifts whilst the cells also require attention. Managing and planning is very important to keep a good work-life balance but is not always easy. Especially when in clinics people fall ill and gaps in planning need to be filled whilst having experiments planned. Luckily, I have colleagues that help out with some creativity.

What is the best advice that you have received as a PhD student, or would like to give to another PhD student?
Never give up. How tough it might get or how many drawbacks you get, it will turn out to be for the good and it is also what makes you remember your PhD program. Perseverance and joy are key.

What goals do you have regarding your career after finalization of your PhD research? Would this be inside or outside academia, and why? Would you consider going abroad?
After finalizing my PhD research, I will continue my road into, hopefully, becoming a pediatric cardiologist. I have been educated in medicine and like taking care of people/ patients too much to leave clinics and fully change my focus to research. This however does not mean I do not want to continue research, but less intensive and more as part of a bigger group.

Please answer the question from the last toxafette PhD-candidate: do you feel that you are conducting PhD research that will be an advantage to your career after you are finished with your PhD?
I do think my PhD-research is advantageous for my career. Not only is it a kind of prerequisite to start a residency, but moreover I am confronted with my topic (statin therapy) on a daily basis.

Could you propose a question for the next PhD-candidate for the Toxafette?
Question: How has your PhD changed your perspective on Academia?

Proefschrift promopraatje

Dear PhD Students and Promotors,

We would like to invite you to share your insights with your fellow toxicologists regarding your almost or recently completed **PhD-research project**.

The TCDD always reserves a spot for thesis promo talks. This section where gives PhD students the opportunity to present their recently completed theses, whether they are scheduled for defence, or have been defended recently.

To participate, we kindly ask the following from you:
- A Word document containing a clear and readable summary of your thesis (approximately 750-1000 words).
- A photograph of yourself, for example during the thesis defense.
- An image of your thesis cover.
- A URL to your thesis if it's available online.

Sharing your insights not only provides an opportunity to celebrate your achievements but also contributes to enriching knowledge within the NVT. We sincerely hope that you are interested in presenting and sharing your research with your fellow toxicologists.

If you would like to participate in our thesis promo talk or need further information, please let us know via redactie@toxicologie.nl

We look forward to celebrating your hard work and welcoming your insights!

‘It is the dose that makes the poison’ – a critical view on dose level setting in extended-one generation reproductive toxicity (EOGRT) studies

By *Joanne G.W. Salverda and Josje H.E. Arts, senior toxicologists at Nouryon Chemicals BV, The Netherlands*

Abstract

Adequate dose level setting is essential for the evaluation of the toxicity of a substance, meaning that the dose level should not be too low to miss the potential of a substance to exert any toxic effects; it should also not be too high to induce various effects related to systemic toxicity that that would hamper a proper evaluation of specific toxicity endpoints such as reproduction and fetal development. This discussion paper is meant to highlight the challenges faced by industry as a result of ECHA’s advice to select high dose levels when conducting EOGRT studies. In our view, in its concern for finding all possible hazards, ECHA is focused on testing at dose levels that may be too high for repeat dose toxicity studies. Such high dose levels may lead to an unethical increase in animal use and animal suffering, an inability to correctly interpret results and unacceptable requests for the repetition of studies leading to an even higher demand for experimental animals.

Introduction

The topic of dose level setting is intrinsically linked to the basic principle of toxicology. When in 1538, Paracelsus expressed the classic toxicology dictum “*Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis macht, dass ein Ding kein Gift ist”* - which is often condensed to *“The dose makes the poison”* - it became already recognized that all chemicals, even water, oxygen and kitchen salt can be toxic if too much is drunk, inhaled or consumed, thereby acknowledging the combination of the intrinsic toxic property of the chemical and the level of (human) exposure, or consumption. Also, the classification of many human health endpoints is based on a combination of the intrinsic toxic properties of a substance (hazard identification, and the amount administered (hazard quantification) most often resulting from data from animal studies. However, whereas regulatory authorities apply this concept to most human health endpoints they make an exception for carcinogenicity and reproductive toxicity where classification is based on the level of evidence and thus hazard identification only, and not on the quantification or potency level, viz. the amount of chemical administered causing the effects.

Recently, the European Chemicals Agency (ECHA) has emphasized the importance of dose level selection in animal studies specifically targeted to investigate reproduction and/or developmental toxicity [ECHA, 2022], which, in our view, is aiming at a classification for these endpoints by default. In their recent report ‘Review of 55 extended-one generation reproductive toxicity (EOGRT) studies under REACH’, ECHA considered insufficient dose level setting as one of the critical issues hampering hazard identification, and thus classification, in 20% (11 out of 55) of the EOGRT studies reviewed [ECHA, 2023].

This discussion paper is meant to highlight the challenges faced by industry and test labs as a result of ECHA’s advice to select high dose levels when conducting EOGRT (OECD TG 443) studies. The same applies to postnatal developmental toxicity studies (OECD TG 414).

What level of toxicity should be accepted for dose level setting?

In the REACH Annexes on information requirements, it is explicitly mentioned in the amendment of 17th June 2021 (Commission Regulation 2021/979) that: *“Where a test method offers flexibility in the study design, for example in relation to the choice of dose-levels, the chosen study design shall ensure that the data generated are adequate for hazard identification and risk assessment. To this end, testing shall be performed at appropriately high dose levels”* [EC, 2021]. The question then is: how should ‘appropriately high’ be defined?

ECHA’s advice on dose level selection mentions that “*Irrespective of the specifications in the OECD TGs regarding the selection of the highest dose, for classification and labelling, it is critical that the tested doses are sufficiently high to also be able to conclude on a lack of clear evidence on reproductive toxic properties warranting a classification as Repr. 1B for the tested parameters”* [ECHA, 2022]. With this statement, it appears as if ECHA is mainly (if not exclusively) interested in substance classification, thereby neglecting the fundamental principle of toxicology that the ultimate toxicity is determined by a combination of the intrinsic properties of a substance in combination with the dose level. Or in other words, studies showing no reproductive findings at the highest dose tested – using doses far in excess of any likely human exposure - could still be relevant for ECHA to use in human risk assessment.

Selection of too high dose levels leads to unacceptable animal suffering

OECD TG 443 for EOGRT studies mentions: *“If dose levels are based on toxicity, the highest dose should be chosen with the aim to induce some systemic toxicity, but not death or severe suffering of the animals”* [OECD, 2018]. However, in another ECHA document on dose level selection for EOGRT studies it was indicated that: “*To be compliant and not rejected due to too low dose-levels, the highest dose level must induce clear evidence of an adverse effect on sexual function and fertility […]”* [ECHA, 2021]. This does not only mean that reproductive toxicity studies may even be rejected when dose levels are considered too low, it also seems to imply that the high-

est dose level of any substance **must** induce clear evidence of an adverse effect on sexual function and fertility, which in fact would mean that– as already concluded by Paracelsus – any chemical could induce reproductive toxicity as long as the dose would be sufficiently high. Indeed, it is quite likely that in case of severe toxicity (including death) mating will be limited if occurring at all. If so, reproductive toxicity testing would even become redundant, and any chemical could actually be classified for reproductive toxicity by default.

