Expert Opinion on Drug Metabolism & Toxicology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iemt20

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To cite this article: Emma E.J. Kasteel & Remco H.S. Westerink (2021) Refining *in vitro* and *in silico* neurotoxicity approaches by accounting for interspecies and interindividual differences in toxicodynamics, Expert Opinion on Drug Metabolism & Toxicology, 17:8, 1007-1017, DOI: 10.1080/17425255.2021.1885647

To link to this article: https://doi.org/10.1080/17425255.2021.1885647

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Published online: 14 Feb 2021.

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EXPERT OPINION ON DRUG METABOLISM & TOXICOLOGY
2021, VOL. 17, NO. 8, 1007–1017
https://doi.org/10.1080/17425255.2021.1885647

REVIEW

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Refining *in vitro* and *in silico* neurotoxicity approaches by accounting for interspecies and interindividual differences in toxicodynamics

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ABSTRACT

Introduction: The process of chemical risk assessment traditionally relies on animal experiments and associated default uncertainty factors to account for interspecies and interindividual differences. To work toward a more precise and personalized risk assessment, these uncertainty factors should be refined and replaced by chemical-specific adjustment factors (CSAFs).

Areas covered: This concise review discusses alternative (*in vitro*/*in silico*) approaches that can be used to assess interspecies and interindividual differences in toxicodynamics, ranging from targeted to more integrated approaches. Although data are available on interspecies differences, the increasing use of human-induced pluripotent stem cell (hiPSC)-derived neurons may provide opportunities to also assess interindividual variability in neurotoxicity. More integrated approaches, like adverse outcome pathways (AOPs) can provide a more quantitative understanding of the toxicodynamics of a chemical.

Expert opinion: To improve chemical risk assessment, refinement of uncertainty factors is crucial. *In vitro* and *in silico* models can facilitate the development of CSAFs, but still these models cannot always capture the complexity of the *in vivo* situation, thereby potentially hampering regulatory acceptance. The combined use of more integrated approaches, like AOPs and physiologically based kinetic models, can aid in structuring data and increasing suitability of alternative approaches for regulatory purposes.

ARTICLE HISTORY
Received 3 November 2020
Accepted 1 February 2021

KEYWORDS
Chemical-specific adjustment factors (CSAFs); *in silico*; interindividual differences; interspecies differences; *in vitro*; neurotoxicity; risk assessment; uncertainty factors (UFs)

1. Introduction

Precision medicine, also referred to as personalized medicine, is becoming increasingly important in drug discovery and safety pharmacology. In neuropharmacology, precision medicine is applied in the treatment of several neurological disorders, including Alzheimer's disease [1], Parkinson's disease [2] and amyotrophic lateral sclerosis (ALS) [3]. Precision medicine refers to 'understanding disease at a deeper level in order to develop more targeted therapy' [4] and aims at understanding variation in the human population, in order to be able to treat (subgroups of) patients more effectively. This human variation can be related to genetic sex, genes, epigenetics, nutrition, exercise, smoking, the microbiome, and others [1,5]. By gaining understanding of these factors, precision medicine can be applied to identify which subgroups benefit most from which treatment. Moreover, in next generation precision medicine, genome editing using CRISPR (Clustered Regularly Interspaces Short Palindromic Repeats) techniques could be useful in treating these neurodegenerative disorders [6].

Variability in the human population is large and (partly) determines responses to treatment. Consequently, the interest in precision medicine is steadily increasing. Human variability is of great importance not only from a pharmacological point of view, but also from a (neuro)toxicological point of view. In working toward a personalized or precision risk (safety) assessment, human variability should be included, sensitive subgroups should be identified and uncertainty must be minimized. This provides challenges and opportunities for more accurate and personalized hazard and risk assessment, associated with less uncertainty. This review is therefore aimed at providing insight into the traditional approaches of (neuro) toxicological risk assessment and associated uncertainty factors to account for interspecies and interindividual differences. Moreover, this review sheds light on *in vitro* and *in silico* approaches to identify interspecies differences and interindividual differences related to neurotoxicological dynamics.

2. Risk assessment and uncertainty factors

Traditionally, the process of (neuro)toxicological risk assessment, especially hazard identification and hazard characterization, depends on animal experiments and involves deriving a 'no observed adverse effect level' (NOAEL) and the application of uncertainty factors (UFs). These factors account for the possible differences in sensitivity between animals and humans (*i.e.* interspecies differences) and between humans (*i.e.* intraspecies differences) [7]. The concept of using UFs was introduced already over 60 years ago as the '100-fold margin of safety' [7,8]. The authors of that study emphasize that this number of 100 is not determined scientifically, but that this factor appears high enough to sufficiently ensure safe exposure to food additives [8].

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Article highlights

- Risk assessment traditionally relies on *in vivo* experiments and involves the application of default uncertainty factors (UFs) to account for interspecies and interindividual differences
- Default UFs should be replaced by chemical-specific adjustment factors (CSAFs) to result in a more confident and more accurate risk assessment, which can be facilitated by *in vitro* and *in silico* approaches
- *In vitro* approaches to refine the interspecies toxicodynamics UF associated with neurotoxicology range from targeted approaches to more integrated approaches and show that interspecies differences can range from 1-1000 and that humans are not always the most sensitive species
- Human-induced pluripotent stem cell (hiPSC)-derived neurons provide opportunities to quantify interindividual differences *in vitro*, but data availability is still poor
- *In silico* approaches like meta-analyses can also elucidate interspecies and interindividual toxicodynamic differences and more integrated approaches, like adverse outcome pathways (AOPs) can structure *in vitro* and *in vivo* data and make data more useful for regulatory purposes
- Integration of approaches, by using AOPs and physiologically based kinetic (PBK) models and incorporating CSAFs, comprises the future of risk assessment.

