Consensus Report

New Approach Methodologies (NAMs) for Human-Relevant Biokinetics Predictions: Meeting the Paradigm Shift in Toxicology Towards an Animal-Free Chemical Risk Assessment

Ans Punt1, Hans Bouwmeester2, Bas J. Blaauboer3, Sandra Coecke4, Betty Hakkert5, Delilah F. G. Hendriks6, Paul Jennings7, Nynke I. Kramer3, Sibylle Neuhoff8, Rosalinde Masereeuw9, Alicia Paini4, Ad A. C. M. Peijnenburg1, Martijn Rooseboom10, Michael L. Shuler11, Ian Sorrell12, Bart Spee13, Marije Strikwold14, Andries D. van der Meer15, Meike van der Zande1, Mathieu Vinken16, Huan Yang17, Peter M. J. Bos5 and Minne B. Heringa5

1WFSR Wageningen Food Safety Research, Wageningen, The Netherlands; 2Division of Toxicology, Wageningen University and Research, Wageningen, The Netherlands; 3Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands; 4European Commission Joint Research Centre, Ispra, Italy; 5RIVM – The National Institute for Public Health and the Environment, Bilthoven, The Netherlands; 6Section of Pharmacogenetics, Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden; 7Division of Molecular and Computational Toxicology, Dept. of Chemistry and Pharmaceutical Sciences, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands; 8Certara UK Ltd, Simcyp Division, Sheffield, UK; 9Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands; 10Shell Health, Shell International B.V., The Hague, The Netherlands; 11Biomedical Engineering, Cornell University, Department of Biomedical Engineering, Ithaca, NY, USA; 12Unilever, Colworth Science Park, Bedfordshire, UK; 13Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; 14Van Hall Larenstein University of Applied Sciences, Leeuwarden, The Netherlands; 15University of Twente, Department of Applied Stem Cell Technologies, Enschede, The Netherlands; 16Department of In Vitro Toxicology and Dermato-Cosmetology, Vrije Universiteit Brussel, Brussels, Belgium; 17Division of Drug Discovery and Safety, Leiden Academic Centre for Drug Research (LACDR)/Leiden University, Leiden, The Netherlands

Abstract

For almost fifteen years, the availability and regulatory acceptance of new approach methodologies (NAMs) to assess the absorption, distribution, metabolism and excretion (ADME/biokinetics) in chemical risk evaluations are a bottleneck. To enhance the field, a team of 24 experts from science, industry, and regulatory bodies, including new generation toxicologists, met at the Lorentz Centre in Leiden, The Netherlands. A range of possibilities for the use of NAMs for biokinetics in risk evaluations were formulated (for example to define species differences and human variation or to perform quantitative in vitro-in vivo extrapolations). To increase the regulatory use and acceptance of NAMs for biokinetics for these ADME considerations within risk evaluations, the development of test guidelines (protocols) and of overarching guidance documents is considered a critical step. To this end, a need for an expert group on biokinetics within the Organisation of Economic Cooperation and Development (OECD) to supervise this process was formulated. The workshop discussions revealed that method development is still required, particularly to adequately capture transporter mediated processes as well as to obtain cell models that reflect the physiology and kinetic characteristics of relevant organs. Developments in the fields of stem cells, organoids and organ-on-a-chip models provide promising tools to meet these research needs in the future.

1 Introduction

There are clear societal and scientific needs for the development and validation of predictive animal-free methods for safety evaluations to prevent adverse effects in humans caused by exposure to chemicals (EU, 2010; OECD, 2018a; Oredsson et al., 2019). Whereas much work has been devoted to the development of in vitro screening methods to capture biological effects (toxicodynamics) of chemicals, insight into the absorption, distribution, metabolism and excretion (i.e., ADME/biokinetics) of chemi-
cals also plays a central role in next-generation risk evaluations that move away from animal experimentation and towards animal-free methods (Albrecht et al., 2019; Bessemens et al., 2014; Coecke et al., 2013; Despréz et al., 2018; Thomas et al., 2019; Wambaugh et al., 2018). These biokinetic data are needed for the adequate design of in vitro toxicity studies with respect to the application of physiologically relevant chemical test concentrations and inclusion of relevant metabolites for testing. In addition, biokinetic data play a crucial role in the process of quantitative in vitro-to-in vivo extrapolation (QIVIVE) of in vitro toxicity results to obtain equivalent oral/skin/inhalation human potency estimates that can be compared with human exposure to define the risk (Blaauboer, 2010; DeJongh et al., 1999; Louisse et al., 2017; Wambaugh et al., 2018; Yoon et al., 2012).

New approach methodologies (NAMs) for biokinetics include both in vitro approaches (experiments using preferably human tissue material or cells), computational (in silico) approaches, and combinations thereof. Examples of different in vitro methods for kinetics that can be performed with animal or human tissue material are: Transwell studies using Caco-2, MDCK or LLC-PK1 monolayers for intestinal absorption and transporter studies, artificial human skin for skin absorption studies, metabolism models with (primary) human or animal (liver) cells or tissue fractions, plasma protein and tissue binding assays, and placenta transport experiments with human BeWo cells (Punt et al., 2017; Strikwold et al., 2017; Wilk-Zasada et al., 2015). Recent research also focusses on the development of organ-on-a-chip models to improve the accuracy of individual methods by better physiological resemblance and to integrate the methods for different organs within one platform (Maschmeyer et al., 2015; McAleer et al., 2019; Prantil-Baun et al., 2018; Santbergen et al., 2019; van der Made et al., 2019).

Different types of computational (in silico) approaches for biokinetics can be distinguished. In silico approaches are first of all used for predictions of the behavior of a chemical in a body based on the structural properties of the chemical. Examples are quantitative structure activity relationships (QSARs) for absorption (Hou et al., 2004) and different prediction models for tissue partitioning (Berezkhovskiy, 2004; Poulin and Theil, 2002; Rodgers and Rowland, 2006). Secondly, computational approaches are very powerful for the integration of the various in vitro and in silico findings with either classical (one- or two-compartment models) or physiologically based kinetic (PBK)/physiologically based pharmacokinetic (PB(P)K) models (Bessemens et al., 2014; Jamei, 2016; Louisse et al., 2017; Prantil-Baun et al., 2018; Punt, 2018). For example, in vitro methods using human cells or tissue fractions are generally used in combination with in silico calculated partition coefficients to parameterize PB(P)K models for predicting human biokinetics (Jamei, 2016, 2020; Jones and Rowland-Yeo, 2013; Wambaugh et al., 2018).