As a result of the required high dose level testing, we are currently facing profound discussions with our contract labs on dose level selection. Together with the labs we feel forced to increase the doses to unethically high levels leading to too much animal suffering, toxicity that is too severe and unnecessary morbidity and mortality, especially in dose-range finding (DRF) studies. However, even this does not provide any guarantee for the main studies because DRF studies are generally of a much shorter duration, viz. 14-28 days versus at least 90 days in an EOGRT study.

We had a recent case of a DRF study in preparation of an EOGRT study where dose levels were selected according to the above-mentioned ECHA requirements. In this DRF study F0 time-mated females were dosed from gestational day 6 until lactation day 20 and F1 selected offspring were dosed between day 21 until 34 of age. During DRF testing it became evident that the test item had a very steep dose-response curve and pups, not dosed before, appeared to be substantially more sensitive than their mothers. In fact, the toxicity observed in the animals was exceeding the acceptable limits under the lab's Animal Welfare License after which the lab was forced to prematurely sacrifice the animals. This resulted in additional DRF testing with several other (lower) dose levels to select the hopefully appropriate dose levels for the scheduled EOGRT study. In our view, the current trend to request higher dose levels leading to more animal suffering cannot be aligned with one of the basic principles of the REACH regulation stating that (Article 25): *"….testing on vertebrate animals for the purposes of this Regulation shall be undertaken only as a last resort"* [EC, 2006]. Moreover, this is also fully in contradiction with the fundamental toxicology 3R's principle and the request from society to ban animal tests. It will also lead to more and probably endless discussions whether findings are the result of the intrinsic property of a substance or the result of general (maternal) toxicity as a consequence of too high dosing.

In a recent paper, van Berlo et al. (2022) suggested that a body weight decrement greater than 10% compared to controls (thus at least a 10% lower body weight than controls) should be the MTD criterion for non-carcinogenicity studies. However, according to OECD Guidance Document (GD) 19 a body weight decrement of 20% compared to controls already qualifies for humane sacrifice due to excessive toxicity [OECD, 2000; Arts et al., 2023a] which was acknowledged in the rebuttal by van Berlo et al. (2023).

But reduced mean BW gain over time can consist of individual days of BW gains and losses and application of the MTD criterion based on BW alone becomes especially challenging for pregnant animals in Developmental and Reproductive Toxicology (DART) studies where even single day(s) of maternal toxicity can have potential consequences on the developing fetuses (Arts et al., 2023a). Thus, if a high dose with 10% lower mean BW compared to controls would not be considered sufficiently excessive or severe, and a high dose with 20% lower mean BW compared to controls would be candidate for humane euthanasia, not much space is left to select the adequate high dose based on a DRF study of shorter duration. Notwithstanding the fact that 10% BW decrement compared to controls is already very large, to end somewhere in the 10-20% range as van Berlo et al. (2023) seem to suggest would in practice require one or more quite extensive DRF studies consisting of several doses and a study duration equal to the duration of an EOGRT study.

Focus on fertility in an EOGRT study by ECHA impairs investigation of other aspects of toxicity such as developmental toxicity

In their clarification on dose level selection for EOGRT studies ECHA claims that "*The focus of the OECD TG 443 study in the REACH annexes is on sexual function and fertility…*' and "*As the study should be designed to ensure adequate assessment of the effects on sexual function and fertility, the dose levels should not be reduced to get enough offspring for the assessment of developmental toxicity"* [ECHA, 2022]. This is a very typical statement, in fact an unfounded opinion, and an intrinsic conflict of the different aims of the EOGRT study – especially when developmental (neuro/immuno)toxicity (DNT/DIT) cohorts need to be added. In our view, addressing the different aims of an EOGRT study cannot be solved without a compromise. While prioritizing for the selection of (higher) dose levels that would allow the identification of potential effects on sexual function and fertility (although according to ECHA "*the highest dose level must induce clear evidence of an adverse effect on sexual function and fertility";* see previous section), this may lead to a significant (but according to ECHA acceptable) reduction in the number of offspring. However, in OECD GD 151 the following is stated regarding the evaluation of DNT: *"…..Interpretation of TG 443 **DNT** test results should take into account available information on mechanisms of action, toxicokinetics, maternal toxicity and potential indirect effects on offspring, as well as any available data on neurotoxic effects of the specific test chemical"* [OECD, 2013, indicating that evaluation of pups should be part of the investigations in an EOGRT study which is impossible in cases where the number of offspring is insufficient for this kind of evaluation. The lack of sufficient animals is even more an issue in the extensive EOGRT studies where additional cohorts and/or testing is requested. Insufficient offspring would mean that under the current REACH requirements there would be no assessment of toxicity possible in the developing offspring. The only other study addressing fetal development is in an OECD TG 414 study but here the development of fetuses is only studied until the end of gestation.

In our view, testing at dose levels that are too high is in clear conflict with the initial requirement of EOGRT studies being able to also capture (subtle) neurobehavioral changes to identify a developmental neurotoxicity hazard. Moreover, with a focus on

classification for reproductive toxicity and its requirement for high dose testing, any findings in pups (if still available) may be mistakenly interpreted as (neuro)developmental toxicity although being the mere consequence of high maternal toxicity resulting in e.g. lack of care by the mothers. Also, as indicated in our earlier example, pups may be more sensitive than adult animals requiring the introduction of asymmetric dosing, whereby adult animals and pups are exposed to different doses. But even more, it should be questioned whether the neurodevelopmental cohorts (as well as the immuno-developmental cohorts) should be dosed at all; not only to be in line with the OECD test guideline to investigate neurodevelopmental toxicity (OECD TG 426) but also to be able to distinguish between developmental neurotoxicity potentially developed during gestation and/or lactation or neurotoxicity caused by direct exposure of the pups after weaning at PND 21, see also our recent paper (Arts et al., 2023b). Also, other agencies may not accept the high dose levels used because of nonlinear kinetics that could occur at high (irrelevant) maternally toxic doses, and could request for additional studies.

Contact with authorities on dose level selection is highly welcomed

At this moment it is uncommon to be in direct contact with the authorities before a REACH dossier is submitted or updated. In fact, all test proposals for higher tier (more advanced) studies as well as any request for studies as part of a compliance check are processed via IUCLID and REACH-IT. In the early days before REACH came into force it was possible to meet with the competent authority to go through testing requirement(s) and discuss any details of the test(s) or substance. In fact, in the case of our earlier example, we asked ECHA for a consult which was actually accepted, and appreciated by both parties. We are in favour of more of such interactions.