This box summarizes key points contained in the article.

Despite the lack of a scientific basis for the factor of 100 [9], this factor is still used to extrapolate results from an animal experiment to an acceptable daily intake (ADI) or equivalent [10]. This factor is subdivided into two factors of 10, one accounting for interspecies differences and one accounting for intraspecies differences or interindividual variability [11] (Figure 1). These factors of 10 are further refined into UFs considering toxicokinetics and toxicodynamics. The differentiation between kinetics and dynamics allows the replacement of UFs with chemical-specific adjustment factors (CSAFs) if knowledge is gained on differences in either toxicokinetics or toxicodynamics and thereby move away from the default factor of 100 [11]. When analyzing (limited) data, more variability was encountered in toxicokinetics than in toxicodynamics when considering interspecies differences, resulting in UFs of 4 and 2.5, respectively [12]. Initially, this distinction was also made in the UFs for interindividual variability, but a more elaborate analysis of data pointed toward an equal subdivision of ~3.16 for both toxicokinetics and toxicodynamics [13]..

The default UF of 100 is presumed 'safe', well accepted and in general adequately protective. However, as the name already implies, the use of UFs in risk assessment brings uncertainty into the process of risk assessment. The default UFs are often (incorrectly) associated with conservatism and reassurance, but they come with lower confidence in the risk assessment and actually involve greater uncertainty than CSAFs [14–16]. It is key to recognize this, as both too low and too high UFs can have consequences with significant societal impact. Alternative approaches, like *in vitro* and *in silico* approaches, can refine UFs, thereby moving toward CSAFs or pathway-specific UFs.

There are multiple concerns when using animals for toxicity testing, including the major ethical issues associated with the large number of animals used, especially for hazard characterization [17]. Besides the ethical issues, economic and scientific aspects are also important when considering these experiments. Animal experiments are expensive and time-consuming and can cost millions of Euros per chemical [18,19]. Furthermore, extrapolating toxic doses from animals to humans can be difficult and involves many uncertainties [17,20]. Last, but not least, animal tests usually do not provide a mechanistic understanding of the observed adverse health outcomes. Animals are 'black box' systems and provide little information about why a compound is toxic to the animal and whether the chemical can be similarly toxic to humans [21]. New international regulations on chemical risk assessment, like REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), require extensive (toxicity) testing of chemicals, resulting in the need for more toxicity tests [22]. Because of the limitations mentioned above, traditional tests are not sufficient to cover all requirements, resulting in the need for alternatives. These alternative approaches become

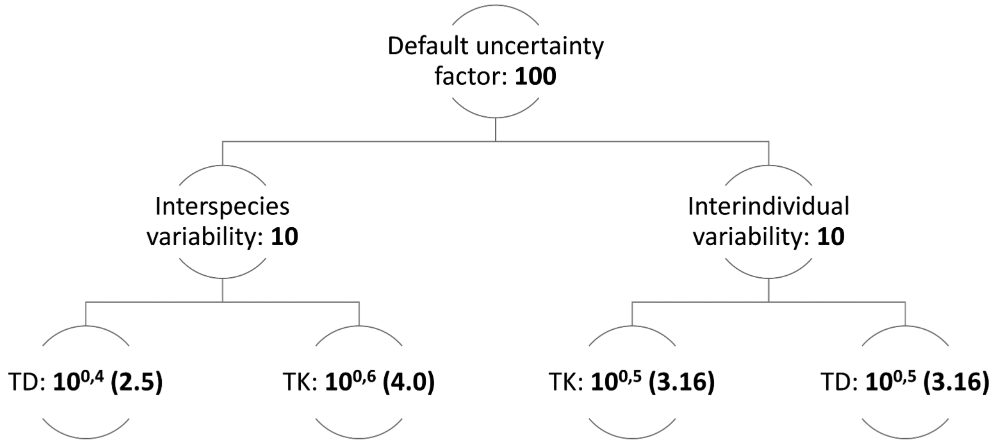

Figure 1. Representation of the uncertainty factors applied in toxicology. The default uncertainty factor is 100, which is divided into two factors of 10, one for interspecies differences and one for interindividual or intraspecies differences. These factors of 10 are again divided into two factors, one for variability in toxicokinetics (TK) and one for variability in toxicodynamics (TD) [11]

increasingly important and can be used to refine UFs associated with interspecies differences and human variability. For example, in *in vitro* assays, human cells can be used and an *in silico* PBK model can be built for humans. By using these approaches, there is no need for the interspecies UF and the uncertainty factor may be reduced from 100 to 10, accounting only for interindividual variability.