Despite the relevance of human- (fully animal-free) and animal-derived in vitro methods and the different in silico methods for biokinetics, the adoption of NAMs for kinetic considerations in regulatory risk evaluations is lagging behind (Gellatly and Sewell, 2019; Punt, 2018; Tan et al., 2018; Zhuang and Lu, 2016). This challenge and the ambition of the Dutch government to become world leading with respect to animal-free research by 2025 formed the starting point for Wageningen Food Safety Research (WFSR) and the National Institute for Public Health and the Environment (RIVM) to organize a workshop on this topic at The Lorentz Centre in Leiden in The Netherlands in October 2017. The general goal of the Lorentz Centre is to grant and facilitate scientific discussions that can lead to ground-breaking changes in any specific field of research. A team of 24 experts from science, industry, and regulatory institutions, including new generation toxicologists, came together to define a strategy to move the field of biokinetics forward. A priori defined goals were (1) to

Fig. 1: Developments that are needed to increase regulatory use and acceptance of NAMs for biokinetics in risk evaluations and support a transition towards next generation risk evaluations that move away from animal experimentation.
make an inventory of ADME considerations within regulatory risk evaluations that can be addressed using NAMs for biokinetics in both current and next generation risk evaluations that move away from animal experimentation, (2) to define what is needed to increase regulatory use and acceptance of available innovative biokinetic approaches, and (3) to define opportunities for new scientific developments. Based on the discussions around these questions and an evaluation of the recent literature, recommendations (see summary Fig. 1) were made on the steps that are needed to achieve a better incorporation of alternative methods for biokinetics in regulatory chemical risk evaluations.

2 Opportunities for NAMs for biokinetics in current and next generation risk evaluations that move away from animal experimentation

As the field of toxicology is moving away from “black box” animal experimentation towards approaches to better understand the internal concentrations of compounds in relation to their mechanisms of toxicity in humans (NRC, 2007; Thomas et al., 2017), the potential of NAMs for biokinetics is increasingly recognized by regulatory bodies (ECHA, 2014; EFSA, 2014; EMA, 2018; FDA, 2020). Formal incorporation of these approaches in regulatory toxicology (for, e.g., plant protection products, pharmaceuticals, chemicals or food additives) is, however, still limited (Gelatly and Sewell, 2019; Punt, 2018; Tan et al., 2018; Zhuang and Lu, 2016). To find means to increase the use of NAMs for biokinetics, an inventory was made of ADME considerations within regulatory risk evaluations that can be addressed with these approaches based on different EU regulations and guidelines in which the use of in vitro and in silico methods for kinetics methods is mentioned (Tab. 1, 2). Such an inventory is key to the future development of Organisation for Economic Co-operation and Development (OECD) guidelines. These can be guidance documents (GD) on how to use alternative methods to inform risk evaluations and test guidelines (TG) on how to perform a specific in vitro biokinetic study, which are generally combined with OECD harmonized templates (OHT; standard data formats for reporting) (OECD, 2018a).

Tab.1: Inventory of ADME considerations within EU regulatory risk evaluations that can be addressed using NAMs for biokinetics

<table>
<thead>
<tr>
<th>ADME consideration</th>
<th>NAMs currently included in guidance documents/regulations</th>
<th>Are biokinetic data required?</th>
</tr>
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<tbody>
<tr>
<td>Species differences</td>
<td>Are there differences in the profile of compound metabolites between the laboratory animals used in the toxicity study and humans?</td>
<td>Yes. However, only the qualitative aspects of species differences in metabolism are evaluated (i.e., differences in types of metabolites), while quantitative differences in rates of metabolic conversion can be just as important.</td>
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<tr>
<td></td>
<td>Are there differences in metabolic rate constants (rate by which a chemical is converted into its metabolites)?</td>
<td></td>
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<td></td>
<td>Is the default uncertainty factor for species differences in kinetics appropriate in risk evaluations?</td>
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<tr>
<td></td>
<td>What is the difference in plasma/tissue concentrations of a parent chemical/toxic metabolite between the laboratory animals of the toxicity study and humans (requires integration of in vitro data in kinetic models)?</td>
<td></td>
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<tr>
<td></td>
<td>Mentioned in (e.g.):</td>
<td></td>
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<tr>
<td></td>
<td>− ECHA endpoint specific guidance, including a fictional example of using PBK modelling and the development of assessment factors (ECHA, 2017b).</td>
<td></td>
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<tr>
<td></td>
<td>− Guidance on the Biocidal Products Regulation Volume III Human Health-Assessment and Evaluation (Parts B+C) (ECHA, 2017a).</td>
<td></td>
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<tr>
<td>Special population, interindividual human variation, drug-drug interactions</td>
<td>What is the expected plasma/tissue concentration of a drug or chemical in children?</td>
<td></td>
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<tr>
<td></td>
<td>Are there any subpopulations potentially at extra risk due to altered kinetics (e.g., ethnic-related polymorphism, as a result of lifestyle factors such as smoking or obesity, or related to diseases like chronic kidney failure)?</td>
<td></td>
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<td></td>
<td>EMA/CHMP/458101/2016 guideline on the reporting of physiologically based pharmacokinetic (PB(P)K) modelling and simulation: “Presently, the main purposes of PB(P)K models in regulatory submissions are to qualitatively and quantitatively predict drug-drug interactions (DDIs) and support initial dose selection in paediatric and first-in-human trials.” (EMA, 2018).</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No, but increasingly included in regulatory dossiers (Jamei, 2016).</td>
<td></td>
</tr>
<tr>
<td>ADME consideration</td>
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</tr>
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<td>----------------------------------------------------------------------------------</td>
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</tbody>
</table>
| Is the default uncertainty factor for human variation in kinetics appropriate in risk evaluations? | - Possibilities for PB(P)K modelling of human variation are mentioned in, e.g.: SCCS Notes of Guidance for the Testing of Cosmetic Ingredients (SCCS, 2018).  
- ECHA endpoint specific guidance, including a fictional example of using PBK modelling and the development of assessment factors (ECHA, 2017b). | No |
| Can any drug-drug interactions be expected?                                      | EMA/CHMP/458101/2016 guideline on the reporting of physiologically based pharmacokinetic (PB(P)K) modelling and simulation: “Presently, the main purposes of PB(P)K models in regulatory submissions are to qualitatively and quantitatively predict drug-drug interactions (DDIs) and support initial dose selection in paediatric and first-in-human trials.” (EMA, 2018). | No, but increasingly included in regulatory dossiers (Jamei, 2016) |
| Read-across                                                                       | Is the selected source chemical appropriate for read-across, having similar kinetics, including similar metabolites?       | No, read-across is voluntary. In case of REACH, considering the possibility for read-across is mandatory. This drives a need for insights into toxicokinetics. |
|                                                                                   | Mentioned in, e.g.:                                                    |                             |
|                                                                                   | - Guidance on the Biocidal Products Regulation Volume III Human Health Assessment and Evaluation (Parts B+C) (ECHA, 2017a). |                             |
| Route-to-route extrapolation                                                       | What is the dermal or inhalation equivalent exposure level of an oral No-Observed-Adverse-Effect Level?                    | No |
|                                                                                   | Mentioned in, e.g.:                                                    |                             |
|                                                                                   | - ECHA endpoint specific guidance, including a fictional example of using PBK modelling and the development of assessment factors (ECHA, 2017b). |                             |
|                                                                                   | - Guidance on the Biocidal Products Regulation Volume III Human Health –Assessment and Evaluation (Parts B+C) (ECHA, 2017a). |                             |
| Internal exposure-based waiving                                                    | Is intestinal/skin/inhalation uptake limited, allowing to waive internal exposure?                                           | No |
|                                                                                   | Mentioned in, e.g.:                                                    |                             |
|                                                                                   | - EFSA guidance for submission for food additive evaluations (EFSA, 2012). |                             |
|                                                                                   | - Specific rules for adaptation provided for, e.g., repfoxt tests in Annex IX of Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). |                             |
|                                                                                   | Selection of chemicals for further testing based on highest expected internal exposure.                                           | No |
|                                                                                   | Mentioned in (e.g.):                                                    |                             |
|                                                                                   | - EFSA guidance on the data required for the risk assessment of flavorings to be used in or on foods (EFSA, 2010). |                             |
| Dose extrapolation                                                                | Extrapolation of effect levels in animal studies obtained at high doses to lower human exposures.                          | No |
|                                                                                   | Mentioned in (e.g.):                                                    |                             |
|                                                                                   | - ECHA endpoint specific guidance, including a fictional example of using PBK modelling and the development of assessment factors (ECHA, 2017b). |                             |
|                                                                                   | - Guidance on the Biocidal Products Regulation Volume III Human Health Assessment and Evaluation (Parts B+C) (ECHA, 2017a). |                             |
Tab. 2: Inventory of regulatory topics in next-generation risk evaluations for which insights into biokinetics (using NAMs) are relevant

