In vitro dosimetry of selected compounds and *in vitro to in vivo* extrapolation (IVIVE) for New Approach Methods (NAM) in toxicology

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The following work was performed from September 2018 until October 2021 at BASF SE in Ludwigshafen am Rhein under the supervision of PD Dr. Robert Landsiedel from the Institute of Pharmacy of the Free University of Berlin and the Department of Experimental Toxicology and Ecology BASF, SE, and Prof. Gerhard Wolber at the Institute of Pharmacy of the Free University of Berlin.

I hereby declare that I have written the present dissertation independently, without any assistance from third parties and without the use of resources other than those indicated. The material, either in full or in part, has not been previously submitted for grading at this or any other academic institution.

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Started from the bottom, now we're here.

Aubrey Drake Graham

List of Abbreviations

3R	Reduction, refinement, replacement
AC	Activity concentration
ADME	Absorption, distribution, metabolism, excretion
AOP	Adverse outcome pathway
APAP	Acetaminophen
BED	Biologically effective dose
BPA	Bisphenol A
CAF	Caffeine
COL	Colchicine
$C_{Cytoplasm}$	Concentration of a substance in the cytosol
$C_{Internal}$	Internal concentration of a substance
C_{Medium}	Concentration of a substance in culture medium
$C_{Membrane}$	Concentration of a substance in the membrane
C_{Nom}	Nominal concentration of a substance
C_{Target}	Concentration of a substance at the target site
FEN	Fenarimol
FLU	Flutamide
f_u	Fraction unbound of a substance
GEN	Genistein
$logP_{ow}$	Octanol-water partition coefficient
KET	Ketoconazole
K_{Air}	Distribution coefficient between air and water
K_{Cell}	Distribution coefficient between cells and water
$K_{Plastic}$	Distribution coefficient between plastic and water
$K_{Serum\ Albumin}$	Distribution coefficient between serum albumin and water
$K_{Serum\ Lipid}$	Distribution coefficient between serum lipids and water
MT	Methyltestosterone
NAM	New Approach Methods
NAPQI	N-acetyl-p-benzo-quinoneimine
OECD	Organisation for economic co-operation and development
PBTK	Physiologically based toxicokinetic model
pKa	Acid dissociation constant
PoD	Point of departure
QIVIVE	Quantitative *in vitro* to *in vivo* extrapolation
QSAR	Quantitative structure-activity relationship
RED	Rapid equilibrium dialysis
RSD	Relative standard deviation
SD	Standard deviation
SPME	Solid phase micro extraction
TAM	Tamoxifen
TRE	Trenbolone
UC	Ultrafiltration
UF	Ultracentrifugation
WAR	Warfarin
YES/YAS	Yeast estrogen/androgen screening assay

List of Publications

1. Dimitrijevic, D., Fabian, E., Nicol, B., Funk-Weyer, D., & Landsiedel, R. (2022). Toward Realistic Dosimetry In Vitro: Determining Effective Concentrations of Test Substances in Cell Culture and Their Prediction by an In Silico Mass Balance Model. *Chemical Research in Toxicology*, *35*(11), 1962-1973. DOI: 10.1021/acs.chemrestox.2c00128.

2. Dimitrijevic, D., Fabian, E., Funk-Weyer, D., & Landsiedel, R. (2023). Rapid equilibrium dialysis, ultrafiltration or ultracentrifugation? Evaluation of methods to quantify the unbound fraction of substances in plasma. *Biochemical and Biophysical Research Communications*. DOI: 10.1016/j.bbrc.2023.02.021.

Table of Contents

1 Introduction

1.1 Implementation of *In Vitro* Toxicity in Chemical Hazard and Risk Assessment

The potential hazard and risk of substances is generally assessed with toxicity tests relied on animal experiments. Standardized and harmonized protocols have been established for apical toxicological endpoints, e.g. reproduction/developmental toxicity (OECD test guideline no. 421) and carcinogenicity (OECD test guideline no. 451) described in the OECD guidelines [1-3].
Based on the outcomes of such studies, dose-response relationships are built to deduce relevant (no) effect doses for humans taking species differences, into account to evaluate substances. However, the ethical criticism towards animal models has grown in the past and strengthened the call for new approach methods. The introduction of the 3R's, the reduction, refinement and replacement of animal tests, was the first proposal to conduct *in vitro* approaches for toxicity testing [4]. The integration of alternative testing strategies for risk assessment is implicitly required including *in vitro* or/and *in silico* toxicity tests for the estimation of hazard and risk of test substances [5, 6]. The application of *in vitro* assays profits of general ethical acceptance, high throughput implementations and lower costs in comparison to animal testing [7, 8]. Numerous *in vitro* methods were already applied in the OECD guidelines for the classification of hazard and risk assessment e.g. defined approaches on skin sensitization and *in vitro* 3T3 neutral red uptake phototoxicity test (OECD test guideline no. 432) [9, 10]. *In silico* approaches e.g. quantitative structure-activity relationships (QSAR) or read-across, allow the prioritization of substance and prediction of toxicity [5, 11]. The actual mechanism of substances on a molecular level leading to adverse effect is well represented by *in vitro* tests and may be addressed in the concept of adverse outcome pathway (AOP) to assess the hazard of substances which got into focus.

In an AOP, events on a molecular level for example receptor activation or protein binding, starts a cascade of biological key events and is causally related to an adverse outcome, like tumor formation, dysfunction of organs. Modes of action are increasingly studied in different test systems to interpret effects leading to adversity. Yet, a proper quantification of the effect and the exposure for the determination of risks is lacking [12]. Many alternatives for the identification of the mechanism of toxicity are available, but the extrapolation of *in vitro* concentrations to *in vivo* doses, also called quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) remains difficult [12, 13]. The applicability highly depends on appropriate input parameters and well characterized systems are a prerequisite for successful QIVIVE. Input parameters, e.g. those describing absorption, distribution, metabolism, and excretion (ADME) of test substances must be well characterized with suitable and robust methods. Appropriate counterparts must be defined to compare effect concentrations derived and measured in tissues, cells, media, and body fluids and extrapolate to relevant toxicological endpoints [14]. The relevance of test concentrations used for *in vitro* testing is not directly comparable to the animal or human external exposure towards test substances. Moreover, test substances underly test substances underly an uptake, distribution and metabolism in the *in vitro* test system. Thus, nominal *in vitro* concentrations (CN_{om}) of test substances, are inaccurate when used to extrapolate to the *in vivo* situation [5, 12].

1.2 Integration of In Vitro Biokinetics in QIVIVE

Performing QIVIVE enables reverse dosimetry: the translation of *in vitro* concentrations to external *in vivo* doses. A conceptual framework describing how data from *in vitro* assay results as well as *in silico* methods can provide input parameters for QIVIVE is represented in figure 1 [15, 16]. Therefore, substance specific data on ADME parameters, e.g. hepatic and/ or renal clearance, protein binding, are either derived from *in vitro* (or *in vivo*) experiments or in silico models. This enables the prediction of tissue and plasma concentrations for the toxicological endpoint of interest [17, 18].

Integrating the effect concentration *in vitro* and the physiologically based toxicokinetic (PBTK) model-derived tissue or plasma concentration, the corresponding in vivo equivalent dose is calculated. Promising results were published predicting nephrotoxicity [19], liver toxicity [20-22], cardiotoxicity [23], developmental toxicity [24-26], neurotoxicity [27], genotoxicity [28] and endocrine disruption [14, 29]. However, deficiencies concerning the experimental determination of metabolic clearance were remarked as well as the assuming that the C_{Nom} of a test substance elicits toxicological effects although the free concentration of substance should be considered [18, 30, 31]. At present, there are two options for the extrapolation of *in vitro* concentrations to *in vivo* doses. Either the concentration in cell culture medium is related to the concentration in the blood of an organism or, concentrations of the test substances in the cultured cells or even subcellular compartments, cellular membranes, receptors or other targets are related to the corresponding compartments in an organism *in vivo* [32, 33].

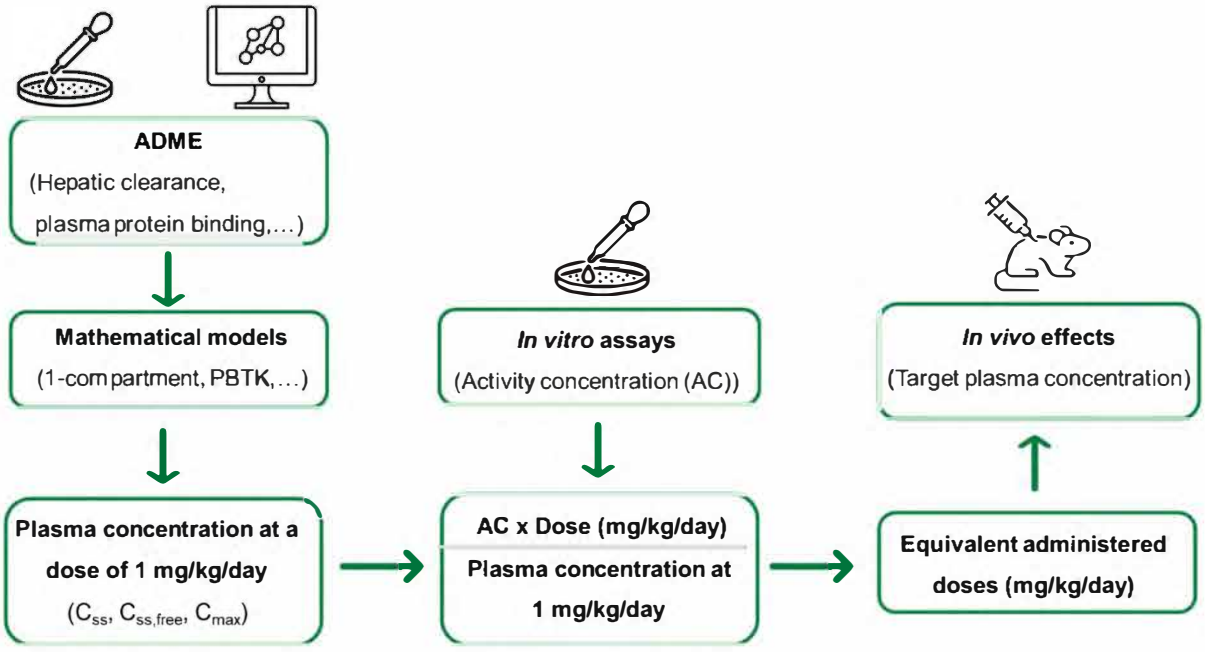

Figure 1: Linear reverse dosimetry concept. *In vitro-in silico* derived assessment of *in vivo* doses (adapted from [15, 16]).