Although with the increasing use of human cells and tissues interspecies differences will become less and less important in risk and safety assessment and eventually will fade out, it can still be important to investigate interspecies differences and compare (human) *in vitro* or *in silico* data with animal data obtained either from *in vitro* or *in vivo* experiments. This is especially necessary to gain confidence in these alternative approaches and facilitate the paradigm shift toward these approaches [23]. Moreover, when human *in vivo* data is scarce and regulatory limits are based on animal *in vivo* data, investigating the interspecies differences can facilitate deriving a CSAF. For example, for the marine neurotoxin tetrodotoxin (TTX), human data is available only from (often fatal) case studies and therefore NOAELs for TTX derived from humans are of limited value. LD_{50} values are available for different animal species, but knowledge on interspecies differences is necessary to translate these results to the human situation [24]. Interspecies differences can be evaluated by comparing *in vitro* tests in rat tissue and human tissue [24,25]. These differences can then be applied to *in vivo* data and the UF for interspecies differences may be altered.

In vitro and *in silico* approaches can also facilitate the evaluation of the intraspecies UF, both for toxicokinetics and toxicodynamics. The use of primary human hepatocytes, for example, can give information on human variability in biotransformation and refine the UF for toxicokinetics. However, human primary cells are difficult to obtain and they have a limited lifespan in culture [26]. Moreover, not every tissue is available and investigating differences in for example the human brain is hardly feasible. On the contrary, stem cells can be differentiated into all kinds of cells. Different types of stem cells (*e.g.* embryonic stem cells, adult human stem cells) are now being used in toxicology and recently, a new class of stem cells is becoming increasingly important: human induced pluripotent stem cells (hiPSCs). hiPSCs are generated from adult reprogrammed somatic cells and are ethically preferred over embryonic and fetal stem cells [27]. These cells have the ability to be reprogrammed into specific types of cells, like cardiomyocytes, neurons and hepatocytes, which provides an opportunity for testing effects of chemicals on different cell types from specific (groups of) people [28]. Human variability can then be measured using hiPSCs from different donors [28,29].

Inter- and intraspecies differences in absorption, distribution, metabolism and excretion in other organs can influence the concentration of a chemical in the brain and thereby influence the risk of a chemical to exert neurotoxicity. Examples of such toxicokinetic processes confined to the central nervous system (CNS) include transport across the blood-brain barrier (BBB) and (oxidative) metabolism of chemicals in the brain. Drug metabolizing enzymes are present throughout the CNS, including the BBB [30] and can significantly influence the neurotoxicity of a chemical. Differences between and within species in these drug metabolizing enzymes and functional BBB properties influence target concentrations of chemicals and are critical in risk assessment. Despite the importance of toxicokinetics, this concise perspective will focus on refining uncertainty factors associated with toxicodynamics.

3. *In vitro* and *in silico* approaches to assess interspecies differences in toxicodynamics

In vitro and *in silico* approaches to better understand toxicodynamic processes are increasingly used to move away from the 'black box' animal system and can provide a refinement of the toxicodynamic UF. If the mechanism of a chemical is known, *in vitro* assays can be used to evaluate interspecies differences and be applied to risk assessment that uses *in vivo* animal data as a point of departure [24]. On the other hand, if the mechanism of a chemical is unknown, human and rat *in vitro* assays can be used to identify both the (neuro)pharmacological or toxicological mode of action and assess interspecies differences [25]. Essential processes in neurodevelopment include glial and neuronal cell proliferation, migration, differentiation into various neuronal and glial cell subtypes, synaptogenesis, pruning, myelination networking and terminal functional neuronal and glial maturation [for review see 31,32]. Different techniques, endpoints and cell models can be used to assess interspecies differences, depending on the (mechanistic) knowledge of a chemical and on the research question asked. These models range from cell lines to primary cells to hiPSCs and differ not only in their origin, but also in their throughput capacity, degree of characterization, complexity and availability [31].

Two neuronal tumor-derived cell lines that are commonly used in neurotoxicity testing are the rat PC12 cell line and the human SH-SY5Y neuroblastoma cell line [33]. The PC12 cell line is the most extensively used neuronal cell line and can be used to study multiple (targeted) endpoints [33]. However, it originates from a rat and therefore interspecies translation is required for human risk assessment. On the other hand, the SH-SY5Y neuroblastoma cell line is of human origin and although less extensively investigated and characterized [33], can provide mechanistic insight into chemicals without the need for assessing interspecies differences. Although many other cell lines and new cell models exist, like the hiPSCs mentioned above, these two cell lines are (historically) most used. Because these approaches are suitable for investigating more targeted endpoints, like neurotransmission and receptor activation, they can give a more detailed insight into the mechanism of action of a chemical. However, more integrated approaches can give more insight into the overall functioning of the neuronal network.

An example of a more integrated *in vitro* assay to assess neuronal network functionality that is increasingly applied is the microelectrode array (MEA). MEA allows for simultaneous

recordings of electrical (neuronal) activity, is noninvasive and can be extrapolated to the *in vivo* situation [34,35]. Although (immortalized) clonal cell lines are unsuitable for addressing neuronal network activity using MEA recordings, different types of tissues can be grown on MEA plates, including primary tissues and slices [35]. The current gold standard for MEA recordings is the rat cortical culture, which is well characterized and easy to culture [34]. However, the use of hiPSC-derived neuronal cultures on MEAs is increasing [36], thereby providing opportunities to assess species differences using this technique [24,25].