<table>
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<tr>
<th>Goal</th>
<th>Regulatory topics that can be addressed a</th>
</tr>
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| Guiding the design of in vitro toxicity studies | – What are physiologically relevant internal exposure conditions of the chemical in humans?  
– Which metabolites need to be taken into account in the in vitro studies?  
– Does the in vitro toxicity assay have the adequate metabolic competence?  
– Which tissues are relevant to include in the toxicity determination?  
– How does the chemical behave in an in vitro system with respect to protein, lipid and plastic binding, and in terms of evaporation?  
– Aiding the development of novel in vitro systems like organ-on-chip systems to determine dimensions and operating conditions. |
| Quantitative in vitro to in vivo extrapolations (QIVIVE) | – What are the internal levels of a chemical that are reached in a certain exposure scenario and how do these relate to in vitro effect concentrations (forward dosimetry approach)?  
– Translation of an in vitro biological effect concentration/exposure (e.g., EC10, BMC10) to an equivalent oral/skin/ inhalation dose/exposure.  
– Improving the interpretation of human biomonitoring studies to reconstruct internal exposure levels for exposure assessment, QIVIVE and IVIVE-PBPK. |

a Topics related to human variation, route-to-route extrapolation, dose-extrapolations, read-across, and exposure-based waiving/priority setting, as described in Table 1 for current risk assessment procedures, remain of importance in next-generation risk evaluations.

2.1 Opportunities in current risk evaluations

Table 1 provides a summary of ADME considerations within regulatory risk evaluations that can be addressed with NAMs for biokinetics. This overview was obtained by exploring EU guidelines for chemical risk evaluations within different domains (e.g., pharmaceuticals, chemical substances, food additives, cosmetics) (ECHA, 2013, 2014, 2016, 2017a-c; EFSA, 2010, 2014; EMA, 2018; EU, 2010; SCCS, 2018).

Of the different in vitro and in silico approaches that are available for biokinetics, PB(P)K modelling is the most frequently mentioned tool. For example, in the field of pharmaceuticals, PB(P)K modelling plays a significant role to support initial dose selection in first-in-human or pediatric trials as well as in the prediction of drug-drug interactions (EMA, 2018; Jamei, 2016; Sato et al., 2017; Shebley et al., 2018; Taskar et al., 2019). PB(P)K models within this context are generally developed bottom-up, starting from in vitro and/or in silico kinetic data, after which the models are evaluated and/or fine-tuned against in vivo kinetic data. The label of several approved drugs already contains information on potential drug-drug interactions obtained by applying such PB(P)K modelling approaches (Jamei, 2016; Sato et al., 2017; Shebley et al., 2018; Taskar et al., 2019). Other documents, such as the guidance for testing of cosmetic ingredients by the European Scientific Committee on Consumer Safety (SCCS, 2018) and the guidance on information requirements and chemical safety assessment by the European Chemicals Agency (ECHA) (ECHA, 2014), refer to (bottom-up) PB(P)K modelling as well. In those cases, possibilities for the use of PB(P)K models are most frequently seen to predict species difference and/or human variation in plasma concentrations for refinement of the default inter/intra-species uncertainty factors for kinetics, or to perform dose or route-to-route extrapolations. However, so far, the actual use of PB(P)K modelling to meet these latter regulatory topics has been limited (Gellatly and Sewell, 2019; Punt et al., 2017; Tan et al., 2018).

Apart from PB(P)K modelling, stand-alone in vitro kinetic data (i.e., not integrated in a kinetic model) can also be requested by regulatory authorities. A recent example is EC Regulation No 1107/2009, which requires the inclusion of in vitro metabolism studies (with microsomes or intact cell systems) in regulatory dossiers of plant protection active substances in order to determine the human relevance of the animal species chosen for the toxicological studies in a dossier. A recent EFSA workshop focused on the key elements to be considered for the interpretation of comparative in vitro metabolism studies and the minimum amount of information that must be obtained to satisfy the data requirement (EFSA, 2019).