Conclusion

In our view, the current mindset at ECHA – backed up by member state competent authorities - is focused on testing at dose levels that are too high for repeat dose toxicity studies. This may lead to an unethical increase in animal use and animal suffering, an inability to correctly interpret the results of such studies and an unacceptable request for the repetition of studies leading to an even higher demand for experimental animals. This does not align with the current trend to promote 3R's and non-animal testing. We also would like to suggest that ECHA re-evaluates the current requirements of an EOGRT study which has as a target to investigate both fertility and sexual behavior as well as development of offspring until sexual maturity, as there are no other REACH Annex VII-X studies in which these endpoints are examined.

We also plea for ECHA to have more interaction with registrants on, among others, dose level selection for repeat dose toxicity Annex IX and X studies. This would create a better understanding of each other's viewpoints and will ultimately result in improved registration dossiers and the use of less animals.

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International Neurotoxicology Association 18th Conference 2023

Durham, North Carolina, USA, May 21-25
Tennessee, the United States, March 19–23

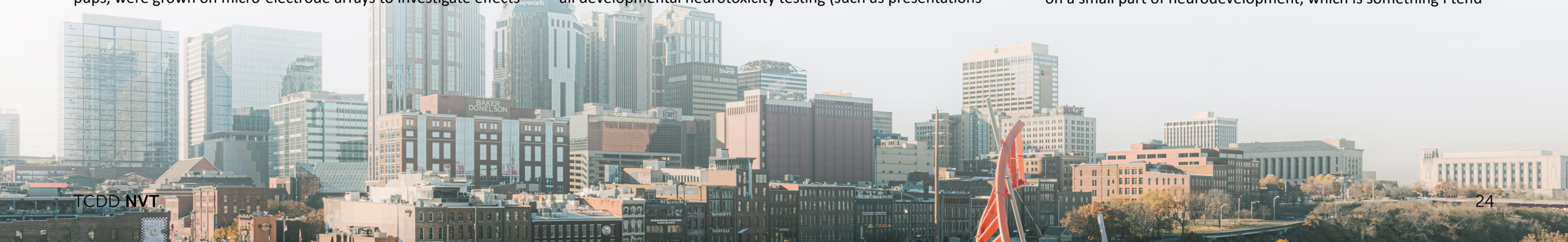
TRAVELER:
Lennart van Melis,
Neurotoxicology Research Group,
Utrecht University

Presenting at the International Neurotoxicology Association conference

During my presentation I showed our findings on the sex-specific effects of acute and developmental exposure to bisphenol-A (BPA) and endosulfan. Human epidemiological and animal *in vivo* data indicate an association between early exposure to environment pollutants and impaired neurodevelopment, with sex differences being rather common in these associations. Our research therefore aimed to assess the sex-specific acute and developmental neurotoxic effects of BPA and endosulfan. To that aim, primary rat cortical cultures, derived from male or female pups, were grown on micro-electrode arrays to investigate effects after acute and developmental exposure. Our data demonstrate that acute exposure to BPA induced a concentration-dependent inhibition of neuronal activity at > 1 µM, with complete cessation of activity at 100 µM. Acute exposure to endosulfan induced a biphasic effect, with excitation at low concentrations (0.1-10 µM) and cessation of activity at 100 µM. Interestingly, endosulfan-induced overexcitation was most pronounced in females. Developmental exposure to 10 µM BPA induced an apparent hyperexcitation from day *in vitro* 21 onwards in both males and females. Exposure to 100 µM BPA induced an inhibition of activity during the entire exposure in males. In females, this initial inhibition was also seen, although activity recovered over time. Interestingly, the clear hyper-excitation observed following acute endosulfan exposure was strongly attenuated during developmental exposure, while exposure to 100 µM still induced an inhibition of activity during the entire exposure in both sexes.

The most interesting learning insights taken from the conference.

For me, the most interesting presentations incorporated either all developmental neurotoxicity testing (such as presentations given by Tim Shafer, Joëlle Ruegg, or Kelly Carstens) or were presentations from people using the same method that I am using, while not being in my research group (such as presentations given by Anke Tukker and Yasunari Kanda). A special highlight was the presentation given by Theodore Slotkin on the developmental neurotoxicity of organophosphates, since he gave some interesting perspectives on the neurotoxicology field. It was very interesting to see all other methods to assess developmental neurotoxicity and to know where the method I am using fits in to the bigger picture. After all, I am only focusing on a small part of neurodevelopment, which is something I tend



to forget while diving deep into my PhD. Also, it was helpful to hear other people presenting the benefits and challenges they experience when working with a micro-electrode array.
During the conference, and especially during the poster session, I talked to different researchers who are working on similar things as I am. Of these, of particular interest was Maren Schenke, who is working at John Hopkins in Baltimore. She is also using the micro-electrode array, and is trying to make their existing brain organoids more male/female-like by a adding different endocrine agonists. This gave us some common ground, since I am working with sex-specific cortical cultures and have been investigating the effects of endocrine (ant)agonists on these compounds. Currently, we are still in (e-mail)contact to help each other doing our research.

During the poster sessions, I was very interested in a new technique presented by Lena Smirnova from John Hopkins, Baltimore. She told me about 3D micro-electrode arrays, which can fold around a brain organoid and measure the neuronal activity in the outer layers of the brain organoids. This would circumvent a big limitation of *in vitro* MEA research compared to *in vivo* research: MEA is usually done in 2D, while *in vivo* research takes place in 3D brains. Being able to implement this in our lab would give (literally) an extra dimension to our current 2D micro-electrode array work.

A “take home message” from this conference
Collaboration is important, since my own PhD research on developmental neurotoxicity does not exist in a vacuum but is connected to other aspects of neurotoxicology.

WUR Scientific PhD Trip

United Kingdom

TRAVELERS:
24 WUR Toxicology PhDs and our Associate Professor Dr. Nynke Kramer

The smell of fresh coffee at 7am in the Eurostar train, the classic British club sandwich with chips for lunch, the thirst-quenching lager ale beer in English pubs, and the iconic red telephone boxes and the London double-decker buses – these are just sneak peeks of our recently organized scientific PhD trip to the United Kingdom.

During our educational 10-day trip, we presented our research with the help of posters, gave short and long talks within our five main fields of scientific expertise: risk assessment of herbal food supplements, mode of action of foodborne and natural toxins, alternatives to animal testing, the role of gut microbiota in the toxicity of foodborne chemicals and environmental toxicology. Some of us are working with high-throughput subcellular fractions, highly complex cellular and chip models, and develop computational kinetic models, while others are performing studies to examine chemical effects on the environment. A comprehensive excursion is only complete when different institutes are involved: industry (Unilever and Syngenta), governmental agencies (UK CEH and UK NC3R) and academics (MRC Toxicology Unit in Cambridge University and Brunel University). The rich program diversity, and its balanced combination of sharing and learning, yielded an excellent scientific excursion program.