The data in Hondebrink, Kasteel [25] show that interspecies differences exist for the new psychoactive substance methoxetamine (MXE) when comparing MEA recordings using primary rat cortical cultures and hiPSC-derived neuronal cultures. Commercially available iCell® neurons and DopaNeurons®, both in the presence and absence of astrocytes, were less sensitive than the primary rat cortical cultures, indicating a toxicodynamic species difference for this compound. Although the use of an interspecies UF presumes that humans are more sensitive than rats or other laboratory animals, the difference here indicates that humans are the less sensitive species in this case. Also other (classes of) substances like endosulfan, methylmercury, pentylenetetrazol and enoxacin appear to be more potent in rat cortical cultures [37] than in hiPSC-derived neuronal cultures [34,36,38]. Contrarily, the data in Kasteel and Westerink [24] show that the marine neurotoxin tetrodotoxin (TTX) is equipotent in primary rat cortical culture and iCell® neurons for inhibiting neuronal activity, pointing toward limited interspecies differences for this compound. Additional studies also indicate that interspecies differences are limited for other chemicals like amphetamine [34,39], whereas others like perfluoroalkyl substances, strychnine, picrotoxin and 4-aminopyridine are either equipotent or more potent in hiPSC-derived neuronal cultures compared to primary rat cortical cultures [40,41].

Also, other assays (both targeted and more integrated) can identify species differences in neurotoxicological responses. For example, interspecies differences in binding selectivity, affinity and agonist potencies have been demonstrated for $P2X_7$ receptors [42] and nicotinic cholinergic receptors [43]. Also, the dopamine transporter shows species differences in the uptake of toxic substances like cocaine and 1-methyl-4-phenylpyridinium (MPP^+), depending on chemical, parameter and species [44]. Dopamine D_1 receptor agonists display different pharmacological characteristics in different species [45]. On the other hand, for methylmercury, species differences appear to be limited using cell viability as endpoint [46], although cell viability assays did show differences between rat and human neuronal cell lines for manganese chloride ($MnCl_2$) and the enantiomers of styrene oxide (SO) [46]. These combined findings are summarized in Table 1 and indicate that considerable interspecies differences exist that are compound-dependent and can result in over- or underestimation of human risk if animal cells are used. It has to be mentioned, however, that besides species differences, differences in culturing conditions (like presence of serum proteins, type of plastic, etc.) and differences in for example metabolic capacity could influence the kinetics of the chemicals and thereby also influence the dynamics. In order to confine the observed interspecies differences to toxicodynamics, ideally cell-associated concentrations of parent chemicals and metabolites should be measured to exclude any potential differences in kinetics.

In silico approaches like meta-analyses can also be used to identify interspecies differences. For example, McPartland, Glass [47] identified interspecies differences in cannabinoid ligand binding affinity and receptor distribution. The meta-analysis identified data heterogeneity due to differences in methods, but also data gaps regarding the affinity of cannabidiol at human cannabinoid receptor type 1, for example. Interspecies differences in mean K_i values for Δ^9-tetrahydrocannabinol (THC) and in mean K_d values for CP55,940, WIN55,212–2 and SR141716A were identified for human and rat cannabinoid receptor type 1 and differences ranged from 1.7–7 times. On the other hand, no statistically significant interspecies differences were found for anandamide, although this could be due to heterogeneity in data. Also receptor distributions differed between human and rat cannabinoid receptor type 1, with human expression more dense in cognitive regions and rat expression more dense in movement-associated regions. By combining all data on these receptors in literature, more valid estimates of K_d and K_i values could be made, which could establish interspecies differences between the binding affinities of natural and synthetic ligands for the cannabinoid receptors.

4. *In vitro* and *in silico* approaches to assess interindividual differences in toxicodynamics

Because of the foreseen paradigm shift from animal experiments to *in silico* approaches and *in vitro* approaches using human cells and tissues, interspecies differences will become less important over time and focus may shift toward assessing human variability. Especially for toxicodynamics, it is hard to derive specific adjustment factors for human variability and few studies have been published on this topic [11,15,48]. Cell lines are often not suitable for assessing human variability, except when using many cell lines of the same cell type [49]. Similar to assessing interspecies differences in toxicodynamics, *in vivo* data are often also unsuitable as the differences in toxicodynamics are influenced by the target concentration of the chemical, which depends on toxicokinetics. Consequently, the use of primary cells or stem cells seems unavoidable to assess human variability in toxicodynamics [48].

In the framework of personalized toxicological risk assessment, it is essential to characterize differences between humans and understand which underlying mechanisms are responsible for these differences. The availability of hiPSC-derived neurons offers the potential to work toward a personalized risk assessment and to identify interindividual differences between humans. Moreover, it provides an opportunity to investigate the effect of both genetic and environmental risk factors and the complex interaction between these

Table 1. Comparison of interspecies differences for a selection of neurotoxicological compounds assessed using *in vitro* assays. The investigated compound, the parameter that is compared, the animal species that was used and the values derived for the animal species and for humans are indicated. Furthermore, the most sensitive species based on that data and the interspecies differences are shown.