1.3 Dosimetry in the *In Vitro* System

Basketter [5] elucidated the main key research areas to support QIVIVE. Amongst others, the accuracy of QIVIVE to derive toxicological endpoints depends on the selection of the point of departure (POD), i.e. the *in vitro* dose metric. Concentrations are relative measures and describe the ratio of a substance and the surrounding system (weight or volume), while doses are an absolute measure and the measured quantity of a substance delivered to a system, e.g. an organism [14, 30]. The selection of doses and concentrations of substance for *in vitro* testing is not arbitrary and depends on the exerted effect: Where scenarios with irreversible effects occur, e.g. covalent binding to cellular targets or oxidative stress of cellular targets [34, 35], in non-equilibrated systems, e.g. due to saturation of targets, instability of substance [36] and uneven distribution of substance in the test system, e.g. accumulation in cells, doses are the evident dose metric. In contrast, reversible effects are reflected by concentrations [37, 38]. Groothuis and colleagues [30] summed up the commonly used dose metrics in *in vitro* toxicology illustrated in figure 2 and table 1. The most prevalent dose metric is C_{Nom}, defined as the amount of a substance divided by the volume of the test solution representing the simplest way to define a test concentration for the quantification of a dose-response relationship in *in vitro* toxicology [30, 39]. Another estimate is the total concentration of a substance which is equal to the analytically measured concentration of a substance in an assay compartment, e.g. culture medium. More independent and comparable dose metric across the different test systems would be represented by the freely dissolved concentration (e.g. Free C_{Medium}) [30, 37-41]. However, the C_{Nom} does not correlate to the biologically effective dose (BED) causing toxicity – the freely dissolved concentration of a chemical is only available to interact with cells or tissues and able to exert a certain response.

On the cellular level, effective concentrations exerting toxic effects would theoretically refer to concentrations at the target site in or on cells, in the cytoplasm, at receptors on enzymes as this concentration on a molecular level triggers a cascade of key events leading to adverse effects. In turn, long exposure durations and cumulative, irreversible doses suggest the quantification via time-dependent exposure metrics, for example area under the curve or time-weighted average [42].

Table 1: *In vitro* dose metrics in toxicology.

	Dose metric	Definition
(1)	C_{Nom}	Amount of a chemical divided by the volume of the culture medium
(2)	Total C_{Medium}	Analytically measurable concentration in culture medium
(3)	Free C_{Medium}	Unbound concentration of a chemical in culture medium
(4)	Total $C_{Internal}$	Concentration of a chemical related with cells
(5)	Total $C_{Membrane}$	Concentration of a chemical in the cell membrane
(6)	Total $C_{Cytoplasm}$	Concentration of a chemical in the cytoplasm
(7)	C_{Target}	Concentration of a chemical at the target site, e.g. receptors, DNA
(8)	Area under the curve	Concentration of a chemical integrated over time
(9)	Time weighted average	Concentration of a chemical averaged over time

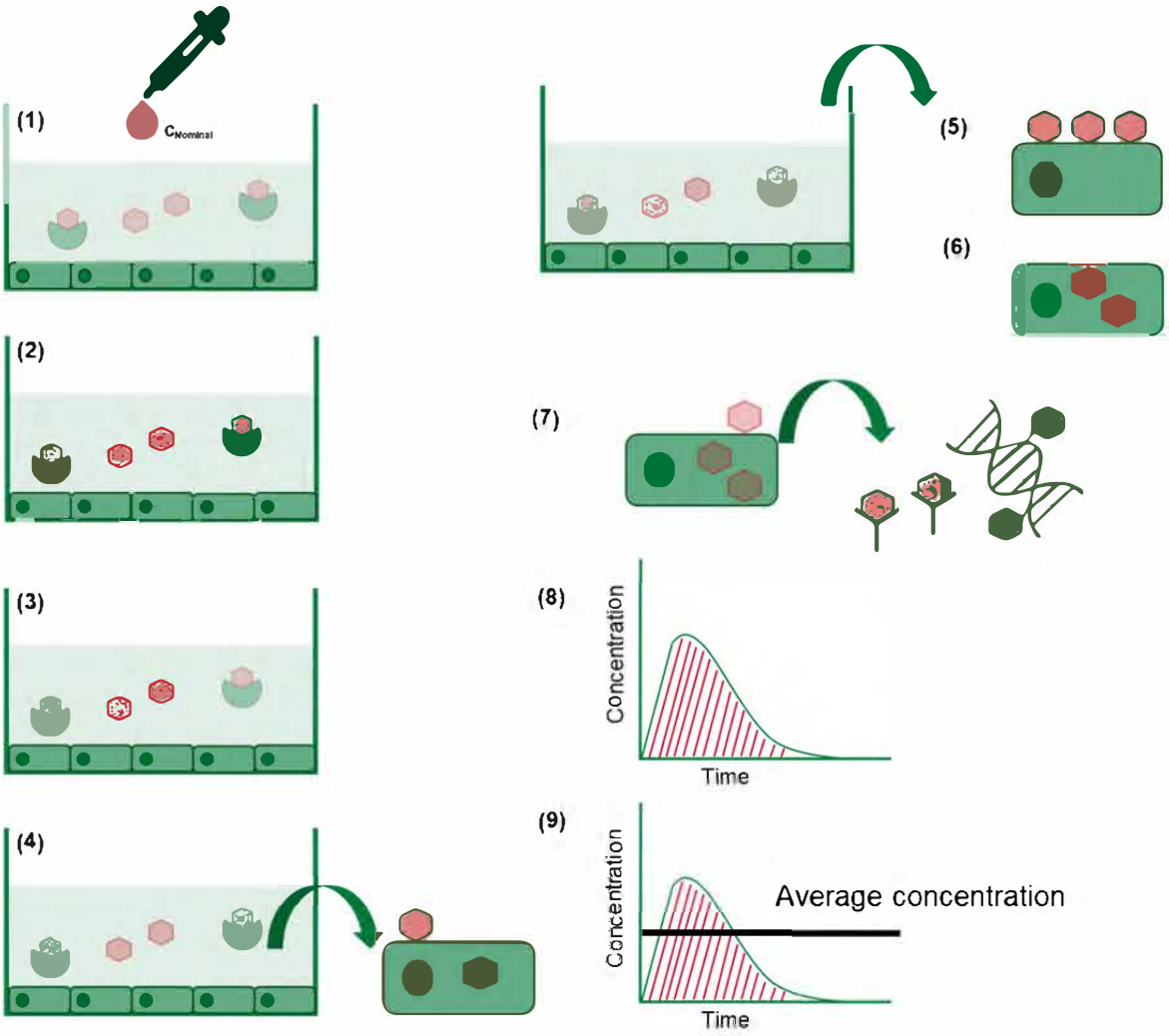

Figure 2: Supplementary figure of table 1 representing the *in vitro* dose metrics.

1.4 Factors Contributing to the *In Vitro* Exposure

An *in vitro* system tries to mimic a miniaturized, whole organism where medium, interpreted as the blood circulation, provides supplements and important nutrients to a certain cell type to maintain their functions. Investigations towards mechanisms of action of test substance, the possibility to perform assays in a high throughput manner and the subsequent time saving demonstrated the importance and the biggest advantages of *in vitro* culture systems as discussed in the previous sections.

Yet, the simplicity in comparison to *in vivo* organism and inter assay variations prohibit a consistent comparison of resulting outcomes and the prediction of *in vivo* toxicity [43]. Apparent deviations of cell culture models to *in vivo* organisms are the lacking interaction of different cell types within an organ and transfer signaling to the nervous and/or endocrine system as well as the determination of local instead of systemic effects. As before mentioned, the exposure in *in vitro* assays is recently related to C_{Nom}. Recent studies postulated that the freely available concentration of substance reflects the actual exposure in *in vitro* assays, is a better estimate to construct concentration-effect relationships and consequently considerations of *in vitro* biokinetics are crucial [12, 30, 37-41, 44]. The resulting concentration of a substance in the test system is driven by three factors (Figure 3):

1) conditions of the experimental setup,
2) the physicochemical properties of the test chemical and
3) the mode of action of the test chemical.