Unilever is a multinational consumer goods company, whereas Syngenta focuses on seeds and pesticides but both companies are utilizing new approach methodologies in assessing chemical

Photo 1: Group photo of our 24 WUR Toxicology PhDs and our Associate Professor Dr. Nynke Kramer in front of the Isaac Newton's derivative apple tree in Cambridge.

safety before these chemicals are released onto the market. At Unilever, health risks of chemicals are categorized based on their bioactivity/exposure ratio, and physiologically based kinetic (PBK) modelling are built on a tier-based approach: starting from a deterministic PBK model up to probabilistic models taking into account variability in population characteristics and experimental errors. At Syngenta, research is ongoing to make the paradigm shift from OECD-based *in vivo* studies, to non-standard *in vivo* studies, *in vitro* studies and lastly PBK mechanistic models. Together with dietary and non-dietary residues exposure, risk assessment yields safety levels such as food maximum residue levels, maximum application rate, minimum time before harvest: pre-harvest interval, maximum number of applications and minimal interval between applications. Finally, we highly valued the career talk from these two companies as options after we graduate.

Moving on to the governmental research agencies, UK CEH focuses more on the science behind chemicals' effects towards water, land and air (i.e. environmental toxicology). The NC3R is directed towards approaches in replacing, reducing and refining animal testing that ultimately shape current regulation and industry's practice to using more new approach methodologies. These two institutes greatly reflect the subdivision of projects that we have at Wageningen University. It is refreshing to visit the lab facilities at UK CEH, and be taught on hands-on procedures to measure pesticide residues in honeys and Covid-19 measurements in wastewater. The hard science is of course important but the NC3R gave us an important soft skill lesson related to scientific communication: We were reminded that society outreach is an important part of science and contributes to its transparency.

Last but not least, the academic institutions and their toxicology departments: Medical Research Council (MRC) Toxicology Unit is an organisation that gathers researchers from different English colleges for mechanistic studies, whereas Brunel University is a leading institute on environmental toxicology. At the MRC Toxicology Unit, we learnt a great deal from Prof. Kiran Patil on the important drug effects towards gut microbiota in relation with antimicrobial resistance because essentially, antimicrobials or not, all drugs pose effects on gut microbiota. At Brunel University, our attention was grabbed by Prof. Andreas Kortenkamp who challenged us to look at mixture toxicity with a different perspective, particularly, on chemicals interaction at low dose levels. During and after the daily symposia, we were given campus walking tours, so we could exchange stories between the PhD' life in Wageningen with those in Cambridge and in Brunel (e.g. Photo 1).

All in all, this PhD trip gave us both: personal and overarching take-away lessons. Personal, because it comes from the interactions we had with one another as well as with the researchers from the institutes we were visiting. We were exposed to new topics during the visit but we were also challenged to reflect on our research with a different perspective. Overarching, because regardless of the different toxicological research institutes, we noticed that we are contributing to current science with our use of new approach methodologies and the assessment of chemical impacts on the environment.

We would like to express our gratitude towards our chair group holder Ivonne Rietjens for encouraging us to organise the trip, as well as her help to financially realise the tour. We also want to thank the NVT, as well as BDS, Shimadzu, VLAG and WIMEK graduate schools for the financial support to make this impressive scientific excursion happen.

WAGENINGEN - UK
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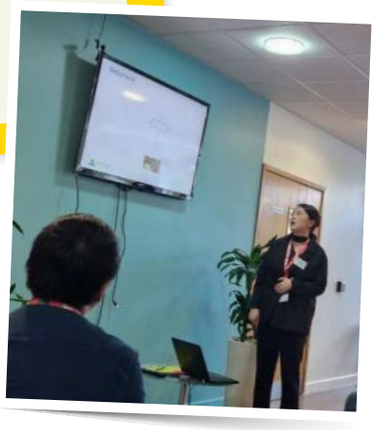
Some personal experiences from participants

TRAVELER:
Edith Etor

At the mini-symposium hosted by Syngenta, UK-Center for Ecology and Hydrology, and at the University of Brunel, I made oral presentations about a chapter in my PhD research. I also had poster presentations Unilever. In my presentations, I compared the sublethal effects and lipid peroxidation in the common cockle exposed in acute toxicity testing to fresh crude oil in a tidal and stagnant system. Benthic invertebrates like the cockle constantly face challenges from anthropogenic inputs like crude oil spillage. Sometimes, ageing of the oil via factors like tide, waves or temperature may exacerbate or reduce the impact of the toxic fractions. Trade-offs like siphoning and burrowing activity are qualities the cockle may exhibit to avoid pollution effects. I was able to show that cockles exposed to fresh crude oil in a tidal environment had significantly higher levels of lipid peroxidation when compared to cockles exposed to fresh crude oil in a stagnant system. The cockles' ability to close their valves and thereby protect themselves from the contaminants in the stagnant system demonstrates their ability to survive. This study suggest that the tide is a natural stressor that may increase toxicity from crude oil spilled in a marine environment.

TRAVELER:
Qiuhui Ren



During the PhD trip, I gave my presentation at Unilever's Safety and Environmental Assurance Centre, our first stop of PhD trip. The presentation is about selection of hydroxyanthraquinones in herbal product and traditional Chinese medicine (TCM) as inducers of Nrf2-EpRE (electrophile-responsive element) mediated gene expression. Hydroxyanthraquinones is naturally occurring in many plants which usually used in traditional Chinese medicine. In previous studies, hydroxyanthraquinones have been reported a lot of biological activities, such as anti-inflammatory, anti-tumor, and anti-bacterial effects. Underlying these beneficial effects, one of important mechanisms is activation of Nrf2-EpRE mediated gene expression. In this study we use the high throughput Nrf2-CALUX reporter gene assay to assess the potencies of Nrf2 activation of sixteen individual hydroxyanthraquinones, eight kinds of single herb-based granules and eight kinds of traditional Chinese medicines. And RT-qPCR was used to validate our method. LC-MS/MS was used to quantify hydroxyanthraquinones in above herbal products and TCMs. The results showed 8 individual hydroxyanthraquinones, 5 extracts of herbal granules and all 8 extracts of TCM showed Nrf2 induction by Nrf2 CALUX assay. The Nrf2 activation by extracts from TCM and herbs can be partially explained by the presence of selected hydroxyanthraquinones.