Compound	Parameter compared	Animal species	Animal species value	Human value	Most sensitive species	Interspecies difference	Reference
BzATP	EC_{50} $P2X_7$	Rat	1.3 µM	5.5 µM	Rat	**4.2**	[42]
BzATP	EC_{50} $P2X_7$	Mouse	83 µM	5.5 µM	Human	**15**	[42]
4-aminopyridine	LOEC MEA	Rat	>100 µM	100 µM	Human	**>1**	[41]
Amphetamine	30 µM MEA	Rat	~90% activity	~65% activity	Human	**1.4**	[34,39]
Amoxapine	LOEC MEA	Rat	1 µM	1 µM	Comparable	**1**	[38]
AT-1001	K_i α3β4 receptor	Rat	1.9 nM	0.092 nM	Human	**21**	[43]
AT-1001	K_i α4β2 receptor	Rat	78 nM	91 nM	Rat	**1.2**	[43]
Chlorpromazine	LOEC MEA	Rat	1 µM	1 µM	Comparable	**1**	[38]
Cocaine ([^{3}H]CFT)	B_{max} (binding)	Rat	156 fmol/10^5 cells	330 fmol/10^5 cells	Human	**2.1**	[44]
Cocaine ([^{3}H]CFT)	B_{max} (binding)	Bovine	35 fmol/10^5 cells	330 fmol/10^5 cells	Human	**9.4**	[44]
Endosulfan	LOEC MEA	Rat	0.3 µM	1 µM	Rat	**3.33**	[34]
Enoxacin	LOEC MEA	Rat	3 µM	>10 µM	Rat	**>3.33**	[38]
Linopirdine	LOEC MEA	Rat	>100 µM	10 µM	Human	**>10**	[38]
Manganese chloride ($MnCl_2$)	EC_{50} cytotoxicity	Rat	1.3 mM; 17 mM	0.71 mM; 5 mM	Human	**1.8–24**	[46]
Methoxetamine (MXE)	IC_{50} MEA	Rat	0.5 µM	>10 µM	Rat	**>20**	[25]
Methylmercury (MeHg)	30 µM MEA	Rat	0% activity	No effect	Rat	**>30***	[36,37]
Methylmercury (MeHg)	EC_{50} cytotoxicity	Rat	2.2 µM	2.1 µM; 8.2 µM	Rat	**<1-3.7**	[46]
MPP^+	Uptake	Rat	68 fmol/10^5 cells	118 fmol/10^5 cells	Human	**1.7**	[44]
MPP^+	Uptake	Bovine	5 fmol/10^5 cells	118 fmol/10^5 cells	Human	**24**	[44]
Pentylenetetrazol	LOEC MEA	Rat	3 mM	>3 mM	Rat	**>1**	[38]
Perfluorooctanoic acid (PFOA)	LOEC MEA	Rat	>100 µM	1 µM	Human	**>100**	[40]
Perfluorooctane sulfonate (PFOS)	LOEC MEA	Rat	100 µM	0.1–100 µM	Human	**1–1000**	[40]
Phenytoin	LOEC MEA	Rat	30 µM	100 µM	Rat	**3.33**	[38]
Picrotoxin	LOEC MEA	Rat	1 µM	1 µM/>10 µM#	Comparable	**1**	[41]
Pilocarpine	LOEC MEA	Rat	>30 µM	1 µM	Human	**>30**	[38]
R-styrene oxide	EC_{50} cytotoxicity	Rat	6.6 µM; 60 µM; 110 µM	29 µM; 20 µM	Human	**<1-5.5**	[46]
Strychnine	LOEC MEA	Rat	3 µM	0.3–30 µM#	Human	**<1-10**	[41]
S-styrene oxide	EC_{50} cytotoxicity	Rat	0.016 mM; 0.12 mM; 0.44 mM	0.58 mM; 0.041 mM	Human	**<1-10**	[46]
Tetrodotoxin (TTX)	IC_{50} MEA	Rat	7 nM	10 nM	Rat	**1.4**	[24]

*The LOEC for the rat was 1 µM; #depending on the model, 2 out of 3 models suggest humans are more sensitive.

factors [50]. Although these cultures are still being characterized and improved, they provide a promising alternative to *in vivo* studies and have great potential for both personalized medicine and personalized risk assessment [36,50].

For example, the use of hiPSC-derived neurons brings the opportunity to investigate genetic and epigenetic mechanisms underlying the development of Parkinon's disease [51]. By taking hiPSC-derived neurons from monozygotic twins that are clinically discordant for Parkinson's disease, biomarkers for this disease could be identified, as well as possible treatment options [51]. Moreover, the use of hiPSC-derived neurons provides the opportunity to link environmental exposures (like metals) to genetic risk factors for Parkinson's disease and investigate the combination of these factors in risk for disease occurrence [50]. However, culture and differentiation protocols need to be further optimized, in order to ensure disease-like phenotypes in cultures [52].

Another possibility to assess human variability directly in tissue, is using another matrix that can function as a surrogate for a brain- and/or CNS-related process. An example is measuring the inhibition of acetylcholinesterase (AChE) in blood. This enzyme is crucial for neuronal functioning, but is also present in blood. Inhibition of AChE is a well-known mechanism of action of multiple pharmaceuticals and pesticides, including carbamates and (metabolites of) organophosphates. Interindividual variability in the inhibition of AChE could be assessed by taking blood from different human donors and measure enzyme activity using a simple fluorescence-based assay [53]. This provides an opportunity to assess interindividual differences in toxicodynamics, without the incorporation of interindividual differences in toxicokinetics that could influence the concentration of the chemical at the target site. The associated CSAFs for interindividual variability for this toxicodynamic endpoint range from 1.2 to 2.2, depending on the compound [53].

The use of dedicated mouse populations may also provide an opportunity to identify variability in human responses to chemical insults. Collaborative Cross and Diversity Outbred (CCDO) mice represent populations of mice that are genetically diverse and can be used for both hazard identification and hazard characterization [54]. These mice thus show larger and more realistic variability that inbred mice. As such, these (CCDO) mice can also be used to identify sensitive subpopulations. However, more research is necessary to identify how to account for human variability using variability in (CCDO) mice.