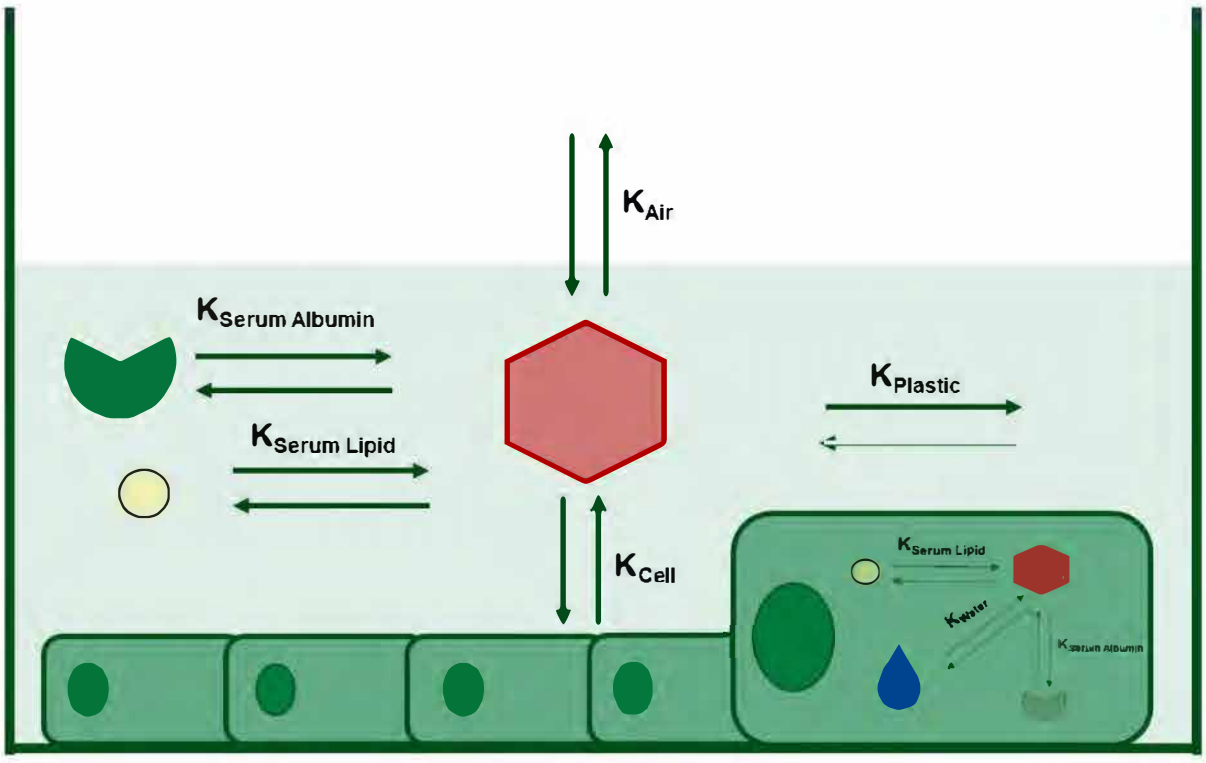

Figure 3: Schematic representation of a generic *in vitro* distribution of a test substance. In a test system comprising a cell layer, the partitioning of the chemical to different system constituents may occur; The main routes are described in the figure, i.e. binding of the substance to culture vessels, culture medium supplements and/ or partitioning to headspace and cells. The processes are expressed as partition coefficients ($K_{Plastic}$, $K_{Serum\ Albumin}$, $K_{Serum\ Lipid}$, K_{Air}, K_{Cell}). Substances undergo the similar processes in the cell itself [40, 45, 46].

Experimental setups, i.e. conditions like temperature, culture medium supplements and duration of experiments, are adjusted in accordance with the cell type, endpoint or mode of action of a toxicodynamic effect. Substances may bind to proteins and lipids derived from culture medium supplements or cells [39, 41], adsorb to the culture vessels, [44, 47] evaporate [40, 48] degrade [49] precipitate or be metabolized depending on the metabolic properties of the cells [50]. The culture conditions are concomitant with the physicochemical properties of a chemical, for example Henry constant, octanol-water-coefficient ($logP_{ow}$) and solubility ensuing changes of concentration levels of substance. Typically, the temperature is set to 37°C which may increase the evaporation of volatile substance indicated by the Henry's law constant of $\geq 10^{-3} \frac{atm \times m^3}{mol}$ [51]. Cross contamination and migration of substance into proximate wells was as well reported [46, 48]. Degradation of substances may cause deviation of C_{Nom} due to the system temperature, pH of the culture medium, photo-oxidation and degrading enzymes like esterases. Supplements, prevalently fetal bovine serum, contain important proteins, hormones, vitamins, and nutrients for an optimum cell growth and account for up to 20 % of the medium volume [38, 52]. Especially the contribution of binding to albumin and serum lipids contained in fetal bovine serum is extensively described as the limiting parameter of the free concentration of substances [30, 40, 46, 53, 54]. However, omitting such supplements is an unfeasible option since optimum cell growth and function must be warranted. Till now, most of the studies focused on the determination of protein binding and developed techniques to derive the free concentration of substances in cell-based assays, i.e. using rapid equilibrium dialysis, ultrafiltration or solid-phase microextraction (SPME) [41, 55]. Adsorption and absorption processes to plastic vessels may be enhanced and increased when the addition of serum lacked, thus, the addition of serum further provides the preservation of an equilibrium between the interacting *in vitro* compartments [37, 38, 44, 46, 56].

Regarding the overall mass balance, the mass of cells make < 1 % of the overall system - however, they cannot be neglected regarding certain scenarios [38, 47]. Considerations on the cell number, metabolic competence, transporter and complexity of the test system must be conducted to assume the substance's fate. From the substance side, the ionization state, lipophilicity, affinity to cellular targets and the corresponding mechanism of action are important parameters driving the cellular uptake.
Possible dose metrics for cells comprise of the free concentration in cytosol/water phase, the membrane bound, protein bound fraction and may include the concentration of chemical acting at the target site. Depending on the mode of action, concentrations may be related to organelles, e.g. when lysosomal trapping occurs [45, 57]. The mode of action of several substances is clearly known as well as the target in cells, e.g. enzymes (azoles binding to CYP3A4) or specific receptors (endocrine disrupting substances binding to estrogen or androgen receptor) [58-60]. However, quantifying such concentrations, e.g. at a receptor or enzyme, is not only challenging but also hard to apply experimentally. Due to the analytical efforts and challenges, cellular uptake of substance was barely addressed [40, 50, 61].

The passage of basic, cationic molecules through the negatively charged cell membrane is facilitated in contrast to anionic, acidic molecules [45]. Neutral molecules pass through the membrane easier by diffusion. Not only the charged membrane drives the uptake of substance into cells – the different pH values in the medium, cells and subcellular compartments promote the uptake of certain ionized

molecules, e.g. through ion trapping of cationic substance in the lysosomes (pH of 5.0) [45, 57, 62]. The ionization state may be neglected if high binding to serum constituents is known and the absence of serum in culture medium is prominent [49, 63]. Active transport needs to be considered as well for the influx and efflux of substance [31, 34].

1.5 Methods for Measuring and Modelling Concentrations *In Vitro*

The prediction of a chemical's fate in in vitro systems avoids extensive analytical measurements, gives a profound understanding on *in vitro* exposure and the possibility to screen compounds. Proenca and colleagues summarized briefly the variety of *in vitro* models existing and implemented until now [46]. The simulations consist of a set of mathematical equations describing partitioning processes with constant rates and partition coefficients in cell-based assays (Figure 3). The complexity in comparison to a living organism is lower as in typical *in vitro* assays consisting of two main compartments, culture medium and cell layer. To simplify matters, it is assumed that medium and cells are described to be composed of proteins, lipids and water which represent the major sorptive fraction in *in vitro* assays [52, 54, 64]. Relevant information like concentrations of supplements, cell number, and partition coefficients are nowadays to be assessed experimentally and adapted to the *in vitro* test scenario. Partition coefficients and physicochemical properties of the substance, e.g. $\log P_{ow}$, Henry's law constant, are related to losses to proteins, lipids or to air due to evaporation estimated and extracted from QSAR or data bases, e.g. ToxCast data base. A steady-state equilibrium, assuming that concentrations stay constant between the compartments over time, is presumed. Meanwhile, dynamic models describing differential equations were implemented and dilated by processes like metabolism [45, 65], degradation and cell growth [66, 67].

Successful correlations were mostly found in the prediction of concentrations [40, 41, 53]. Even though the applicability of such models is highly recommended and needed, many factors were not described yet to fully implement them into chemical risk assessment. The cytoskeleton in cells comprised of structurally different proteins and bovine serum albumin might not be the closest assumption to cellular proteins as well as lipids; the closest applied approach is the determination of binding to storage lipids, triglycerides, and membrane lipids, e.g. phospholipids and sterols [68]. Saturable processes, unspecific binding to cellular structures and predicting the mode of action of substance based on the simulations is hard to obtain [53, 68]. Furthermore, explicit knowledge on the fate of substances from experiments is missing – whether the chemical is found in the membrane, cytosolic fraction, binds to specific cellular structures or is taken up via transporters. Furthermore, such pathways are difficult to implement in models [30]. The validation of *in vitro* models based on experimental data is still required. In this context, appropriate analytical techniques are essential to gather experimental data of *in vitro* assays which may complement a full validation on computational models. Nevertheless, experimental data is rarely reported to predict free concentration in cells due to extensive experimental work and analytical challenges [69]. Nowadays, robust predictions are performed for neutral, acidic, basic or/and organic as well as for several volatile molecules. Challenges arise when it comes to the prediction of *in vitro* concentrations of nanoparticles, inorganic and volatile molecules where migration and cross-contamination was observed in the semi-closed test systems [48]. Most of the *in vitro* models were

generated for scenarios containing monolayers but must consider advanced test systems like spheroids and organ-on-a-chip which might be relevant in future testing.

1.6 Test Substances

In this work, twelve substances with a wide-ranging physicochemical properties and modes of action were selected and summarized in table 2. The selection of the test substances is further explained in the following section 2.