Stand alone in vitro/in silico kinetic data may also be used for exposure-based waiving of toxicity tests. An example can be found in the EFSA “Guidance for submission for food additive evaluations” (EFSA, 2012). In this guideline, it is indicated that “a negligible metabolic conversion of an additive by gastrointestinal fluids or the gut microbiota (in vitro) and negligible absorption (in vitro), together with absence of genotoxicity, provides scientific justification for not undertaking higher-tier kinetic and toxicological studies.” Another example of the use of kinetic data for exposure-based waiving can be found within the European REACH legislation for industrial chemicals for some cases. Within REACH, there is no specific requirement to generate biokinetic data (ECHA, 2017b). For chemicals that are manufactured or imported in quantities of 10 tons or more, an assessment needs to be made of the biokinetic behavior of the substance to the extent that can be derived from the relevant available information (Annex IX Regulation (EC) No 1907/2006). These kinetic data, which may include data from NAMs, can be used to guide the design of appropriate toxicity studies or to waive studies. For example, no reproductive toxicity tests are required if a substance “is of low toxicological activity (no evidence of toxicity seen in any of the tests available), it can be proven from biokinetic data that no systemic absorption occurs via relevant routes of exposure (e.g., plasma/blood concentrations below detection limit using a sensitive method and absence of the substance and of metabolites of the substance in urine, bile or exhaled air) and there is no or...
no significant human exposure” (Annex IX Regulation (EC) No 1907/2006). Given that all three requirements need to be met as a waiver for reproductive toxicity tests, the impact of the biokinetic data as such will remain in context.

A final area in which NAMs for biokinetics are emerging is the justification of read-across. The principle of read-across is to predict endpoint information for a data-poor substance by using data from (an)other related substance(s) (Cronin and Yoon, 2019; Escher et al., 2019). The role of biokinetics in read-across becomes clear from different ECHA read-across guidelines for REACH as well as the biocidal product regulation (ECHA, 2013, 2017a, 2017b). According to these guidelines, the similarity in the biokinetics (e.g., type of metabolites and speed of metabolism) between the target and source substance(s) needs to be considered in the justification of the read-across approach (ECHA, 2013).

2.2 Opportunities in next generation risk evaluations that move away from animal experimentation

Compared with current risk evaluation procedures (Tab. 1), an even more important role of biokinetics is foreseen in next-generation risk evaluations that move away from animal experimentation (summarized in Tab. 2). Questions related to the extrapolation of animal data to humans are expected to become less important, but methodologies that incorporate human variation and allow route-to-route extrapolation, read-across and exposure-based waiving/priority-setting without the use of experimental animals will remain and may even become more important.

In addition, new applications of biokinetics will emerge. For example, NAMs for biokinetics will play a crucial role in the design of in vitro toxicity experiments. Particularly, given that not all in vitro biological effect assays include metabolism, it is important to characterize the metabolites that are formed in the human body and, if possible, to subsequently assess the potential toxic effects of these metabolites (Escher et al., 2019; Wilk-Zasadna et al., 2015). In addition, biokinetic information provides insights into whether specific exposure scenarios, such as irregular peak exposure concentrations, may lead to different cellular responses than continuous exposure to lower dose levels. In some cases there can also be a significant time delay between in vitro medium concentrations and the internal cell concentration (McAleeer et al., 2019; Rozman and Doull, 2000). Such temporal concentration effects can be important to consider in the design of an in vitro toxicodynamic experiment and the subsequent translation of the results to actual human exposure scenarios. Finally, PB(P)K modelling can also be used as a tool to guide the design of in vitro experiments, for example by providing insight into dimensions and operating conditions for organ-on-a-chip systems (Abaci and Shuler, 2015; Hartung et al., 2017; Sung et al., 2014).

Another new role of biokinetics (and particularly PB(P)K modelling) in next-generation risk evaluations is to put in vitro biological activity data in the context of human exposure, also called QIVIVE (quantitative in vitro to in vivo extrapolations). This can be done based on a forward dosimetry approach (i.e., comparing predicted or observed internal plasma or tissue levels with the in vitro concentration-response curves) or based on a reverse dosimetry approach (i.e., extrapolating the in vitro effect concentrations to equivalent oral exposures) (Dancik et al., 2013; Rostami-Hodjegan, 2012; Yoon et al., 2012). An example of forward dosimetry is the comparison of an internal exposure with in vitro biological effect concentrations in so-called exposure-to-activity ratios (EARs) (Becker et al., 2014; Dent et al., 2019). This approach provides predictions on whether certain in vitro biological effects are likely to be induced in a certain exposure scenario and provides a means to prioritize the key biological targets, focusing on those that are elicited at exposures (concentration, duration, frequency, delay) that are relevant for the expected internal levels. Furthermore, EARs of different compounds can be compared to place the exposure-activity data of a chemical relative to a known reference compound (Becker et al., 2014; Dent et al., 2019). In contrast to forward dosimetry, reverse dosimetry approaches focus on the extrapolation of in vitro effective or benchmark concentrations (e.g., EC10, BCM10) into equivalent in vivo exposure-response curves (Louisse et al., 2017; Wetmore, 2015; Yoon et al., 2012). Kinetic modelling (including PB(P)K modelling) plays an important role in obtaining the required insights into the relation between external and internal exposure for QIVIVE, based either on forward or reverse dosimetry. A prerequisite within the context of animal-free testing strategies is that these models are developed based on in vitro and/or in silico input data themselves (Rostami-Hodjegan, 2012). Furthermore, good PB(P)K modelling practice as well as the quantification of uncertainty and variability within PB(P)K is of importance to gain confidence in the predictions and to estimate the credible interval around the predicted external dose levels that are linked to the in vitro effect concentrations (Jamei et al., 2009a; McNally et al., 2012, 2018; Paini et al., 2019; Wambaugh et al., 2019). Alternatively to kinetic (computer) modelling, exposure measurements from human biomonitoring studies (blood, urine, or milk) could be used for QIVIVE, though only for chemicals that are already marketed (Becker et al., 2014). Also then, PB(P)K modelling can be used for the interpretation of the human biomonitoring data, for example to translate a urinary concentration back to the corresponding blood concentration (Clewell et al., 2008; McNally et al., 2012, 2019; Rostami-Hodjegan, 2012).