Meta-analyses can also be used to assess interindividual variability. For example, Terrazzino, Argyriou [55] identified genetic determinants of neurotoxicity induced by oxaliplatin and combined these results with two other studies in a meta-analysis. Interindividual variability in susceptibility to chronic oxaliplatin-induced peripheral neurotoxicity (OXAIPN) is partly explained by clinical factors like preexisting disease, dosing and schedule, but genetic factors may also be involved. However, none of the polymorphisms investigated in the study were associated with chronic OXAIPN (≥ grade 2), which was in contrast with results of a previously performed genome-wide association study. This emphasizes that although this study did not find interindividual variability in OXAIPN due to genetic factors, performing a meta-analysis can provide more information on initial results found in single studies and can either confirm or contradict these results.

(1) **Integrated and *in silico* approaches to assess interspecies and interindividual differences in toxicodynamics**

In silico approaches, such as (quantitative) structure–activity relationships ((Q)SARs) or quantitative structure–toxicity relationships (QSTRs), read-across, molecular docking, meta-analyses and physiologically based kinetic (PBK) models can be complementary to *in vivo* and *in vitro* data for mechanism-based risk assessment and can also provide insights into toxicological potencies of (metabolites of) chemicals. Using QSARs for example, the toxicological profile of a chemical can be predicted, based on its chemical structure, indicating that chemical characteristics such as hydrophobicity, aqueous solubility and volatility can be predictive of neurotoxicity [56]. This can help in prioritizing chemicals for hazard characterization, thereby limiting the need for extensive toxicity testing of each chemical. This was, for example, used in combination with read-across to predict the developmental- and neurotoxicity of pesticide metabolites and degradation products and to distinguish between toxic and nontoxic chemicals, without the need for expensive (*in vitro* or *in vivo*) testing [57]. Also, QSARs can be used in combination with high-throughput screening data to both classify chemicals and predict effect concentrations [58]. In the study of Chushak, Shows [58], QSARs for molecular interactions were linked to the more integrated cellular responses measured with MEAs. Another study used QSARs to simultaneously predict the anticonvulsant activity and neurotoxicity of succinimides, with relatively high accuracy (R^2 = 0.87) [59]. Other studies regarding QSARs and neurotoxicity [60] investigated, for example, neurotoxicity of solvents [56,61], triazole fungicides [62], polychlorinated biphenyl congeners (PCBs) [63,64] and AChE inhibitors [65].

The studies mentioned above on QSARs are not designed to identify interspecies differences or interindividual variability, but are merely *in silico* tools to identify toxicity. Models like QSARs can be used to identify interspecies relationships, using interspecies quantitative structure-toxicity-toxicity relationships (QSTTR) or interspecies quantitative toxicity–toxicity relationships (iQTTR) models. These models are mainly applied in ecotoxicology to extrapolate data between (aquatic) species [66], but recently also for extrapolation between mammals [67]. In this study, the toxicity of nitroaromatic compounds was predicted using the iQTTR model to rats using mouse toxicity data and vice versa [67]. Thereby, a data gap of toxicity data for one species could be filled using toxicity data from another species. Although the focus of these models is not to identify interspecies differences, but rather to correlate species toxicity data, these models can shed light on both the differences and similarities between two species in their toxic response.

On the other hand, molecular docking studies can provide information on interspecies differences and interindividual differences. For example, Kamal, Lim [68] identified the effect of several human cytochrome P450 single nucleotide polymorphisms (SNPs) on the binding of methadone using molecular docking. By using this *in silico* method, the binding affinity of the compound for the receptor or enzyme is calculated and thereby indicates the substrate metabolism activity [68]. In this way, it was identified that some SNPs have a significant effect on binding affinity to methadone. In a comparable manner, molecular docking can provide insight into the active site of an enzyme and the differences herein between species [69]. In the study of Choughule, Joswig-Jones [69], molecular docking was used to model the binding of methotrexate into the active site of aldehyde oxidase in rabbits and humans. This identified important residues in the active site that could be important in binding, thereby explaining observed species differences.

For regulatory purposes, approaches should preferably be more integrated and cover all processes in the human body, instead of assessing a single endpoint. An example of such an integrated approach is the adverse outcome pathway (AOP). AOPs link a molecular initiating event (MIE) to an adverse outcome (AO) via different key events (KE) and key event relationships (KERs), and cover empirical (toxicodynamic) evidence from prototypic chemicals [70]. An AOP describes the entire pathway of toxicity, and it thus includes and connects multiple levels of biological organization (molecular, organelle, cellular, tissue, organ, organism, population) [71]. This implies that different types of information can be used, ranging from *in silico*, to *in vitro, in vivo* and even clinical and epidemiological data, thereby making effective use of all existing data on a chemical [72]. Moreover, AOPs are chemical agnostic and can thus be applied to multiple chemicals with the same mechanism [72,73]. The description of an AOP is usually qualitative, meaning that the KERs are only described and not quantified. This is useful for hazard identification, but a quantitative dose- or concentration–response relationship is required for application in risk assessment [23]. Quantitative AOPs (qAOPs) can quantitatively relate KEs to one another and describe how severe the AO is at a certain perturbation of the MIE and/or KEs [74,75]. By then combining AOPs with, for example, toxicity tests with hiPSCs, they can provide detailed mechanistic information and serve as an integrated approach in risk assessment, while incorporating human variability.