Acetaminophen (APAP) is an analgesic and antipyretic drug. Despite the inhibition and suppression of the synthesis of prostaglandins, the exact mechanism is not fully discovered. In the liver, APAP is transformed by Cytochrome P450 enzymes to the electrophilic mediate N-acetyl-p-benzo-quinone imine (NAPQI), which is detoxified via conjugation with glutathione, sulfate or UDP-glucuronosyltransferase and eliminated via urine as mercapturic acids [70-72]. However, an overdose of APAP (> 2 g per day) promotes depletion of glutathione leading; the mediate NAPQI can bind to cellular proteins and cause irreversible damages of the hepatocytes, e.g. necrosis. The toxicological endpoint is attributed to hepatotoxicity [71].

Bisphenol A (BPA) is commonly used to produce polycarbonates, as antioxidant of plasticizer, coating material for cans. As an estrogen receptor agonist, it triggers the estrogenic pathway and may interact with the reproductive and neuroendocrine system. BPA is metabolized mainly in the liver to inactive, glucuronidated conjugates and eliminated fast [58, 73, 74].

Caffeine (CAF) belongs to the class of alkaloids and naturally occurs in coffee beans, tea and chocolate, blocks the adenosine receptor and stimulates the central nervous system. Adverse effects, e.g. anxiety, tremors, tachycardia, insomnia and dehydration are reported toxicological endpoints [75]. CAF is metabolized by CYP 1A2 to theobromine, theophylline and 1,3,7-trimethyluric acid [76, 77].

Another alkaloid analogue, Colchicine (COL) from the autumn crocus, was also selected as test substance. The therapeutical use is limited to, for example, the treatment of gout. The binding to tubulin, the inhibition of microtubules polymerization and resulting inhibition of mitosis is defined as the mechanism of action of COL. The reported adverse outcomes are rhabdomyolysis and neuromuscular toxicity. COL is metabolized via acetylation in the liver [74, 76, 78].

Fenarimol (FEN) acts as an organic chlorinated fungicide against rusts, blackspot and mildew fungi and blocks the CYP51 enzyme in fungi. By blocking the enzyme, the synthesis of steroid molecules ergosterol is disrupted. However, the treatment of fruits, ornamental plants and trees with pesticides containing FEN was banned in 2009 because of the attributed xenoestrogen effects. FEN is thus known as a modulator of the androgen and estrogen receptor which promotes the proliferation in breast cancer cells [74, 79]. Furthermore, FEN inhibits CYP19, an aromatase which converts steroids to estrogens in the adrenal glands. FEN underlies an extensive metabolism, but is mainly oxidated and glucuronidated [74].

The nonsteroidal antiandrogen Flutamide (FLU) is used against metastatic prostate carcinoma since it is a selective antagonist of androgen receptors and consequently reducing binding of androgens. The compound is mainly hydroxylated in the liver by CYP1A2 to 2-Hydroxyflutamide and undergoes a rapid and extensive metabolism [76, 80].

Genistein (GEN) belongs to the group of isoflavones and naturally occurs in plants, e.g. soybeans and fava beans. The substance is weakly modulating the estrogen and androgen receptor [76, 81]. Furthermore, GEN undergoes hepatic and intestinal conjugation to glucuronides and inhibits cellular tyrosine kinases and interacts with estrogen receptor β [58].

Products containing the azole ketoconazole (KET) are used to treat fungal infections by interacting with 14 α-sterol demethylase. The substance is either metabolized by CYP3A4 or inhibits the enzyme and undergoes biliary excretion. The substance is attributed to cause liver toxicity [14, 76, 82].

Methyltestosterone (MT) is an anabolic steroid and structurally related to the hormone testosterone and reported as a moderate agonist of the androgen receptor. Furthermore, MT may also activate certain estrogen receptors [83]. MT underlies a hepatic metabolism; MT is hydroxylated followed by a glucuronidation or sulfation. A medication with MT is prescribed when constitutional delay in growth and absence or deficiency of endogenous androgen. Reported adverse hepatic effects are cholestasis, hepatitis and jaundice [76].

The prevention of breast cancer may be medicated with Tamoxifen (TAM) that elicits antiestrogenic effects in breasts, estrogenic effects in the uterus. As a selective estrogen receptor modulator, TAM activates estrogen receptors and initiates the conversion of androgen to estradiol [71, 84]. Common metabolites are endoxifen, desmethyl-tamoxifen, tamoxifen-N-oxide transformed by the enzymes CYP3A4, CYP2C9, CYP2D6. After conjugation with sulfate and glucuronides. At higher doses, respiratory difficulties, convulsions, neurotoxic effects were observed in cancer patients [76].

Like MT, trenbolone (TRE) is an androgen and anabolic steroid. The acetate form is prevalently used in veterinary medicine to increase muscle growth and appetite, but also illegally used as doping drug in sports. TRE has a high affinity to progesterone receptors. Aromatase and 5α-reductase transform trenbolone into estrogenic compounds [76].

The compound warfarin (WAR) is an anticoagulant with anti-vitamin K activity and utilized as rodenticide. The mechanism of action is the antagonistic interaction with the vitamin K epoxide reductase which inhibits the production of vitamin K. Numerous metabolites, mostly hydroxy-warfarin derivates, are transformed in the liver by CYP2C9, CYPC18, CYP1A2, CYP3A4 and CYP2C8 and conjugated with sulfate or glucuronide for elimination [76, 85].

Table 2: Test substances and their physico-chemical properties.

Test substance	CAS-No.	Molecular weight [g/mol]	$LogP_{ow}$	pKa	Plasma protein binding [%]	Solubility [M]	References
Acetaminophen	103-90-2	151.16	0.46	9.47	20.1	93.0	[66-68, 82, 83]
Bisphenol A	80-05-7	228.29	3.32	9.69	93.6	5.26×10^{-4}	[54, 82]
Caffeine	58-08-2	194.19	-0.07	14.00	39.0	1.10	[82-86]
Colchicine	64-86-8	399.44	1.30	1.85	39.0	0.11	[82, 83, 87]
Fenarimol	60168-88-9	331.20	3.60	12.30	93.5	4.18×10^{-5}	[82, 83]
Flutamide	1311-84-7	276.21	3.35	13.12	93.5	3.26×10^{-5}	[82, 83]
Genistein	446-72-0	270.24	2.85	6.51/ 7.72	83.5	4.55×10^{-4}	[54, 80, 82-84]
Ketoconazole	65277-42-1	531.43	4.35	4.46	96.7	1.69×10^{-4}	[67, 82, 83]
Methyltestosterone	58-18-4	302.45	3.36	19.05	97.5	0.11	[82, 83]
Tamoxifen	10540-29-1	371.51	6.84	8.82	98.5	4.58×10^{-4}	[67, 82, 83]
Trenbolone	10161-33-8	270.37	2.59	14.73	98.0	2.18×10^{-4}	[83]
Warfarin	81-81-2	308.33	2.70	5.72	95.3	0.06	[67, 68, 82, 84]

2 Aims & Objectives

Modern toxicology aims at the use of effect concentrations from non-animal testing strategies for hazard- and risk-assessment. Looking at the effects, biochemical and cellular interactions of the test substance in *in vitro* models need to be translated to toxic effects *in vivo*. This relation is established by AOPs. To obtain quantitative dose information in addition to the qualitative description of effects, *in vitro* effect concentrations need to be translated to *in vivo* effect doses. This reverse dosimetry can be performed by QIVIVE. QIVIVE is based PBPK models which needs input parameters describing the ADME of a test substance *in vivo*. There are, however, also processes *in vitro*, like cellular uptake, distribution and metabolism, which affect the effective concentration *in vivo*.

To address differential binding processes occurring and reducing the actual available concentration of substances in *in vitro* assays, the relevant doses in *in vitro* assays should be established in the course of this work to support QIVIVE in terms of dose metrics. The work was divided into three main sections to accomplish the aims.

(1) Protein binding was elucidated as one limiting system parameter. Suitable and robust techniques are necessary to determine the fraction unbound (f_u) of substance in *in vitro* assays. In the first objective, the most prevalently used experimental methods, rapid equilibrium dialysis (RED), ultrafiltration (UF) and ultracentrifugation (UC) were performed and the f_u of twelve test substances determined with validated analytical methods (**manuscript no. 1, section 3.1.1**).

(2) In the second section, concentrations in culture medium and cells were quantified in an assay setup with mouse fibroblasts, Balb/c 3T3 cells, where the distribution and uptake of twelve substances in the diffusion-based cell model was investigated. The aim was to obtain C_{Nom}, the total concentration of the substances in culture medium and cells as well as to determine the free concentration. To promote the application and proof of reliability of prediction models, the experimentally derived data were compared with a mass balance model (**manuscript no. 2, section 3.1.3**).

(3) In the last objective, the concept of *in vitro* dosimetry was transferred to predict the endocrine potential of selected test substance based on analytically measured concentrations *in vitro*. Reverse dosimetry was applied to determine external *in vivo* doses based on the *in vitro-in silico* approach. Analytical concentrations in the yeast estrogen/ androgen screening (YES/YAS-) and steroidogenesis assay were determined and an *in vitro-in silico* extrapolation was conducted based on the measured total and free concentrations of the analytes using a published PBTK model (**manuscript no. 3, section 3.2.1**) [14].