TRAVELER:
Véronique de Bruijn

During the MRC-TOX mini-symposium, I presented my research comparing hepatic *in vitro* models for studying cholestasis. Current models have limited predictive accuracy for cholestatic drug-induced liver injury. Cholestasis obstructs bile flow from the liver, leading to toxic bile acid accumulation. To address this, we compared HepaRG cells, SCHHs, and organoids, analyzing bile acid composition and gene expression. Our findings showed that organoids produced significantly lower bile acid levels compared to HepaRG cells and SCHHs. Furthermore, the gene expression profiles of the organoids differed more from a liver biopsy than those of HepaRG cells or SCHHs. Thus, organoids do not prove to be a superior *in vitro* alternative for cholestasis testing compared to the other models. This research highlights the need for improved *in vitro* models that can replicate human liver functionality to enhance the predictive accuracy of cholestatic drug-induced liver injury studies. By understanding the limitations and potentials of different models, we can refine our approaches and develop more reliable tools for studying cholestasis.
At Brunel University, I gave a longer presentation about my PhD project. Besides the work about hepatic *in vitro* models, I

also presented about our strategies to predict cholestasis using physiologically based kinetic modelling. In this work, the focus was on the Molecular Initiating Event (MIE) in the Adverse Outcome Pathway (AOP) of cholestasis, namely inhibition of Inhibition of the hepatic Bile Salt Efflux Pump (BSEP), which facilitates the transport of bile acids into the bile canaliculi. Previous research showed that the potential of a chemical to inhibit bile acid efflux alone is insufficient to prioritize its risk for cholestasis. To enhance the prioritization of chemicals based on their cholestasis risk, we hypothesized that considering external dose and toxicokinetics would be beneficial. Based on our predictions we ranked the chemicals in line with FDA's DILI concern classification associated with these therapeutics. These results offer a proof-of-principle for an animal-free PBK-facilitated risk prioritization of potential cholestatic therapeutics. Our results demonstrate that, in addition to dose and the IC50 for bile acid efflux inhibition, the kinetics of the therapeutics should be considered to make accurate predictions.

TRAVELER:
Jing Fang

On 2nd of June, we visited the MRC Toxicology Unit of Cambridge. I gave a presentation of the first experimental chapter of my PhD project which is about *in vitro* developmental toxicity testing of unsubstituted and methylated polycyclic aromatic hydrocarbons (PAHs) using the zebrafish embryo toxicity test (ZET). PAHs are a group of organic compounds comprised of only carbon and hydrogen atoms. Exposure to PAHs has been linked to developmental toxicity in various animal species, including humans. However, the current understanding of developmental toxicity primarily revolves around unsubstituted PAHs, while knowledge regarding alkylated PAHs (such as methylated PAHs) remains limited. Research suggests that the addition of a methyl group to the aromatic ring of PAHs may influence their developmental toxicity. Thus, the objective of this study is to examine the hypothesis that the developmental toxicity of methylated PAHs, compared to their unsubstituted parent PAH, is influenced by the position of the methyl substituent on the aromatic ring. To accomplish this, *in vitro* assays such as the ZET will be employed to evaluate *in vitro* developmental toxicity.
As a toxicology PhD student, this PhD trip emphasized the critical need of collaboration and integration of diverse evidence, and the dissemination of scientific knowledge in advancing our understanding of toxicology and promoting evidence-based approaches to safeguard human health and the environment.

TRAVELER:
Hugo Brouwer

During the PhD trip I had the opportunity to present my research entitled "*In vitro* digestion increases micro and nanoplastic uptake in THP-1 derived macrophages" at Cambridge university, the UK-center for ecology and hydrology and syngenta. Humans are continually exposed to micro and nanoplastics(MNPs) with unknown health consequences for the gastrointestinal tract. During ingestion the surface of the MNPs strongly binds to surrounding digestive proteins which may alter their toxicity but is currently rarely taken into account. During my presentation I have shown that polystyrene particles between 50 and 1000nm of various charges generate a stable particle specific digestive protein corona that is retained if it comes into contact with serum-containing medium. THP-1 derived macrophages were used as a model for intestinal macrophages and were exposed to *in vitro* digested and undigested MNPs. For nanoplastics the digestion caused a significant increase in uptake compared to serum-treated particles, however this was not seen for larger or charged particles. Using LC-MS-MS based proteomics we have shown that apolipoproteins A-II, C, and E as well as calcium-binding protein S-100-A1 were significantly correlated with uptake, forming a proof of principle that the protein corona should be considered when performing nanotoxicity testing but may be less important for microplastics.

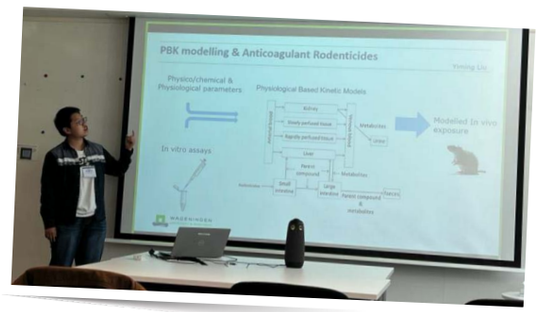
TRAVELER:
Germaine Aalderink

We, the division of Toxicology at WUR have organised a scientific trip to England with 27 PhDs and one t the mini-symposium at Unilever, I presented my PhD research chapter about *Lipid transport across the intestine within an in vitro microfluidic device.* During my presentation, I discussed the current lack of knowledge we have about the lymphatic system and representative models. My research is focused on the development of such a representative model for the lymphatic system, in combination with an intestinal barrier, to look at the absorption of (novel) drugs. In the human intestine, most nutrients are taken up by the blood vessels and pass through the portal vein to be metabolized by the liver. However, long-chain fatty acids are taken up by the enterocytes of the intestine and formed into chylomicrons. Chylomicrons (75-1200 nm diameter), are excluded by the blood vessels due to their size and enter the lymphatic system instead, where they re-enter the blood system through the thoracic duct. Potential drug candidates, therefore, circumvent the first-pass effect of the liver. Representative models for the intestinal barrier in connection to the blood and lymphatic system could therefore give insight in the uptake of potential drug candidates. The first part of my research was devoted to developing an intestinal model from iPSC-derived intestinal cells. These iPSC-derived intestinal cells were grown directly on Transwells to generate a barrier model for exposure and transport studies. Chylomicron formation was promoted by the addition of digested Palm oil that included the compounds of interest. So far, we have been able to produce a functional intestinal model of Transwells that can produce chylomicrons for lipophilic compound transport.