A final role for biokinetics in next-generation risk evaluations is to establish freely available concentrations in in vitro assays. Irrespective of whether the internal predicted exposure levels are compared with the in vitro biological activity data based on forward dosimetry or based on reverse dosimetry, it is important to account for potential differences between the free concentration of the chemical in plasma (or the relevant organ or even cell under consideration) vs. the chemical in the in vitro toxicity test. Apart from protein and lipid binding, the free concentration of a chemical in an in vitro system can also be impacted by evaporation and/or binding to plastic or filter material (e.g., microtiter plates), reviewed in Kramer et al. (2015). Calculation tools to predict the free available concentration in an in vitro experiment (Armitage et al., 2014; Fischer et al., 2017; Fisher et al., 2019; Groothuis et al., 2015; Kramer, 2010; Zaldivar Comenges et al., 2017) and the fraction unbound in plasma (Lobell and Sivarajah, 2003; Yamazaki and Kanaoka, 2004) will therefore become crucial tools in next-generation risk evaluations.
Increasing the regulatory acceptance and use

Though the possibilities for NAMs for biokinetics are manifold (see above evaluation), formal inclusion is still limited due, amongst others, to the lack of robust and reproducible methods (Punt et al., 2017; Sewell et al., 2017; Tan et al., 2018; Zhuang and Lu, 2016). To this end, the experts at the workshop recommended to set up a continuous OECD expert group on biokinetics, analogous to the expert groups on, e.g., genotoxicity and skin sensitization, to stimulate and guide the generation of TGs for methods to generate biokinetic parameters in a robust and reproducible way and of GDs for their application in risk assessment procedures (see summary Fig. 1). In the meantime, this working group has been formally established. Within the context of the OECD, a GD focuses on how experimental results can be used to inform risk evaluations (i.e., to answer a specific regulatory question), and a TG focuses on how to perform a specific study (OECD, 2009). In the area of biokinetics, currently OECD TG 417 (with relative harmonized template OHT58) describes in vivo studies, providing the protocol for the conduct of studies on mass balance, absorption, bioavailability, tissue distribution, metabolism, excretion, and basic kinetic parameters (e.g., $C_{\text{max}}$, AUC) (OECD, 2010).

3.1 Standardization and development of TGs

The experimental conditions of in vitro kinetic studies can have a significant impact on the kinetic parameters that are obtained (e.g., metabolic clearance, absorption rates). This leads to a high variability in the estimates for these parameters for the same chemical among different studies as recently shown for metabolic clearance experiments (Louisse et al., 2020; Ring et al., 2011). One currently needs to evaluate each study in detail to find out whether the results are in agreement with what is expected in vivo and reliable enough to use for, e.g., PB(P)K modelling (e.g., is the intrinsic clearance measured at concentrations below the $K_m$, are the Michaelis-Menten kinetics ($K_m$ and $V_{\text{max}}$) obtained at incubation conditions in which not too much of the substrate is depleted, which solvents were used as vehicle and at which concentration ranges. Expression and/or activity of intestinal transporters, such as P-gp (Nishimuta et al., 2013), CYP (Martinez et al., 2019; Nishimuta et al., 2013), UGT (Chiu and Huskey, 1998; De Guchi et al., 2011) and SULT (Punt et al., 2007; Wang et al., 2009) are differently expressed/active in rat, dog, monkey and mouse compared to humans. By measuring metabolic conversions with GIVIMP guidance into account with respect to the critical aspects that need to be considered in the design of in vitro kinetic experiments in general. For regulatory use, the TGs should furthermore provide specific boundaries (test concentrations, time-point, etc.) within which experiments need to be performed to obtain results that are relevant to the human situation.

3.2 Development of GDs

Providing that the kinetic data are obtained with protocols that lead to robust and reproducible results, an important second step is to develop GDs that aid to systematically interpret submitted kinetic data (e.g., in vitro-in vivo scaling, defining uncertainties) and to define acceptance criteria (e.g., biological and technical considerations like maximum incubation times and acceptable variability in the estimates for these parameters for the same chemical among different studies as recently shown for metabolic clearance experiments (Louisse et al., 2020; Ring et al., 2011). One currently needs to evaluate each study in detail to find out whether the results are in agreement with what is expected in vivo and reliable enough to use for, e.g., PB(P)K modelling (e.g., is the intrinsic clearance measured at concentrations below the $K_m$, are the Michaelis-Menten kinetics ($K_m$ and $V_{\text{max}}$) obtained at incubation conditions in which not too much of the substrate is depleted, which solvents were used as vehicle and at which concentration ranges. Expression and/or activity of intestinal transporters, such as P-gp (Nishimuta et al., 2013), CYP (Martinez et al., 2019; Nishimuta et al., 2013), UGT (Chiu and Huskey, 1998; De Guchi et al., 2011) and SULT (Punt et al., 2007; Wang et al., 2009) are differently expressed/active in rat, dog, monkey and mouse compared to humans. By measuring metabolic conversions with GIVIMP guidance into account with respect to the critical aspects that need to be considered in the design of in vitro kinetic experiments in general. For regulatory use, the TGs should furthermore provide specific boundaries (test concentrations, time-point, etc.) within which experiments need to be performed to obtain results that are relevant to the human situation.