3 Results

3.1 Published Results

3.1.1 Rapid Equilibrium Dialysis, Ultrafiltration or Ultracentrifugation? Evaluation of Methods to Quantify the Unbound Fraction of Substances in Plasma

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In pharmacokinetics plasma protein binding (PPB) is a well-established parameter impacting drug disposition. The unbound fraction (f_u) is arguably regarded the effective concentration at the target site. Pharmacology and toxicology, increasingly use in vitro models. The translation of in vitro concentrations to *in vivo* doses can be supported by toxicokinetic modelling, e.g. physiologically based toxicokinetic models (PBTK). PPB of a test substance is an input parameter for PBTK.

We compared three methods to quantify f_u: rapid equilibrium dialysis (RED), ultrafiltration (UF) and ultracentrifugation (UC) using twelve substances covering a wide range of Log P_{ow} (−0.1 to 6.8) and molecular weights (151 and 531 g/mol): Acetaminophen, Bisphenol A, Caffeine, Colchicine, Fenarimol, Flutamide, Genistein, Ketoconazole, α-Methyltestosterone, Tamoxifen, Trenbolone and Warfarin.

After RED and UF separation, three polar substances (Log P_{ow} < 2) were largely unbound (f_u > 70%), while more lipophilic substances were largely bound (f_u < 33%). Compared to RED or UF, UC resulted in a generally higher f_u of lipophilic substances. f_u obtained after RED and UF were more consistent with published data. For half of the substances, UC resulted in f_u higher than the reference data. UF, RED and both UF and UC, resulted in lower f_u of Flutamide, Ketoconazole and Colchicine, respectively.

For f_u quantifications, the separation method should be selected according to the test substance's properties. Based on our data, RED is suitable for a broader range of substances while UC and UF are suitable for polar substances.

3.1.2 Toward Realistic Dosimetry *In Vitro*: Determining Effective Concentration of Test Substances in Cell Culture and Their Prediction by an *In Silico* Mass Balance Model

Dimitrijevic D, Fabian E, Nicol B, Funk-Weyer D, Landsiedel R

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Abstract:

Nominal concentrations (C_{Nom}) in cell culture media are routinely used to define concentration–effect relationships in the in vitro toxicology. The actual concentration in the medium (C_{Medium}) can be affected by adsorption processes, evaporation, or degradation of chemicals. Therefore, we measured the total and free concentration of 12 chemicals, covering a wide range of lipophilicity (log KOW −0.07–6.84), in the culture medium (C_{Medium}) and cells (C_{Cell}) after incubation with Balb/c 3T3 cells for up to 48 h. Measured values were compared to predictions using an as yet unpublished in silico mass balance model that combined relevant equations from similar models published by others. The total C_{Medium} for all chemicals except tamoxifen (TAM) were similar to the C_{Nom}. This was attributed to the cellular uptake of TAM and accumulation into lysosomes. The free (i.e., unbound) C_{Medium} for the low/no protein binding chemicals were similar to the C_{Nom}, whereas values of all moderately to highly protein-bound chemicals were less than 30% of the C_{Nom}. Of the 12 chemicals, the two most hydrophilic chemicals, acetaminophen (APAP) and caffeine (CAF), were the only ones for which the C_{Cell} was the same as the C_{Nom}. The C_{Cell} for all other chemicals tended to increase over time and were all 2- to 274-fold higher than C_{Nom}. Measurements of $C_{Cytosol}$, using a digitonin method to release cytosol, compared well with C_{Cell} (using a freeze–thaw method) for four chemicals (CAF, APAP, FLU, and KET), indicating that both methods could be used. The mass balance model predicted the total C_{Medium} within 30% of the measured values for 11 chemicals. The free C_{Medium} of all 12 chemicals were predicted within 3-fold of the measured values. There was a poorer prediction of C_{Cell} values, with a median overprediction of 3- to 4-fold. In conclusion, while the number of chemicals in the study is limited, it demonstrates the large differences between C_{Nom} and total and free C_{Medium} and C_{Cell}, which were also relatively well predicted by the mass balance model.

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Article

Toward Realistic Dosimetry *In Vitro*: Determining Effective Concentrations of Test Substances in Cell Culture and Their Prediction by an *In Silico* Mass Balance Model

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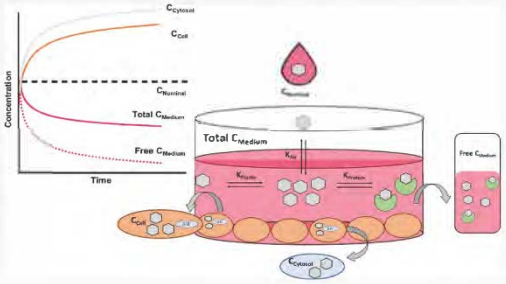
ABSTRACT: Nominal concentrations (C_{Nom}) in cell culture media are routinely used to define concentration−effect relationships in the *in vitro* toxicology. The actual concentration in the medium (C_{Medium}) can be affected by adsorption processes, evaporation, or degradation of chemicals. Therefore, we measured the total and free concentration of 12 chemicals, covering a wide range of lipophilicity ($\log K_{OW}$ −0.07−6.84), in the culture medium (C_{Medium}) and cells (C_{Cell}) after incubation with Balb/c 3T3 cells for up to 48 h. Measured values were compared to predictions using an as yet unpublished *in silico* mass balance model that combined relevant equations from similar models published by others. The total C_{Medium} for all chemicals except tamoxifen (TAM) were similar to the C_{Nom}. This was attributed to the cellular uptake of TAM and accumulation into lysosomes. The free (i.e., unbound) C_{Medium} for the low/no protein binding chemicals were similar to the C_{Nom}, whereas values of all moderately to highly protein-bound chemicals were less than 30% of the C_{Nom}. Of the 12 chemicals, the two most hydrophilic chemicals, acetaminophen (APAP) and caffeine (CAF), were the only ones for which the C_{Cell} was the same as the C_{Nom}. The C_{Cell} for all other chemicals tended to increase over time and were all 2- to 274-fold higher than C_{Nom}. Measurements of $C_{Cytosol}$ using a digitonin method to release cytosol, compared well with C_{Cell} (using a freeze−thaw method) for four chemicals (CAF, APAP, FLU, and KET), indicating that both methods could be used. The mass balance model predicted the total C_{Medium} within 30% of the measured values for 11 chemicals. The free C_{Medium} of all 12 chemicals were predicted within 3-fold of the measured values. There was a poorer prediction of C_{Cell} values, with a median overprediction of 3- to 4-fold. In conclusion, while the number of chemicals in the study is limited, it demonstrates the large differences between C_{Nom} and total and free C_{Medium} and C_{Cell}, which were also relatively well predicted by the mass balance model.



INTRODUCTION

Modern toxicological methods aim at the reduction, refinement, and replacement of animal tests while providing reliable data for risk and hazard characterization of chemicals.[1−3] Key events observed *in vitro* are linked to *in vivo* adverse outcomes, and the corresponding concentrations *in vitro* and doses *in vivo* can be linked by "quantitative *in vitro* to *in vivo* extrapolation" (QIVIVE). Information on *in vitro* biokinetics and dosimetry of test chemicals in cell-based test systems is helpful to define toxicological effects and no-effect levels based from *in vitro* studies.[4−6] *In vitro*-derived toxicological endpoints generally relate to the nominal concentration (C_{Nom}), defined as the amount of a chemical added to the test system divided by the volume of the culture medium.[7−10] However, C_{Nom} might deviate considerably from the actual concentrations in the medium and, importantly, the cellular concentrations at the target that exerts toxic effects.[11,12] Therefore, the biologically effective concentration of a chemical should more accurately correlate to plasma and tissue concentrations *in vivo* to enable more accurate QIVIVE.[13−15]

There are multiple factors that can alter the distribution and concentration of free concentrations of chemicals in the *in vitro* assays. These include adsorption of test chemicals, e.g., binding to vessels of culture flasks[16] and/or serum proteins and lipids,[5,17,18] evaporation, or spontaneous and enzymatic degradation of the test chemical. Other phenomena govern the uptake of chemicals into cells, including their ionization state and affinity to cellular targets such as binding to receptors and cell membranes, as well as accumulation into lysosomes.[10,19−21] The extent of these processes depends on the

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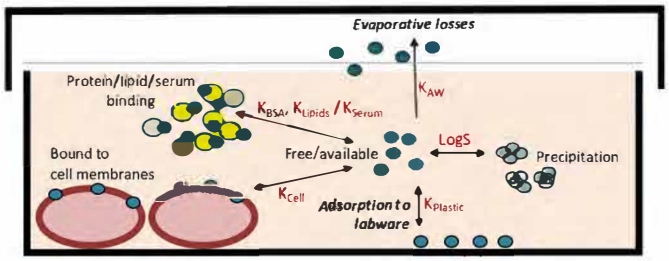

Figure 1. Partitioning within the test system used to describe the mass balance model. Schematic representation of an *in vitro* system and an example cell type, including the processes influencing the concentration of a substance and partitioning within the test system (Adapted with permission from Kramer et al. "Quantifying processes determining the free concentration of phenanthrene in basal cytotoxicity assays." Chemical Research in Toxicology, 25(2), 436−445. Copyright 2012, American Chemical Society[12]).

test system (e.g., the constituents of the culture medium, material of the vessels, coatings), as well as incubation conditions such as gas atmosphere and temperature,[12,22,23] the metabolic competence of the cells, and the physicochemical properties of the test chemical.[12,24−26]

Numerous studies recommend the total ("C_{Medium}") and unbound freely dissolved ("free C_{Medium}") concentrations in the culture medium to describe *in vitro* concentrations.[5,12,23] Several methods are available to separate free C_{Medium} and the fraction bound to proteins: equilibrium dialysis, ultracentrifugation, ultrafiltration, and solid phase microextraction (SPME), with the latter being the most prominent and established method.[1,5,12,23,27] Only a few *in vitro* studies have estimated the intracellular concentrations of test chemicals.[12,13,24] Obtaining cellular concentrations (C_{Cell}) presents analytical challenges, while the measurement of C_{Medium} is well implemented.[20] Measuring the intracellular distribution of chemicals in other compartments, such as cytosol, membranes, or receptors are even more difficult to assess. Estimating cellular concentrations by more simple concentration concepts is applicable when interactions between the chemical and intracellular targets are noncovalent, reversible, and where the *in vitro* system reaches steady state. By contrast, irreversible reactions,[28] transporter-mediated uptake,[29] accumulation in cells,[30] and instability of the test chemicals in the *in vitro* system[31,32] require more refined methods to estimate C_{Cell}. Due to the various technical difficulties in measuring chemical concentrations in multiple cell compartments, the work here focused on overall cell concentrations (C_{Cell}), as well as free and total C_{Medium}.