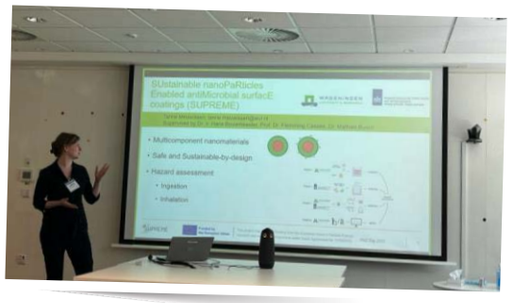
At Unilever, I gave one of several microfluidic presentations and it was very helpful to talk to other people with similar problems. While I use the TissUse model for my microfluidic work, several scientists at Unilever had experiences with the Mimetas microfluidic platform. Our platforms differ in their use, as the former concentrates on multi-organ culturing, whereas the latter focuses on single organs with many replicates. As Unilever tests a large number of compounds, a higher number of potential replicates for quantitative measurements are more interesting to them. After the presentations, we addressed the fact that most organs-on-chips are made from PDMS, which binds lipophilic compounds. Since I am interested in lipophilic compound uptake and distribution between the blood and lymph, it will be important for me to address what fraction of my compounds are taken up by the PDMS.

TRAVELER:
Yiming Liu

At the mini symposium at Brunel University London, I have presented my PhD research project about developing a PBK model to predict exposure of small mammals to anti-coagulant rodenticides (ARs). ARs are the most widely used rodenticide nowadays, which may potentially affect non-target species. Hence, a specific monitoring system is required in order to evaluate the exposure of non-target organisms to ARs and assess associated risks. However, currently used monitoring methods may underestimate the potentials risks because the possible metabolism of ARs were not included. In this project, we developed a physiologically-based kinetic (PBK) modelling to quantify actual exposure of small mammals to ARs by measuring the concentration of parent compounds and associated metabolites in liver and blood. Model simulations were compared with *in vivo* exposure data from literature to further verify the model. At the end of this project, the developed PBK models were used to predict actual exposure levels to ARs for non-target species based on tissue concentration. Results can be further used to perform risk assessments of ARs at selected field locations.



TRAVELER:
Tanne Meuwissen



I took the opportunity to outline my proposed framework for my PhD during flash presentations at Unilever and the UK Centre for Ecology & Hydrology (UK-CEH). My research is part of the Horizon Europe project SUPREME which aims to use multicomponent nanomaterials such as core/shell nanomaterials to develop such a coating. Specifically, my PhD will focus on performing hazard assessments for the developed novel nanomaterials ensure effectiveness against bacteria, viruses, and fungi, while prioritising safety for humans. Four different tasks of my proposed PhD thesis were outlined. Initially, oral and inhalation exposure to the pristine nanomaterials will be studied by comparing multiple models per exposure route. In addition, the developed coating as well as its aged and weathered products will be studied using the same models. Finally, I will use Physiologically Based Kinetic (PBK) modelling to extrapolate *in vitro* findings to *in vivo* benchmark doses.

TRAVELER:
Liang Zheng

At Brunel University London, I have presented part of my PhD research on the role of glutathione and flavonoids in protection against cellular toxicity induced by α-dicarbonyl compounds: endogenous and food-borne perspectives. I introduced the adverse effects of α-dicarbonyl compounds in food products and also in the human body, with an emphasis on how they induce the formation of advanced glycation end products. Furthermore, I presented my work on the investigation of the role of intracellular glutathione in protecting cells against the electrophilic reactivity of α-dicarbonyl compounds reflected by their activity to induce Nrf2-mediated gene expression. The study showed that the cytotoxicity and induction of the Nrf2-mediated pathway by dicarbonyls was significantly enhanced by the depletion of GSH, while a decrease in Nrf2-activation was observed upon an increase of the cellular GSH levels. Then I elaborated on the comparison of the methylglyoxal (MGO) scavenging effects of kaempferol and glutathione and their influence on the toxicity of methylglyoxal in SH-SY5Y cells. This work showed that kaempferol can form stable adducts with MGO, while the GSH adduct was reversible. Furthermore, the scavenging of MGO by kaempferol significantly decreased MGO-induced cytotoxicity and provided better protection than GSH against extracellular MGO. Taking all together, It is concluded that flavonoids like kaempferol provide better scavengers for food-borne MGO than thiol-based scavengers such as GSH, while, given the endogenous concentrations of both scavengers and the detoxification of the GSH-MGO adduct by the glyoxalase system for intracellular MGO protection GSH will be dominant.

The study trip in the UK highlighted the importance of next-generation risk assessment, new approach methodologies, gut microbiota's response to drugs, refining techniques, potential regulatory changes, and understanding mixture toxicity and chemical interactions.

TRAVELER:
Katharina Nitsche

At the Syngenta- WUR symposium, I presented one of my PhD chapters concerning chemical non- specific binding to system components within the framework of evaluating organ- on- chip (OoC) technology for a Next- Generation Risk Assessment (NGRA). OoC is expected to revolutionise cell culturing by introducing (micro)fluidic flow and shear stress which has demonstrated in several recent studies to improve tissue morphology and function. However, before conducting chemical safety assessments with OoC there is a need to build confidence and experience in their use as these *in vitro* models exhibit particular distribution processes. To address this, I presented how we programmed a mass prediction model and developed an experimental approach that allows us to understand chemical kinetics after on- chip exposure and how both can contribute to calculate nominal system concentrations - because only a chemical fraction actually reaches the cell. These nominal effect variations are due to non- specific binding of especially hydrophobic chemicals. This is increasingly acknowledged as concern as it hampers the translatability of these models to *in vivo* and other *in vitro* systems. I concluded that our computational model can contribute to the validation of OoC but that there are still more (biological) challenges to tackle in order for OoC to provide data for decision- making processes.

TRAVELER:
Frances Widjaja

At UK Center for Ecology and Hydrology (UK CEH), I have presented my PhD on the relative potency values of plant toxin pyrrolizidine alkaloid N-oxides. In 3 minutes, I began with explaining the relevance of this toxin in our food and environment (i.e. not collecting the beautiful toxic flowers in the slide for father's day just because they can be found almost everywhere in nature), the two opposite opinions in viewing relative potency, and the use of physiologically based kinetic (PBK) modeling to derive such values that depend on both dose and endpoint. The generic model for rat was well-validated with *in vivo* data, and so the same model can be extended to human situation or to another pyrrolizidine alkaloid N-oxides. The output shows that at high dose levels, relative potency values are affected by dose and endpoint. Yet, at low dose levels that are more relevant to human exposure, these values are higher than at high dose levels and they remain unaffected by dose and endpoint. All in all, physiologically based kinetic modeling is shown to be a powerful tool in predicting relative potency values at low dose levels and for human, both of which are practically impossible with animal studies.

Save the date!

Sections Pharmaceutical Toxicology and Genetic Toxicology

"Afternoon co-symposium of the NVT Sections Pharmaceutical Toxicology and Genetic Toxicology with Topic: "Genotoxic impurities, e.g., nitrosamines, in pharmaceuticals". Date: **24 April 2024**. More information will follow."