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Providing that the kinetic data are obtained with protocols that lead to robust and reproducible results, an important second step is to develop GDs that aid to systematically interpret submitted kinetic data (e.g., in vitro-in vivo scaling, defining uncertainties) and to define acceptance criteria (e.g., biological and technical considerations like maximum incubation times and acceptable variability in the estimates for these parameters for the same chemical among different studies as recently shown for metabolic clearance experiments (Louisse et al., 2020; Ring et al., 2011). One currently needs to evaluate each study in detail to find out whether the results are in agreement with what is expected in vivo and reliable enough to use for, e.g., PB(P)K modelling (e.g., is the intrinsic clearance measured at concentrations below the $K_m$, are the Michaelis-Menten kinetics ($K_m$ and $V_{\text{max}}$) obtained at incubation conditions in which not too much of the substrate is depleted, which solvents were used as vehicle and at which concentration ranges. Expression and/or activity of intestinal transporters, such as P-gp (Nishimuta et al., 2013), CYP (Martinez et al., 2019; Nishimuta et al., 2013), UGT (Chiu and Huskey, 1998; De Guchi et al., 2011) and SULT (Punt et al., 2007; Wang et al., 2009) are differently expressed/active in rat, dog, monkey and mouse compared to humans. By measuring metabolic conversions with GIVIMP guidance into account with respect to the critical aspects that need to be considered in the design of in vitro kinetic experiments in general. For regulatory use, the TGs should furthermore provide specific boundaries (test concentrations, time-point, etc.) within which experiments need to be performed to obtain results that are relevant to the human situation.
mans only express one MDR1-encoded protein (P-glycoprotein, P-gp), and BCRP expression is high in rodent kidneys but low in humans (Chu et al., 2013). Differences in physiology may need to be accounted for as well when predicting absorption on the basis of NAMs, including differences in the small intestinal transit time (Sutton, 2004), the intestinal pH and radius (Dressman and Yama-
da, 1991), microbiome composition (Behr, 2019), and differences in the structure of the gastro-intestinal tract, e.g., no circular folds in the dog (Slatter, 2003) that affect the surface area. Differences in distribution in rat, mouse, dog and human have been associat-
ed with differences in protein binding and active transport into or-
gans (Grover and Benet, 2009). In case of renal clearance, species differences can range from 1.6- to 13-fold as a result of differen-
tes in passive renal clearance alone (Walton et al., 2004). Ideally, all these kinetic processes are integrated in a PB(P)K model to al-
low evaluation of species differences on plasma concentrations of the chemical (Musther et al., 2017). Though PB(P)K models can be made as complex as needed, in practice a balance must be found between accuracy (and therefore complexity) and sim-
plicity (ease of use) (Bois, 2010). This also needs to be kept in mind for the development of GDs. Rather than directly focusing on covering all sources of species differences in kinetics, it will be important first to enhance the use of individual (human-based) NAMs for kinetics and to make sure that these are sufficiently un-
derstood and provide robust results for application in a regula-
ory context. In case of hepatic clearance studies, for example, the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) recently established a systematic frame-
work to characterize in vitro methods for human hepatic meta-
Bolic clearance in terms of their design, applicability and perform-
ance (Gouliarmou et al., 2018). In addition, guidelines are need-
ed on the integration of in vitro kinetic data into in silico kinetic models (Paini et al., 2019).

4 Availability of NAMs for human-relevant biokinetics and the opportunities of new scientific developments

4.1 Methods that are considered ready for the development of TGs

In Table 3, priorities are set for the methods available for different kinetic processes for standardization and the development of TGs and research needs. Particularly, the generation of standardized approaches and a TG for hepatic metabolic clearance with primary hepatocytes or hepatic microsomes/S9 is considered a high priori-
ty. Gouliarmou et al. (2018) already made a start by establishing a systematic framework to characterize in vitro methods for human hepatic metabolic clearance to provide a basis for harmonization within the OECD. A TG for hepatic clearance can potentially be developed further into TGs for metabo-lite identification and extra-hepatic (i.e., intestine, kidney, lung, skin) clearance.

The development of TGs for different in vitro and/or in silico approaches that predict partition coefficients, fraction unbound in plasma, blood-plasma ratio, and in vitro biokinetics (free fraction as a result of protein binding, plastic binding or evaporation) is also considered relevant. Based on ample experience with the use of these in silico approaches in kinetic model development and QIVIVE (Armitage et al., 2014; Fischer et al., 2017; Fisher et al., 2019; Kramer, 2010; Zaldivar Comenges et al., 2017), guidance on their use and applicability domain effectively can be devel-
oped. A final priority can be given to developing a technical guide-
line for Caco-2 (MDCK, LLC-PK1, or PAMPA) absorption studies to measure passive intestinal permeability. Though alternatives to Caco-2 permeability experiments (co-cultures, 3D, medium flow, peristaltic stretching) have been developed (Costa and Ahlu-
walla, 2019), Caco-2 transwell studies remain the best established among different protocols for permeability measurement (Hu-
batsch et al., 2007) and can serve as a starting point for technical guidance development. Opportunities for the development of TGs for lung absorption experiments and transporter studies (intestine, kidney, lungs) are currently considered to be limited as further sci-
entific research is needed to identify physiologically relevant cells and define scaling methods (see Section 4.2). An OECD TG al-
ready exists for in vitro dermal absorption studies (OECD, 2004), and a TG on measuring induction and inhibition processes of meta-
bolic enzymes is currently being developed (Bernasconi et al.,
2019; OECD, 2019).

4.2 Opportunities for new scientific developments

Whereas much can be achieved to increase regulatory acceptance and use of NAMs for biokinetics by standardization and the de-
vopment of guidelines, this is currently not possible for all as-
pects of biokinetics. Especially capturing transporter-mediated absorption (intestine), distribution (e.g., blood-brain barrier, pla-
enta, organ uptake) or excretion (e.g., active renal excretion, bili-
ary secretion) with NAMs is still difficult (Clerbaux et al., 2018,
2019; Taskar et al., 2019). Clerbaux et al. (2018) performed a survey among seventy-three experts in the field of transporter ki-
netics to evaluate the applicability of transporter data for ADME con-
siderations in regulatory risk evaluations. Respondents high-
lighted the complex interplay with metabolic enzymes and other transporters, species differences, lack of specific transporter sub-
strates and inhibitors, loss of cell polarity, lack of negative con-
trols, problems of inter-laboratory variability, and lack of stan-
dardized protocols as key challenges of transporter models (Cler-
baux et al., 2018, 2019). For the use of transporter data in PB(P)K models, scaling approaches also are needed to account for the quantitative difference of transporters in cell lines versus tissues (Clerbaux et al., 2018; Galetin et al., 2017; Taskar et al., 2019).

Apart from the specific challenges with transporter kinetics, there is also a general need for new cell models to overcome many of the hurdles of the classically used tumor-derived cell lines as well as primary cell cultures. Widely used tumor-derived cell lines (e.g., Caco-2 intestinal cells, HepaRG or HepG2 liv-
er cells and A549 lung cells) all have shortcomings to some ex-
tent. These either relate to the expression of metabolic enzymes and transporters or to the barrier properties (e.g., the ability to grow to confluence, polarized cell layer(s), mucus secretion) (Ehrhardt et al., 2017; Van Breemen and Li, 2005; Wilk-Zasada et al., 2015). These shortcomings affect not only kinetic studies (measurements of metabolism or permeability rates) but also in vitro toxicodynamic studies that require, for example, metabolic activation. In contrast to tumor-derived cell lines, primary cells closely mimic the physiological state of cells in vivo but can on-
Tab. 3: Overview of the current availability of NAMs for different kinetic parameters and research needs