In addition to the experimental methods, *in silico* models have been established and used to predict *in vitro*-derived concentrations.[33,34] Commonly, these models assume steady state and an equilibrated partitioning between the compartment culture medium, cells, headspace, and plastics. Different elements such as spontaneous and enzymatic degradation, ionization of test chemicals, or the pH of different compartments were implemented in these models.[21,33,35−38] More comprehensive models for predicting a test chemical's fate in the *in vitro* test systems are recommended but not yet sufficiently established, mainly due to the lack of experimental data to validate them.[12,14] We have developed an as yet unpublished refined mass balance model using equations from versions developed by Armitage et al.,[36] Fischer et al.,[33] and Kramer et al.[12] While the equations used within the current model are not new, the combination of all of them is. The model assumes instantaneous equilibrium and is based on mass balance equations describing the partitioning between five compartments of an *in vitro* test system: headspace, serum components (proteins and lipids), cells, water phase (free), and plastic (Figure 1). The model also removes the chemical that is added to the system above the solubility limit to a "precipitate" fraction.

This manuscript describes a comprehensive experimental method to characterize the cell test system and to quantify the total and free C_{Medium} and C_{Cell} of 12 test chemicals (acetaminophen (APAP), bisphenol A (BPA), caffeine (CAF), colchicine (COL), fenarimol (FEN), flutamide (FLU), genistein (GEN), ketoconazole (KET), 17α-methyltestosterone (MT), tamoxifen (TAM), trenbolone (TRE), and warfarin (WAR)) over time in culture. The structures of the chemicals are shown in Figure S1. These chemicals were suitable for HPLC-MS analysis and represented a wide range of lipophilicities, i.e., $\log P_{ow}$ of −0.07 to 6.84, which is considered to be a key parameter that drives the cellular uptake of chemicals. Balb/c 3T3 cells were used since they are routinely used in incubations of up to 48 h in several *in vitro* toxicity assays, e.g., the *in vitro* neutral red uptake phototoxicity test (OECD guideline no. 432) and the embryonic stem cell test. This study therefore provides a robust evaluation of the comparison of predictions using the refined *in silico* mass balance model with a set of measured data generated under the same conditions.

MATERIALS AND METHODS

Chemicals and Materials. All chemicals were of the highest purity. The suppliers of the main chemicals and materials used in the experiments are listed in Supporting Information S1.

Chemicals and Cell Culture. Embryonic murine fibroblasts, clone A31 (Balb/c 3T3 cells) were obtained from the European Collection of Authenticated Cell Cultures. Cells were cultured in 150 cm^2 flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% newborn calf serum, described as "culture medium", and incubated at 37 °C, 90% humidity, and 5% CO_2. Experiments with Balb/c 3T3 cells were performed with cells at passages 5−14.

Characterization of the Transporter Expression in Balb/c 3T3 Cells. Balb/c 3T3 cells were characterized according to the doubling time (cell number) and cell size (see Supporting Information (SI) Table S1). The expression levels of membrane transporters in Balb/c 3T3 cells were measured using mRNA sequencing. To generate cell samples, cells were washed twice with 10 mL of phosphate buffer saline (PBS) and harvested using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid. The cell number was determined before the suspension was centrifuged at 300 *g* for 5 min at room temperature (RT) for mRNA extraction. For the

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purification and isolation of mRNA, cell samples were prepared as described in the user manual.[39] Raw reads were checked for quality using FastQC. Transcript sequences were mapped to the genome of mouse (GRCm38) accessed from the National Center for Biotechnology Information to derive Transcript abundance values (Program: kallisto 0.44.0). Reads were normalized for sequencing depth and gene length by dividing the read counts with the length of each gene in kilobases to give reads per kilobase (RPK). All RPK values were normalized to cell number ("per million cells") to give transcripts per million (TPM).

Cytotoxicity. The cell viability after incubation of a range of test chemical concentrations was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[40] (SI Table S2). The assay was performed as described by Kramer et al.[12] with slight modifications: 3.2×10^4 Balb/c 3T3 cells/well were seeded in 24-well plates. After 24 h, the cells were exposed to five test concentrations per test chemical (in 0.2% dimethyl sulfoxide, DMSO) for 48 h. After exposure, the cells were incubated with 0.5 mL/well culture medium containing 1 mg/mL MTT for 40 min at 37 °C. Formazan was extracted with 0.5 mL/well 100% DMSO for 5 min. The absorbance was measured at 570 nm and normalized against the control.

Exposure of Balb/c 3T3 Cells with the Test Chemicals. C_{Nom} (SI Table S3) were based on the viability in Balb/c 3T3 cells (concentrations of test chemicals resulting in ≥80% cell viability according to the MTT assay or the maximum solubility in the solvent (DMSO)). This criterion was not valid for COL, for which a cell viability of 80% was only observed at 0.2 μmol/L (data not shown). Due to analytical limitations, a higher test concentration was selected for COL. Stock solutions of the test chemicals in DMSO were diluted in culture medium (500× the final concentration) and stirred on a magnetic stirrer for 24 h at 840 rpm, 43 °C to ensure homogeneity. One million Balb/c 3T3 cells were seeded in Petri dishes (60 cm^2) with 15 mL of the culture medium. Test chemicals were added 24 h after seeding for 6, 24, and 48 h. After incubation, the culture medium was transferred to 15 mL tubes. The cell layer was washed twice with 10 mL of PBS and harvested using trypsin. Culture medium and cell lysate samples were stored at −20 °C until analysis. Cell lysate samples underwent three thaw and freeze cycles to destroy the cellular membrane and release the cytosolic fraction from the intercellular space.[41,42]

Determination of the Unbound Fraction of Test Chemicals in the Culture Medium. RED was performed as described by the manufacturer[43] to determine the fraction unbound (f_u) in culture medium. Briefly, the culture medium was spiked with the test chemicals at a final C_{Nom} of 5 μmol/L medium, 1% DMSO. A volume of 300 μL of spiked culture medium and 500 μL of PBS were transferred to the sample chamber of the inserts. The RED base plate with the samples was incubated for 6 h, at 37 °C, 5% CO_2, on an orbital shaker at 250 rpm. After dialysis, 200 μL of each chamber and an equal volume of PBS were added. The samples were frozen at −20 °C until analysis. The assay was performed in triplicates. The f_u was calculated using eq 1, where C_{PBS} is the concentration in PBS (buffer chamber) and C_{Medium} is the concentration of the test chemical in the culture medium (sample chamber)

$$f_u\ [\%] = \frac{C_{PBS}}{C_{Medium}} \times 100\% \quad (1)$$

Recovery was determined with $C_{Medium(initial/end)}$, $V_{Medium(initial/end)}$, $C_{PBS(end)}$, and $V_{PBS(end)}$. The terms "initial" and "end" indicate the concentrations before (0 h) and after the experiment (6 h). Acceptable thresholds for recovery tend from 70 to 130%

$$\text{recovery}\ [\%] = \frac{V_{Medium(end)}C_{Medium(end)} + V_{PBS(end)}C_{PBS(end)}}{V_{Medium(inital)}C_{Medium(initial)}} \times 100\% \quad (2)$$

The recoveries of all test chemicals were all within the acceptance criterion (see SI Table S4).

The total concentration in culture medium, C_{Medium}, was corrected by the f_u determined via RED to obtain the free concentration of each test chemical in the culture medium, free C_{Medium} (eq 3)

$$\text{free}C_{Medium} = f_u C_{Medium} \quad (3)$$

Determination of the Cell Number According to the Protein Content. The protein content of the treated Balb/c 3T3 cells was determined using the bicinchoninic acid (BCA) assay[44] as a marker for the number of cells. The culture medium was removed and the cell layer was washed twice with 10 mL of PBS before the addition of 3 mL of Triton-X (0.5% in PBS). After 45 min incubation at 37 °C, the cell lysate was collected and centrifuged at 1000 rpm, RT for 5 min. The supernatant of the lysate was stored at −80 °C until analysis according to the user manual.[45]

Calculation of Intracellular Concentrations. A generic diameter ("d" in μm) of Balb/c 3T3 cells was determined at each incubation time point to calculate the cellular volume of treated and untreated Balb/c 3T3 cells (V_{Cell} in μL) using the Casy Cell Counter (Roche, Germany). Assuming a spherical shape, together with the diameter and cell number (n_{Cell}) using the BCA assay, the V_{Cell} was calculated using eq 4

$$V_{Cell}\ [\mu L] = \left(\frac{d}{2}\right)^3 \pi \frac{4}{3} n_{Cell} \quad (4)$$

The concentration of the test chemicals in the cell lysate, C_{Lysate}, was measured with the appropriate analytical method and corrected by the added volume of water and trypsin (V_{Water}, 0.004 L). The intracellular concentration (C_{Cell}) was calculated using eq 5 and V_{Cell}

$$C_{Cell}\ [\mu mol/L\ cell] = \frac{C_{Lysate}\ [\mu mol/L] \times V_{Water}\ [L]}{V_{Cell}\ [\mu L]} \quad (5)$$

Determination of the Concentration of Test Chemicals in Cytosol. For potential differentiation between the intracellular and membrane-bound test chemical, an additional experiment was performed with APAP, CAF, FLU, and KET as model compounds adapted from Deusser et al.[46] and Kaiser et al.[47] Balb/c 3T3 cells were treated with the same concentrations of APAP, CAF, FLU, and KET for 48 h as described in the previous section. After 48 h of incubation, the culture medium was removed, and the cell layer washed twice with 10 mL of PBS. Then, 5 mL of digitonin solution (20 mg/L in PBS) was incubated with the cells for 5 min at RT and then on ice for 30 min to release the cytosol. The supernatants were collected and stored at −20 °C until analysis. The volume of the cytosol was based on generic calculations and assumptions. The volume of Balb/c 3T3 cells was measured (see Results section). It was assumed that cells consist of 70% water and the distribution between medium and cells occurs in the water phase. Although organelles in cells contribute to the total volume of the cell and also contain water, we applied a simplified assumption in which the volume of the cytosol in Balb/c 3T3 cells was set to be 30% lower than the total cell volume.