Inschrijving TiO

Voorletters	Achternaam	Opleider	Datum inschrijving
M.C.	Bouwmeester	Prof.dr. M. van den Berg	05-05-2023
M.	Kloukinioti	Prof.dr. P.J.A. Borm	20-06-2023
G.A.A.	AlNoaimi	Prof.dr.ir. N. van den Brink	20-06-2023
H.T.	Chien	Prof.dr. F.G.M. Russel	20-06-2023
A.E.T.	van den Berg	Prof.dr. M.B.M. van Duursen	06-07-2023
J.J.	Meerman	Prof.dr. F.G.M. Russel	05-09-2023
C.	Pachoulide	Prof.dr.ir. I.M.C.M. Rietjens	05-09-2023

Inschrijving Register

Voorletters	Achternaam	Datum inschrijving	Datum afloop registratie
C.G.G.M.	Pauwels	13-06-2023	12-06-2028
L.	Chen	23-06-2023	22-06-2028
D.T.	Garcia Mendoza	21-06-2023	20-06-2028
A.	Hussein Bakheit Adam	31-08-2023	30-08-2028

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EXPERT OPINION AND COMMENTARY

Joanne G.W. Salverda and Josje H.E. Arts

'It is the dose that makes the poison' – a critical view on dose level setting in extended-one generation reproductive toxicity (EOGRT) studies

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‘It is the dose that makes the poison’ – a critical view on dose level setting in extended-one generation reproductive toxicity (EOGRT) studies

Joanne G.W. Salverda and Josje H.E. Arts, senior toxicologists at Nouryon Chemicals BV, The Netherlands

Abstract

Adequate dose level setting is essential for the evaluation of the toxicity of a substance, meaning that the dose level should not be too low to miss the potential of a substance to exert any toxic effects; it should also not be too high to induce various effects related to systemic toxicity that that would hamper a proper evaluation of specific toxicity endpoints such as reproduction and fetal development. This discussion paper is meant to highlight the challenges faced by industry as a result of ECHA’s advice to select high dose levels when conducting EOGRT studies. In our view, in its concern for finding all possible hazards, ECHA is focused on testing at dose levels that may be too high for repeat dose toxicity studies. Such high dose levels may lead to an unethical increase in animal use and animal suffering, an inability to correctly interpret results and unacceptable requests for the repetition of studies leading to an even higher demand for experimental animals.

Introduction

The topic of dose level setting is intrinsically linked to the basic principle of toxicology. When in 1538, Paracelsus expressed the classic toxicology dictum “*Alle Dinge sind Gift, und nichts ist ohne Gift; allein die Dosis macht, dass ein Ding kein Gift ist”* - which is often condensed to *“The dose makes the poison”* - it became already recognized that all chemicals, even water, oxygen and kitchen salt can be toxic if too much is drunk, inhaled or consumed, thereby acknowledging the combination of the intrinsic toxic property of the chemical and the level of (human) exposure, or consumption. Also, the classification of many human health endpoints is based on a combination of the intrinsic toxic properties of a substance (hazard identification, and the amount administered (hazard quantification) most often resulting from data from animal studies. However, whereas regulatory authorities apply this concept to most human health endpoints they make an exception for carcinogenicity and reproductive toxicity where classification is based on the level of evidence and thus hazard identification only, and not on the quantification or potency level, viz. the amount of chemical administered causing the effects.

Recently, the European Chemicals Agency (ECHA) has emphasized the importance of dose level selection in animal studies specifically targeted to investigate reproduction and/or developmental toxicity [ECHA, 2022], which, in our view, is aiming at a classification for these endpoints by default. In their recent report ‘Review of 55 extended-one generation reproductive toxicity (EOGRT) studies under REACH’, ECHA considered insufficient dose level setting as one of the critical issues hampering hazard identification, and thus classification, in 20% (11 out of 55) of the EOGRT studies reviewed [ECHA, 2023].

This discussion paper is meant to highlight the challenges faced by industry and test labs as a result of ECHA’s advice to select high dose levels when conducting EOGRT (OECD TG 443) studies. The same applies to postnatal developmental toxicity studies (OECD TG 414).

What level of toxicity should be accepted for dose level setting?

In the REACH Annexes on information requirements, it is explicitly mentioned in the amendment of 17th June 2021 (Commission Regulation 2021/979) that: *“Where a test method offers flexibility in the study design, for example in relation to the choice of dose-levels, the chosen study design shall ensure that the data generated are adequate for hazard identification and risk assessment. To this end, testing shall be performed at appropriately high dose levels”* [EC, 2021]. The question then is: how should ‘appropriately high’ be defined?

ECHA’s advice on dose level selection mentions that “*Irrespective of the specifications in the OECD TGs regarding the selection of the highest dose, for classification and labelling, it is critical that the tested doses are sufficiently high to also be able to conclude on a*

lack of clear evidence on reproductive toxic properties warranting a classification as Repr. 1B for the tested parameters" [ECHA, 2022]. With this statement, it appears as if ECHA is mainly (if not exclusively) interested in substance classification, thereby neglecting the fundamental principle of toxicology that the ultimate toxicity is determined by a combination of the intrinsic properties of a substance in combination with the dose level. Or in other words, studies showing no reproductive findings at the highest dose tested – using doses far in excess of any likely human exposure - could still be relevant for ECHA to use in human risk assessment.

Selection of too high dose levels leads to unacceptable animal suffering

OECD TG 443 for EOGRT studies mentions: *"If dose levels are based on toxicity, the highest dose should be chosen with the aim to induce some systemic toxicity, but not death or severe suffering of the animals"* [OECD, 2018]. However, in another ECHA document on dose level selection for EOGRT studies it was indicated that: "*To be compliant and not rejected due to too low dose-levels, the highest dose level must induce clear evidence of an adverse effect on sexual function and fertility […]"* [ECHA, 2021]. This does not only mean that reproductive toxicity studies may even be rejected when dose levels are considered too low, it also seems to imply that the highest dose level of any substance **must** induce clear evidence of an adverse effect on sexual function and fertility, which in fact would mean that– as already concluded by Paracelsus – any chemical could induce reproductive toxicity as long as the dose would be sufficiently high. Indeed, it is quite likely that in case of severe toxicity (including death) mating will be limited if occurring at all. If so, reproductive toxicity testing would even become redundant, and any chemical could actually be classified for reproductive toxicity by default.

As a result of the required high dose level testing, we are currently facing profound discussions with our contract labs on dose level selection. Together with the labs we feel forced to increase the doses to unethically high levels leading to too much animal suffering, toxicity that is too severe and unnecessary morbidity and mortality, especially in dose-range finding (DRF) studies. However, even this does not provide any guarantee for the main studies because DRF studies are generally of a much shorter duration, viz. 14-28 days versus at least 90 days in an EOGRT study.