AOPs can be applied as a framework to develop integrated approaches to testing and assessment (IATA). IATA is, similar to the AOP concept, an approach that integrates the use of different types of data (like physicochemical, *in silico, in vitro*, etc.) to perform hazard characterizations and risk assessments on chemicals. It relies on the integrated analysis of all existing data and combining this with the generation of new information using testing strategies [76]. Ideally, an IATA is mechanistically informed and this mechanistic understanding can be provided by AOPs.

The application of the AOP concept in neurotoxicity is increasing. Several AOPs have been developed for (developmental) neurotoxicity, ranging from the (acute) inhibition of voltage-gated sodium channels to chronic antagonism of N-methyl-D-aspartate receptors (https://aopwiki.org/aops/12) and others (https://aopwiki.org/aops/48; https://aopwiki.org/aops/10; https://aopwiki.org/aops/3) [for review see 77]. An AOP network that links multiple linear AOPs has been developed for human neurotoxicity [78]. In this study, linear AOPs from AOP-Wiki (https://aopwiki.org/) relevant for neurotoxicity were identified and an AOP network was developed. Different MIEs were included, like antagonistic binding to NMDA receptors and agonistic binding to ionotropic glutamate receptors. Moreover, multiple AOs were identified, including neurodegeneration, Parkinsonian motor deficits and impairment of learning and memory/decrease of cognitive function. Multiple common KEs were identified that trigger multiple other downstream KEs, with the most central KE being cell injury/death. This KE is triggered by several KEs that are present in multiple linear AOPs. The AOP network was characterized using several (network) analytics.

A major advantage of using AOPs instead of standalone *in vitro* or *in silico* approaches is the possibility to generate response–response curves. From a risk assessment point of view and the associated improvement/refinement of UFs, AOPs can provide detailed knowledge on the mechanism (*i.e.* toxicodynamics) of a chemical, which is necessary to derive toxicodynamic CSAFs. According to the guidance document of the WHO on deriving CSAFs 'There is usually also no detailed knowledge on how the dose/concentration–effect relationship of one step at the effect site is related to the dose/concentration–effect relationship of the next step' [11]. This is exactly where qAOPs can provide a solution. By deriving response–response curves, KERs are quantified [74]. These KERs describe the level of response of a downstream KE based on the level of response of an upstream KE. KERs relate *in vitro* KEs, but they can also relate an *in vitro* KE to an *in vivo* KE, which are generally more downstream. This is directly useful for risk assessment as the response–response curves can then be used to predict the toxicity of other chemicals that perturbate the same AOP. Moreover, as AOPs are chemical agnostic, this will reduce the amount of toxicity testing needed for a chemical.

Although AOPs are useful tools in next generation risk assessment and can provide a more quantitative understanding of the toxicodynamics of a chemical, they are still simplistic. Especially single, linear, unidirectional AOPs are probably of limited use to (regulatory) risk assessors [79]. In other words, AOPs should be able to capture at least partly the complexity of the *in vivo* situation. This can be accomplished by the development of AOP networks (as mentioned above) and/or branching AOPs, but also by including compensatory mechanisms and modulators [79,80]. It also needs to be considered that AOPs can be biased by focusing just on the known adverse outcome, thereby missing (other) important KEs [81]. Moreover, the thresholds associated with KEs and the development of a quantitative AOP is a requirement for effective use of AOPs in risk assessment. Research should also focus on developing more AOPs. For example, an AOP for acute inhibition of AChE exists, but AOPs for chronic and/or developmental neurotoxicity associated with AChE inhibition still need to be developed. Until now, only relatively few AOPs have been developed and research should focus on increasing the quality and quantity of AOPs. The use of high-throughput screening can facilitate the development of AOPs and the identification of potential KEs, including those that are not represented in the existing data, assuring that all potential targets of the chemical are captured [81,82].

5. Conclusions

Humans are exposed to thousands of chemicals, but only a relatively small part of these have been properly examined for their (neuro)toxicological potency. To ensure human safety, the risks of chemical exposure should be examined properly and rapidly. Although the use of default UFs that account for both interspecies and interindividual differences is still common, the refinement of UFs is a crucial step in improving (neurotoxicological) risk assessment. Debate about the use of the default factors started already over 35 years ago and is still ongoing [13,15,83–85] and refinement of UFs can facilitate a more reliable risk assessment of chemicals, with less uncertainty and toward a pivotal evolution away from the default UFs [15,81]. The derivation of more accurate CSAFs is feasible using *in vitro* and *in silico* methods, both for interspecies and intraspecies differences.

In vitro and *in silico* approaches that range from very targeted (for example *in vitro* assays and QSAR *in silico* approaches for receptor or enzyme inhibition) to more integrated (*in vitro* MEA measurements, AOPs) can characterize neurotoxicological differences between animals and humans and between individuals. Clearly, much more data is currently available on interspecies differences than on human interindividual differences. Interindividual toxicodynamic differences in humans are hard to investigate, especially for a complicated organ(system) like the CNS, with limited primary tissue available. However, hiPSCs or surrogate matrices (like enzymes in blood) provide opportunities to identify interindividual differences in neurotoxicity and work toward refined uncertainty factors.

Interspecies differences and the associated uncertainty factors can vary greatly. Differences between 1 and 1000 (Table 1) have been reported and indicate that in some cases humans are the more sensitive species, whereas in other

cases the test animal is more sensitive than human. Furthermore, as (human) brain models are not always well established and the use of *in vitro* and *in silico* methods provides the opportunity to derive multiple parameters, the magnitude of this difference can highly depend on the model and/or parameter chosen.