Determination of the Effect of Washing on Chemical Distribution. APAP, CAF, COL, and FLU were incubated for 6, 24, and 48 h, after which the cell monolayer was washed twice with 10 mL of PBS, as described above. In this experiment, the PBS wash samples were also collected after both steps. The test chemicals were measured in the culture medium, the two PBS wash samples, and in Balb/c 3T3 cells.

Sample Preparation and HPLC-MS/MS Analysis. The concentrations of the test chemicals in the culture medium, Balb/c 3T3 cell lysate, and RED samples were quantified with a high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Details of the HPLC method, the generic tune files of the mass spectrometer, and the transitions monitored in parallel reaction monitoring are summarized in Supporting Information S2, Tables S5−S12. The samples were prepared by adding 10 μL of the respective deuterated or ^{13}C-labeled internal standard (ISTD) and 4 mL of acetonitrile to 1 mL of the samples. After centrifugation at 4000g for 20 min, the supernatant was analyzed. The culture medium (50 μL) and buffer samples from the

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RED assay were mixed with 10 μL of ISTD and 200 μL of cold acetonitrile. Samples were centrifuged at 4000*g*, RT for 20 min and the supernatant analyzed. The concentrations were calculated using calibration standards containing the same matrix as the samples. Detailed parameters, e.g., concentration of ISTD, linearity range, limit of detection, and quantification can be found in Supporting Information S2, Tables S13−S18.

***In Silico* Mass Balance Model.** The refined mass balance model used several equations developed by Armitage et al.,[36] Fischer et al.,[33] and Kramer et al.[12] The free fraction of the initial amount of chemical in the aqueous phase of the medium was calculated as follows

$$F_{free} = \frac{1}{1 + K_{serum}\frac{V_{serum}}{V_{water}} + K_{cell}\frac{V_{cell}}{V_{water}} + K_{plastic}\frac{A_{plastic}}{V_{water}} + K_{air}\frac{V_{air}}{V_{water}}} \quad (6)$$

or

$$1 \Big/ \left[1 + K_{protein}\frac{V_{serum\text{-}proteins}}{V_{water}} + K_{lipid}\frac{V_{serum\text{-}lipids}}{V_{water}} + K_{cell}\frac{V_{cell}}{V_{water}} + K_{plastic}\frac{A_{plastic}}{V_{water}} + K_{air}\frac{V_{air}}{V_{water}}\right] \quad (7)$$

where

F_{free} is the fraction of chemical free in the aqueous media phase;

K_{serum} is the distribution coefficient between the serum matrix (lipid, protein) and water expressed as [L/L serum albumin];

V_{serum}/V_{Water} is the volume ratio of the serum matrix (proteins + lipids) to media water;

$K_{protein}$ is the distribution coefficient between proteins and water [expressed as L/L];

$V_{serum\ proteins}/V_{Water}$ is the volume ratio of serum proteins to media water;

K_{lipid} is the distribution coefficient between lipid and water [expressed as L/L];

$V_{serum\ lipids}/V_{Water}$ is the volume ratio of serum lipids to media water;

K_{cell} is the distribution coefficient between cells and water expressed as [L/L cells];

V_{Cell}/V_{Water} is the volume ratio of cells to media water;

$K_{plastic}$ is the distribution coefficient between plastic and water [expressed as m^3/m^2];

$A_{plastic}/V_{Water}$ is the ratio between exposed area of plastic [m^2] and media water volume [m^3];

K_{air} is the distribution coefficient between air and media water [L/L]; and

V_{air}/V_{Water} is the volume ratio between the headspace in well and media water.

Details of the mass balance model can be found in Supporting Information S3.

Data Evaluation. For the quantification and qualification of the analytes, data were handled with Xcalibur and Chromeleon 7.2. Data were analyzed with Microsoft Excel and GraphPad Prism version 9.4.1.

RESULTS

Characterization of the Applied Cells. The diameters of harvested untreated cells were 17.6 ± 0.5, 16.3 ± 0.2, and 16.0 ± 0.5 μm after 6, 24, and 48 h (≥9 biological replicates). The respective V_{Cell} were 2.9 ± 0.2, 2.3 ± 0.1, and 2.0 ± 0.1 μL/10^6 cells, assuming a spherical shape of the cells. Balb/c 3T3 cells contain 0.5 ± 0.2 mg protein/10^6 cells. The mRNA expression of membrane transporters in Balb/c 3T3 cells is presented in SI Figure S2. None of the expression levels exceeded 300 TPM that is assessed to represent a low expression. Membrane transporters of the solute carrier family (Slc) showed the highest expression, e.g., solute carrier transporters Slc7a5 (255.42 TPM), Slc3a2 (203.20 TPM), and Slc39a7 (137.60). The expression of other SLC transporters ranged from 30 to 90 TPM. Two transporters of the ATP binding cassette (ABC) family were prominent Abcf1 (106.18) and Abcf2 (98.69), and the other transporters of the ABC family were expressed at <40 TPM.

Measured Concentrations in the Culture Medium (Total and Free C_{Medium}). The initial measured concentrations of test chemicals in the culture medium at t = 0, i.e., before adding to the cells, were comparable to the C_{Nom} (with only up to 26% deviation) (Figure 2 and SI Table S19).

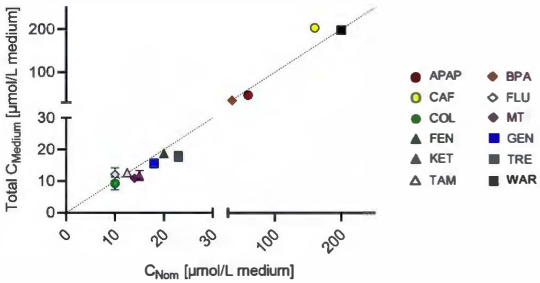

Figure 2. Measured initial total C_{Medium} of test chemicals before addition to Balb/c 3T3 cells (t = 0) compared to C_{Nom}. Each icon denotes one test chemical where circles represent hydrophilic (log P_{ow} −0.07−1.30), rhombus and squares represent moderate lipophilic (log P_{ow} 2.59−3.36), and triangles represent lipophilic (log P_{ow} > 3.60) test chemicals. Data are represented as mean in μmol/L (standard deviation, SD, if n = 3 or mean difference between individual values, if n = 2*).

Figure 3 shows the values of f_u, C_{Cell}, and total and free C_{Medium} for all test chemicals and compares them with their C_{Nom} (concentrations are also listed in SI Table S20). The highest f_u values were observed for the most hydrophilic test chemicals APAP, CAF, and COL (88.3−108.6%). MT, TRE, and WAR were moderately bound to proteins (f_u was 35−52%), and BPA, FEN, GEN, KET, and TAM were more highly bound to medium proteins (f_u values were ≤22%), especially TAM, which exhibited the lowest f_u of 1% and the highest lipophilicity.

The total C_{Medium} for all chemicals except TAM were similar to the C_{Nom} and remained constant over the 48 h incubation. The free C_{Medium} for the low (APAP) or no (CAF and COL) protein binding chemicals were similar to the C_{Nom} and remained constant over the 48 h incubation period (Figure 3A−C). The free C_{Medium} values of all other chemicals remained stable but all were less than 30% of the C_{Nom}. This was especially noticeable for TAM (Figure 3L), the total and free C_{Medium} of which decreased to 50% of the initial test concentration after 48 h of exposure.