We had a recent case of a DRF study in preparation of an EOGRT study where dose levels were selected according to the abovementioned ECHA requirements. In this DRF study F0 timemated females were dosed from gestational day 6 until lactation day 20 and F1 selected offspring were dosed between day 21 until 34 of age. During DRF testing it became evident that the test item had a very steep dose-response curve and pups, not dosed before, appeared to be substantially more sensitive than their mothers. In fact, the toxicity observed in the animals was exceeding the acceptable limits under the lab's Animal Welfare License after which the lab was forced to prematurely sacrifice the animals. This resulted in additional DRF testing with several other (lower) dose levels to select the hopefully appropriate dose levels for the scheduled EOGRT study. In our view, the current trend to request higher dose levels leading to more animal suffering cannot be aligned with one of the basic principles of the REACH regulation stating that (Article 25): *"….testing on vertebrate animals for the purposes of this Regulation shall be undertaken only as a last resort"* [EC, 2006]. Moreover, this is also fully in contradiction with the fundamental toxicology 3R's principle and the request from society to ban animal tests. It will also lead to more and probably endless discussions whether findings are the result of the intrinsic property of a substance or the result of general (maternal) toxicity as a consequence of too high dosing.

In a recent paper, van Berlo et al. (2022) suggested that a body weight decrement greater than 10% compared to controls (thus at least a 10% lower body weight than controls) should be the MTD criterion for non-carcinogenicity studies. However, according to OECD Guidance Document (GD) 19 a body weight decrement of 20% compared to controls already qualifies for humane sacrifice due to excessive toxicity [OECD, 2000; Arts et al., 2023a] which was acknowledged in the rebuttal by van Berlo et al. (2023).

But reduced mean BW gain over time can consist of individual days of BW gains and losses and application of the MTD criterion based on BW alone becomes especially challenging for pregnant animals in Developmental and Reproductive Toxicology (DART) studies where even single day(s) of maternal toxicity can have potential consequences on the developing fetuses (Arts et al., 2023a). Thus, if a high dose with 10% lower mean BW compared to controls would not be considered sufficiently excessive or severe, and a high dose with 20% lower mean BW compared to controls would be candidate for humane euthanasia, not much space is left to select the adequate high dose based on a DRF study of shorter duration. Notwithstanding the fact that 10% BW decrement compared to controls is already very large, to end somewhere in the 10-20% range as van Berlo et al. (2023) seem to suggest would in practice require one or more quite extensive DRF studies consisting of several doses and a study duration equal to the duration of an EOGRT study.

Focus on fertility in an EOGRT study by ECHA impairs investigation of other aspects of toxicity such as developmental toxicity

In their clarification on dose level selection for EOGRT studies ECHA claims that "*The focus of the OECD TG 443 study in the REACH annexes is on sexual function and fertility…*' and "*As the study should be designed to ensure adequate assessment of the effects on sexual function and fertility, the dose levels should not be reduced to get enough offspring for the assessment of devel-*

opmental toxicity" [ECHA, 2022]. This is a very typical statement, in fact an unfounded opinion, and an intrinsic conflict of the different aims of the EOGRT study – especially when developmental (neuro/immuno)toxicity (DNT/DIT) cohorts need to be added. In our view, addressing the different aims of an EOGRT study cannot be solved without a compromise. While prioritizing for the selection of (higher) dose levels that would allow the identification of potential effects on sexual function and fertility (although according to ECHA "*the highest dose level must induce clear evidence of an adverse effect on sexual function and fertility";* see previous section), this may lead to a significant (but according to ECHA acceptable) reduction in the number of offspring. However, in OECD GD 151 the following is stated regarding the evaluation of DNT: *"…..Interpretation of TG 443* ***DNT*** *test results should take into account available information on mechanisms of action, toxicokinetics, maternal toxicity and potential indirect effects on offspring, as well as any available data on neurotoxic effects of the specific test chemical"* [OECD, 2013, indicating that evaluation of pups should be part of the investigations in an EOGRT study which is impossible in cases where the number of offspring is insufficient for this kind of evaluation. The lack of sufficient animals is even more an issue in the extensive EOGRT studies where additional cohorts and/or testing is requested. Insufficient offspring would mean that under the current REACH requirements there would be no assessment of toxicity possible in the developing offspring. The only other study addressing fetal development is in an OECD TG 414 study but here the development of fetuses is only studied until the end of gestation.

In our view, testing at dose levels that are too high is in clear conflict with the initial requirement of EOGRT studies being able to also capture (subtle) neurobehavioral changes to identify a developmental neurotoxicity hazard. Moreover, with a focus on classification for reproductive toxicity and its requirement for high dose testing, any findings in pups (if still available) may be mistakenly interpreted as (neuro)developmental toxicity although being the mere consequence of high maternal toxicity resulting in e.g. lack of care by the mothers. Also, as indicated in our earlier example, pups may be more sensitive than adult animals requiring the introduction of asymmetric dosing, whereby adult animals and pups are exposed to different doses. But even more, it should be questioned whether the neurodevelopmental cohorts (as well as the immuno-developmental cohorts) should be dosed at all; not only to be in line with the OECD test guideline to investigate neurodevelopmental toxicity (OECD TG 426) but also to be able to distinguish between developmental neurotoxicity potentially developed during gestation and/or lactation or neurotoxicity caused by direct exposure of the pups after weaning at PND 21, see also our recent paper (Arts et al., 2023b). Also, other agencies may not accept the high dose levels used because of nonlinear kinetics that could occur at high (irrelevant) maternally toxic doses, and could request for additional studies.

Contact with authorities on dose level selection is highly welcomed

At this moment it is uncommon to be in direct contact with the authorities before a REACH dossier is submitted or updated. In fact, all test proposals for higher tier (more advanced) studies as well as any request for studies as part of a compliance check are processed via IUCLID and REACH-IT. In the early days before REACH came into force it was possible to meet with the competent authority to go through testing requirement(s) and discuss any details of the test(s) or substance. In fact, in the case of our earlier example, we asked ECHA for a consult which was actually accepted, and appreciated by both parties. We are in favour of more of such interactions.

Conclusion

In our view, the current mindset at ECHA – backed up by member state competent authorities - is focused on testing at dose levels that are too high for repeat dose toxicity studies. This may lead to an unethical increase in animal use and animal suffering, an inability to correctly interpret the results of such studies and an unacceptable request for the repetition of studies leading to an even higher demand for experimental animals. This does not align with the current trend to promote 3R's and non-animal testing. We also would like to suggest that ECHA re-evaluates the current requirements of an EOGRT study which has as a target to investigate both fertility and sexual behavior as well as development of offspring until sexual maturity, as there are no other REACH Annex VII-X studies in which these endpoints are examined.

We also plea for ECHA to have more interaction with registrants on, among others, dose level selection for repeat dose toxicity Annex IX and X studies. This would create a better understanding of each other's viewpoints and will ultimately result in improved registration dossiers and the use of less animals.

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