6. Expert opinion

In many cases, the use of default UFs for toxicodynamics is (more than) adequate, although there are also numerous cases in which default UFs provide insufficient protection. To improve chemical risk assessment, the improvement of UFs is therefore essential. Both too low and too high UFs can have consequences with significant societal impact. For example, when the default UF that is used to derive a 'safe' dose for a potentially life-saving chemical (for example, a flame retardant) is too high, the use of the chemical may be restricted. On the other hand, if the UF used to derive a 'safe' dose for a chemical is too low, it can result in an acceptable/tolerable daily intake that is not adequately protective, resulting in health risks for humans. This means that the application of CSAFs in (neuro)toxicology is crucial when the default UFs are not protective enough, but equally important when UFs are lower than the default simply because they are more precise, chemical-specific and have a more quantitative nature [15]. *In vitro* and *in silico* approaches can facilitate the shift toward a more mechanism-based risk assessment and thereby provide the opportunity for deriving CSAFs. The improvement of UFs can impact real-world outcomes by reducing the uncertainty around reference values like the ADI and although regulatory acceptance is still developing, confidence in alternative methods is growing. Furthermore, more integrated approaches like AOPs can integrate multiple (types of) data and can provide even more knowledge on the mechanism of action than standalone *in vitro* or *in silico* models.

In the use of alternatives for risk assessment and the development of CSAFs, it has to be recognized that although *in vitro* and *in silico* models continuously evolve to better represent human physiology and toxicology, they remain models that cannot always capture the full complexity of an *in vivo* situation. In an *in vivo* situation, many interactions take place that usually are not all present in the *in silico* or *in vitro* model. The development of AOP networks helps in trying to capture this complexity from a toxicodynamic point of view. However, the question remains how much complexity is necessary for these models to adequately assess the risk of chemical exposures to humans. To increase the usefulness of alternative methods for regulatory purposes, research into the required complexity of alternative models is necessary. This is highly dependent on the research question asked. For instance, for more generic questions a 2D *in vitro* model may be appropriate and can provide a simple, cheap and fast alternative to a costly and complex 3D model. Essentially, a cost-benefit analysis should be made to decide how complex the *in vitro* or *in silico* model should be. This certainly also depends on the available knowledge of the mechanism/AOP of the chemical, which is required to decide which targets should be represented in the model.

In general, the acceptance of *in vitro* and *in silico* approaches in regulatory risk assessment is falling behind [16,23,79,86,87]. This may have several reasons, which include more familiarity with animal tests and lack of understanding between risk assessors, modelers and scientists [16]. The fact that animal tests are more familiar may result in a (misplaced) trust in these experiments and therefore a restraint on alternative methods. Future research in this area should focus on more integration of data and establish a link between all chemical data (*in vitro, in silico, in vivo, in chemico*) available in order to provide a solid base for risk assessment of chemicals. The use of AOPs can structure data and make the data more suitable for regulatory purposes. Moreover, the development and elaboration of more complex *in vitro* systems and frameworks and guidance for (q)AOPs are crucial in moving toward a personalized 'next generation' risk assessment. Also, for personalized risk assessment, research into genetic polymorphisms should be intensified, as these are major contributors to interindividual variability, not only in toxicokinetics, but also in toxicodynamics [88]. In the light of personalized medicine, polymorphisms are researched more thoroughly, also from a pharmacodynamic point of view [89,90]. Toxicological research should make effective use of these data to identify polymorphisms that could affect the toxicodynamics of a chemical.

To provide the most accurate risk assessment, it is important to deal with (unavoidable) uncertainties in the best way possible and reduce as much as can be reasonably expected. In other words, uncertainty should be characterized and quantified. Therefore, the future of risk assessment lies in the ultimate integration of approaches, using AOPs and PBK models. The latter are *in silico* models that describe the kinetics of a chemical in an organism using mathematical equations. In human risk assessment, they can be applied to predict the kinetics (*i.e.* absorption, distribution, metabolism and excretion) of chemicals, resulting in estimated plasma- and organ concentrations. The use of PBK models in risk assessment is promising, as PBK models can link toxicokinetic and toxicodynamic data. PBK models are beneficial in dose- and route extrapolation and can describe the target concentration of a chemical, which is useful for deriving an intraspecies CSAF for toxicokinetics.

Toxicodynamic data generated in an AOP framework, including response–response curves and incorporating human variability by using primary cells or human iPSCs, can serve as a base for addressing toxicodynamic human variability. A quantitative *in vitro* – *in vivo* extrapolation (QIVIVE) by applying, for example, reverse dosimetry in a PBK model can shed light on the corresponding *in vivo* dose, incorporating the aforementioned variability in toxicodynamics and variability in toxicokinetics, assessed using the PBK model. This ultimate PBTK-TD model, based on good quality *in vitro* and *in silico* approaches, can identify susceptible populations and provide a quantitative next generation risk assessment, without the need for animal tests. This model

can elucidate both interspecies and interindividual differences and can provide and incorporate CSAFs, which will be the future of risk assessment. The ultimate challenge for a 'next-generation' risk assessment would be the incorporation of chemical mixtures on top of single chemical exposures, especially for chemicals with dissimilar mechanisms that activate different AOPs.

Funding

This work was funded by the Faculty of Veterinary Medicine (Utrecht University).

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

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