<table>
<thead>
<tr>
<th>Kinetic processes</th>
<th>Research needs</th>
<th>Priority for OECD TG development</th>
<th>Related regulatory topic (see Tab. 1, 2 and references therein)</th>
</tr>
</thead>
</table>
| Liver clearance   | – Standardization of incubation methods with (cryopreserved) primary human hepatocytes or S9/microsomes.  
                   | – Evaluation of the applicability of non-primary cells (e.g., HepaRG™ cells).                        | Hepatic metabolic clearance studies with (cryopreserved) primary human hepatocytes or S9/microsomes are considered a high priority for the development of a TG. Well-established protocols are available. Hepatic clearance data are relevant to a large range of regulatory topics (see Tab. 1, 2) as a major determinant of blood and tissue concentrations (Cao et al., 2006; Musther et al., 2014). Potential for extension of the TG to HepaRG clearance studies as an alternative to primary cells, which are of limited availability. | Species differences  
                   |                                                                                 |                                                                                                     | Human variation  
                   |                                                                                 |                                                                                                     | Route-to-route extrapolations  
                   |                                                                                 |                                                                                                     | QIVIVE |
| Metabolite        | – Standardization of methods that are used based on incubations with S9/microsomes/ (cryopreserved) primary hepatocytes.  
                   | – Higher sensitivity and standardization of analytical chemical techniques and improved methods to characterize metabolites.  
                   | – Applicability of non-primary cell cultures (e.g., HepaRG™).                                        | TGs for comparative metabolite studies with S9/microsomes/ (cryopreserved) primary hepatocytes are considered a high priority, particularly as comparative metabolite studies are a data requirement within the EU regulation on plant protection products (EC, 2013). Potential for extension of the TG to HepaRG clearance studies as an alternative to primary cells, which are of limited availability. | Species differences in type of metabolites (required for plant protection products)  
                   | identification |                                                                                 | Read-across  
                   |                                                                                 |                                                                                                     | Guiding in vitro toxicity test design  
                   |                                                                                 |                                                                                                     | Identification of toxic agent |
| Extrahepatic      | – Show proof of principle with S9/microsomes/ (cryopreserved) primary cell cultures of extrahepatic tissues (with a focus on kidney, lung, intestine and skin).  
                   | – Development of non-primary cell cultures with physiologically relevant metabolic capacity.  
                   | – Contribution of the gut microbiota to the metabolic clearance of chemicals.                         | When TGs for hepatic metabolic clearance and metabolic formation are established, these can serve as a template for clearance measurements with S9/microsomes/ (cryopreserved) primary cell cultures of extrahepatic tissues (with a focus on kidney, lung, intestine and skin). | Species differences  
                   | metabolic       |                                                                                 | Human variation  
                   | clearance and    |                                                                                 | Route-to-route extrapolations  
                   | metabolite       |                                                                                 | QIVIVE |
                   | formation        |                                                                                 | Guiding in vitro toxicity test design  
                   |                                                                                 |                                                                                                     | Identification of toxic agent |
| (Passive)         | – Standardization of transwell absorption experiments (e.g., Caco-2).  
                   | – Exploring value of added complexity (co-cultures, 3D, medium flow, peristaltic stretching).      | Although protocols for Caco-2 transwell absorption studies are well established (Hubatsch et al., 2007), the applicability domain of a TG for these studies will be limited to passive absorption as challenges still exist in the adequate scaling of transporter-mediated influx/efflux processes (Clerbaux et al., 2018). | Route-to-route extrapolations  
                   | intestinal       |                                                                                 | Exposure based waiving and priority setting  
                   | absorption       |                                                                                 | QIVIVE |
                   |                                                                                 |                                                                                                     | Guiding in vitro toxicity test design |
| Tissue binding    | – Standardization and the development of guidance documents of available in vitro and/or in silico methods. | Well-established in vitro methods and in silico calculation tools (Peters, 2012) can form the basis for the development of TGs. | Species differences  
                   | (fraction        |                                                                                 | QIVIVE  
                   | unbound in plasma, |                                                                                 | Guiding in vitro toxicity test design |
                   | partition         |                                                                                 |                                                                                                     | methods)  
                   | coefficients)    |                                                                                 |                                                                                                     | |

a The mentioned in vitro and in silico methods for different kinetic processes should be considered as providing parts of the information that is needed to answer the mentioned regulatory topics. An integrated approach (combined in vitro and in silico approaches) will be needed to cover the regulatory topics as a whole.
ly be kept in culture for a limited time. Hence, developments are needed to overcome these different challenges.

New technologies involving telomerase overexpression in primary cells, induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs), or adult stem cells (ASCs) as well as new culturing techniques like 3D spheroid or organoid cultures and organ-on-a-chip technology are expected to provide novel solutions in the field of NAMs for biokinetics.

Relevant examples include the following developments:

- a non-cancerous human renal proximal tubular cell line, RPTEC/TERT1, that has been utilized successfully for several long-term kinetic studies of cyclosporine A, cisplatin and ade-fovir, showing promising results due to the expression of many relevant transporters and metabolizing enzymes (Aschauer et al., 2015; Wilmes et al., 2015),

<table>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro biokinetics (fate of a compound in an in vitro assay)</strong></td>
<td>– Standardization and development of guidance documents for available calculators based on physicochemical properties</td>
<td>Various in silico tools are available for animals and humans with different degrees of complexity but outcomes may vary depending on the applicability domain (Groothuis et al., 2015).</td>
<td>– QIVIVE&lt;br&gt;– Guiding in vitro toxicity test design</td>
</tr>
<tr>
<td><strong>(Passive) lung absorption</strong></td>
<td>– Development of non-physiologically relevant primary cell cultures with physiologically relevant transporter activity and metabolic capacity.</td>
<td>Availability of in vitro tools is still limited. Calu-3 monolayer assays are currently the most promising for regulatory use (Ong et al., 2013).</td>
<td>– Route-to-route extrapolations&lt;br&gt;– Exposure based waiving and priority setting&lt;br&gt;– QIVIVE&lt;br&gt;– Guiding in vitro toxicity test design</td>
</tr>
<tr>
<td><strong>Transporter mediated absorption or excretion processes</strong></td>
<td>– Development of in vitro methods that contain physiologically relevant transporter activity (required for intestine, kidney, liver, placenta, blood-brain barrier). – Development of adequate scaling methods.</td>
<td>Low priority due to limitations in currently available methods as described in Section 4.2 (Clerbaux et al., 2018, 2019), current capabilities and challenges are highlighted in a recent review (Taskar et al., 2019).</td>
<td>– Species differences&lt;br&gt;– Human variation&lt;br&gt;– Route-to-route extrapolations&lt;br&gt;– QIVIVE&lt;br&gt;– Guiding in vitro toxicity test design</td>
</tr>
<tr>
<td><strong>Induction and inhibition processes of metabolic enzymes</strong></td>
<td>Currently being developed (Bernasconi et al., 2019; OECD, 2019b). CYP induction/inhibition can be seen as biomarker for metabolic competence of cells and as potential mechanism of action.</td>
<td>– Human variation, including drug-drug interactions&lt;br&gt;– Species differences</td>
<td></td>
</tr>
<tr>
<td><strong>Dermal absorption</strong></td>
<td>OECD TG 428 already exists.</td>
<td>– Route-to-route extrapolations&lt;br&gt;– QIVIVE&lt;br&gt;– Guiding in vitro toxicity test design</td>
<td></td>
</tr>
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</table>
Although these examples show that in vitro approaches that better reflect human physiology are emerging fast, the applicability of these approaches to obtain kinetic parameters still needs be explored (see summary Fig. 1).

5 Integration of NAMs for biokinetics in a next-generation risk assessment framework

New developments in animal-free methods for biokinetics should not stand by themselves. Efforts are needed to seamlessly connect different approaches within next-generation risk assessment, particularly to link the biokinetics and the adverse effects (toxicodynamics) of a chemical. With next generation risk assessment frameworks, methods like high-throughput transcriptomic and high-content imaging followed by more specific assays play a crucial role in the screening of chemicals for biological effects (Thomas et al., 2019). As long as the results of these assays provide quantitative information (e.g., concentration/exposure response curves), kinetic data can be linked to these biological effect assays to obtain equivalent oral/skin/ inhalation human potency estimates that can be compared with human exposure estimates to define the risk.

For the use of in vitro biological effect data and QIVIVE, in vitro toxicity studies should be able to pick up all relevant interactions with biological targets or pathways. The adverse outcome pathway (AOP) framework currently provides the most effective framework to organize and describe biological effects in terms of molecular initiating events (MIEs), key events (KEs), and key event relationships (KERs) in relation to an adverse outcome (Leist et al., 2017; Vinken et al., 2017). At present, a direct link between biokinetics (including QIVIVE) and AOPs is not straightforward. Consideration of biokinetics is not incorporated directly into an AOP description, particularly given that AOPs are not chemical-specific. In addition, AOPs are currently mainly qualitative in nature, providing (narrative) descriptions of the KERs, i.e., the relation between MIEs, KEs, and an adverse outcome (Edwards et al., 2015; Vinken, 2018). Though qualitative AOPs are relevant for hazard identification, moving towards the use of AOPs in risk evaluations also requires quantitative dose-response and time-course information. A relevant new development in this respect are so-called quantitative AOP (qAOP) approaches that focus on the simulation of the dynamic processes linking a MIE with an adverse outcome using different modelling approaches (Conolly et al., 2017; Schultz and Watanabe, 2018; Zgheib et al., 2019). Such qAOPs can be linked to PB(P)K modelling results. The in vitro exposure that is expected to perturb a MIE or KE would then provide a starting point to estimate an external exposure scenario of possible concern. Therefore, the development of qAOPs will be an important next step to link biokinetics and toxicodynamics within next-generation risk assessment (Edwards et al., 2015; Vinken, 2018).

6 Conclusion

Figure 1 provides a schematic overview of the workshop results. Overall, it can be concluded that opportunities for the implementation of NAMs for biokinetics in current regulatory risk assessment are to

– evaluate interspecies differences,
– simulate special populations and human interindividual variability,
– perform read-across,
– perform route-to-route extrapolations,
– set priorities (e.g., exposure-based waiving),
– perform dose extrapolations, and
– guide in vitro toxicity test selection and design.

When switching to a fully animal-free regulatory risk assessment for humans in the future, these methods will be necessary to also

– guide in vitro toxicity test selection and design, and
– enable QIVIVE.

Given the importance of NAMs for biokinetics in a successful transition towards next-generation toxicity testing strategies, both regulatory and scientific experience with in vitro/in silico biokinetic approaches must increase. To facilitate the use of human-relevant NAMs for biokinetics, GDs are needed that provide information on

– how NAMs for biokinetics should be designed to address a regulatory topic, including, for example, how the different parameters should be scaled and integrated in a kinetic model,
– the essential kinetic parameters that are needed to address the regulatory topic, and
– the applicability domain of the different methods available for each of these parameters,
– the use of different existing in silico tools to calculate, e.g., partition coefficient, fraction unbound plasma or blood:plasma ratios, and
– how to link NAMs for biokinetics and for toxicodynamics for risk assessment purposes.

In addition, the validation of NAMs for biokinetics and the development of OECD TGs (on how to perform a specific biokinetic study) is important. At the workshop, the development of OECD TGs was prioritized based on the availability of current methods and research needs. It was recognized that OECD TGs are required for:

– intrinsic clearance and identification of metabolites (liver and, at later stages, also other organs),
– in vitro approaches for tissue binding (e.g., fraction unbound plasma, blood:plasma ratio), and
– (passive) intestinal absorption (at later stages also passive and active permeation into other organs). In case of in vitro skin absorption, a TG already exists, which may need to be developed further.

Before TGs can be developed for each of these kinetic processes, protocols are needed that lead to robust and reproducible results. Moreover, it is advised to have a continuous OECD expert group to perform this work on GDs and TGs. In the meantime, the OECD Expert Group on Toxicokinetics (previously Ad hoc OECD Expert Group on Biotransformation) has been established.

Furthermore, it was concluded that method development is required to

– adequately address transporter mediated absorption (intestine, lung), distribution (e.g., blood-brain barrier, placenta, organ

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uptake) or excretion (e.g., active renal excretion, biliary secretion),
– obtain cell models that reflect the physiology and kinetic characteristics of different organs, e.g., expression of transporters and metabolizing enzymes.
Recent progress in the fields of immortalized primary cells, iPSCs, ASCs or ESCs, 3D organoids and body-on-a-chip systems provide promising tools to meet these research needs. To facilitate the transition towards next-generation risk evaluations, NAMs within the field of biokinetics should be linked to conceptual frameworks, such as (quantitative) AOPs.

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**Conflict of interest**

The authors declare that they have no conflicts of interest.

**Acknowledgements**

This work was financed by the Lorentz Centre (16118@O), the Dutch Ministry of Agriculture, Nature and Food Quality (project WOT-02-002-003), and ZonMW (project 114024503). We thank Dr Jean Lou Dorne (EFSA) for his contribution to the workshop discussions.