Test chemicals could be measured in all samples, except for BPA in cell lysates after 6 h of incubation, in which C_{Cell} was below the LOQ. Of the 12 chemicals, the two most hydrophilic chemicals, APAP and CAF, were the only ones for which the C_{Cell} was the same as the C_{Nom} at t = 6 h and then decreased over the remaining time (down to 38 and 28% of the 6 h concentration, respectively). The C_{Cell} for all other chemicals tended to increase over time and were all higher than the C_{Nom}, with values 2- to 13-fold higher than C_{Nom} for six chemicals (COL, TRE, WAR, MT, FLU, and GEN (Figure 3C−H)) and

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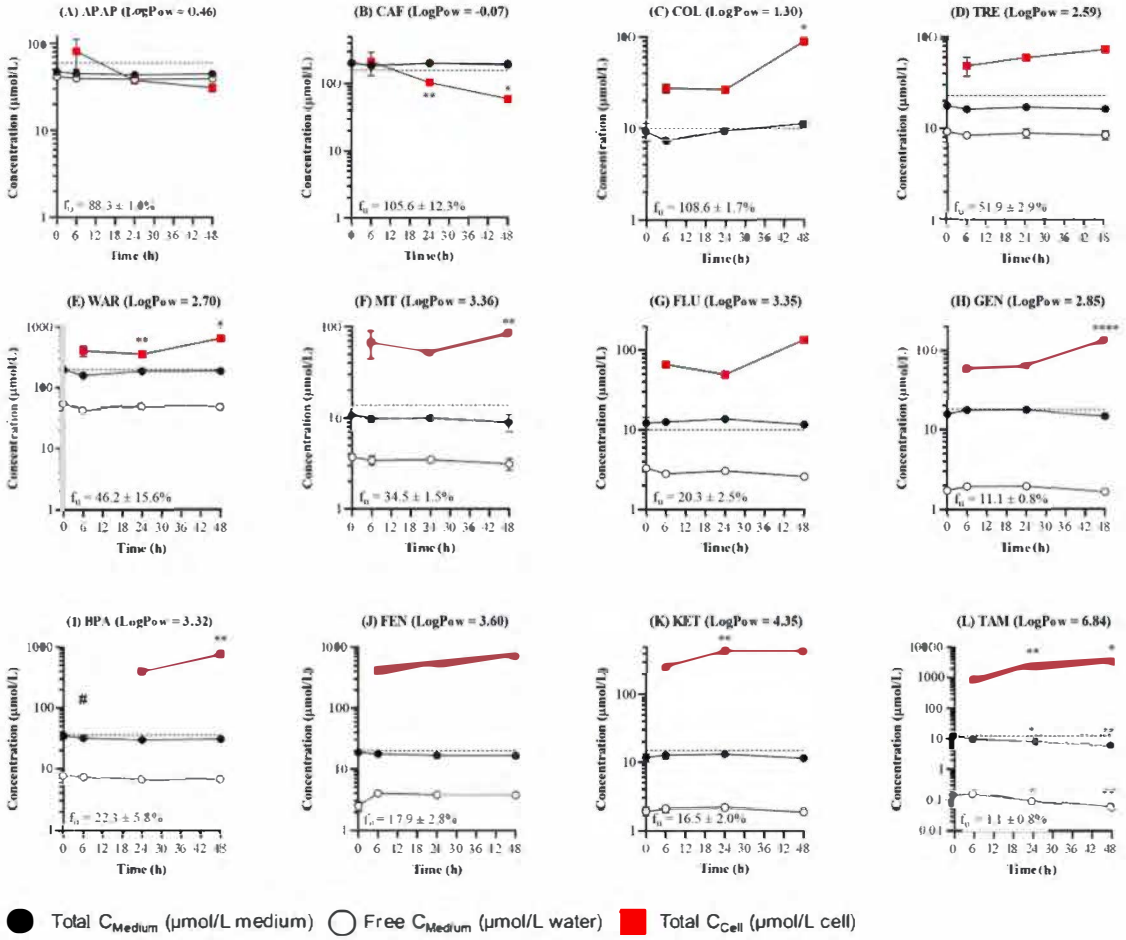

Figure 3. Measured values of f_u, C_{Cell}, and total and free C_{Medium} for all chemicals. The C_{Nom} is denoted by the dotted line, total C_{Medium} by black circles, free C_{Medium} by white circles, and the C_{Cell} by red squares. Data are represented as mean in μmol/L (SD if $n = 3$ or mean difference between individual values if $n = 2$; Welch t test where * indicates $p < 0.01$ and **$p < 0.005$). The concentration of BPA could not be detected after 6 h of incubation (#).

11- to 274-fold higher than C_{Nom} for four chemicals (BPA, FEN, KET, and TAM (Figure 3I–L)).

Table 1 shows the ratios of C_{Cell}/C_{Medium} for each chemical, along with their molecular weights, Log P_{ow}, and ionization state at pH 7.4 and measured values for f_u in the medium. Chemicals that were neutral at pH 7.4 with a low log P_{ow} and a high f_u tended not to accumulate in the cells, e.g., CAF and APAP (C_{Cell}/C_{Medium} ratios were close to 1). The C_{Cell}/C_{Medium} tended to increase as the log P_{ow} increased and the f_u decreased. The highest cellular accumulation was observed for KET and TAM, which were lipophilic, highly protein-bound, as well as partly ionized (positively charged).

Comparison of C_{Cell} and $C_{Cytosol}$. Figure 4 shows the comparison of $C_{Cytosol}$ with C_{Cell} at 48 h for four test chemicals (CAF, APAP, FLU, and KET) covering a range of lipophilicities (Log P_{ow} of −0.07–4.35). The concentrations were the same in cell lysates and cytosol from incubations with CAF and KET. $C_{Cytosol}$ values were statistically significantly higher than C_{Cell} after incubation with APAP (3.8-fold higher) and FLU (3.2-fold higher), although they were of the same order of magnitude.

Effect of Washing on Chemical Distribution. The total C_{Medium} and C_{Cell} after 6, 24, and 48 h measured in the repeat experiment (Table 2) were in accordance with those of the first experiment (SI Table S20). Approximately 50–90% of the chemicals were recovered in culture medium compared to only 0.04–6.4% in the cells, depending on the lipophilicity of the test chemical. Test chemicals were detected in the PBS after the first washing step and this amount represented 0.5–6.7% of the total C_{Medium} at $t = 0$. The concentrations of test chemicals in PBS after the second washing step for all timepoints were almost all below the LOQ for APAP, CAF, COL, and FLU, accounting for <1.1, <0.5, <2.3, and <0.3% of the total C_{Medium} at $t = 0$, respectively. Exceptions of these findings are the results in the second PBS wash for CAF and FLU after 6 h of incubation, representing 1.0 and 1.5% of the total C_{Medium}, respectively.

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Table 1. Physicochemical Properties of Test Chemicals and Measured f_u and C_{Cell}/C_{Medium} Ratios after 6, 24, and 48 h Incubation[a]

test chemical	MW [g/mol]	speciation at pH 7.4	Log P_{ow}	f_u	C_{Cell}/C_{Medium} ratio 6 h	24 h	48 h
CAF	194.19	4.91 × 10^{-7}% [neutral]	−0.07	105.6	1.1	0.5	0.3
APAP	151.16	0.86% [neutral]	0.46	88.3	1.8	0.9	0.7
COL	399.44	2.20 × 10^{-5}% [neutral]	1.30	108.6	3.8	2.9	8.0
TRE	270.37	2.96 × 10^{-8}% [neutral]	2.59	51.9	3.0	3.5	4.5
WAR	308.33	78.1% [acidic]	2.70	46.2	2.6	1.9	3.5
GEN	270.24	58.4% [neutral, acidic]	2.85	11.1	3.4	3.6	9.1
BPA	228.29	0.42% [neutral]	3.32	22.3	NA	13.3	25.2
FLU	276.21	1.69 × 10^{-4}% [neutral]	3.35	20.3	5.3	3.6	11.6
MT	302.45	1.86 × 10^{-8}% [neutral]	3.36	34.5	6.9	5.3	9.5
FEN	331.20	1.88 × 10^{-3}% [neutral]	3.60	17.9	23.5	32.5	43.7
KET	531.43	18.2% [neutral, basic]	4.35	16.5	20.0	33.0	37.6
TAM	371.51	95.9% [neutral, basic]	6.84	1.1	93.8	1.9	597.5

[a]Information about the molecular weight (MW) and log P_{ow} were obtained from the U.S. Environmental Protection Agency CompTox Chemicals Dashboard,[38] and speciation at pH 7.4 was calculated with Chemaxon. The C_{Cell}/C_{Medium} ratio was calculated by dividing the C_{Cell} value by the total C_{Medium} measured at each time point. The value for BPA after 6 h is not applicable (NA) due to the concentration in cell lysates being below the LOQ.

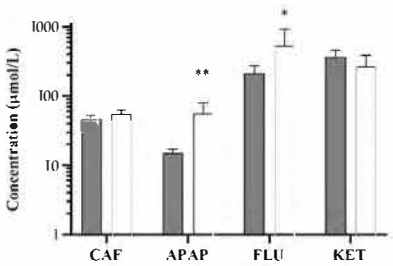

Figure 4. Comparison of C_{Cell} and $C_{Cytosol}$ for APAP, CAF, FLU, and KET. The bars show the total C_{Cell} (gray bars) and the $C_{Cytosol}$ (white bars) after 48 h of incubation. Data are represented as mean in µmol/L (SD of n = 3 experiments with triplicates; Welch t test, $p < 0.05$).

Predictions by the Mass Balance Model. The comparisons of the predicted and measured values for total and free C_{Medium} and total C_{Cell} after 6, 24, and 48 h are shown in Figure 5 and SI Table S20. Values of total C_{Medium} at 6, 24, and 48 h were well predicted by the model (Figure 5A–C), with values for 11 of 12 chemicals predicted to be within 30% of the measured values, and a median ratio of predicted/measured values of 1.0. The exception to this was for TAM, for which the model predicted much lower concentrations (0.23–0.46 µmol/L) than were measured (5.7–9.8 µmol/L) at different timepoints. While the total C_{Medium} of TAM was not well predicted, the predicted free C_{Medium} of this chemical was within 2-fold of the measured values (Figure 5D–F). Indeed, the free C_{Medium} of all 12 chemicals were relatively well predicted, with a median ratio of measured/predicted values of 1.1 at all three timepoints. The maximum overprediction was for GEN, which was overpredicted by 2.9-fold at 48 h, and the

Table 2. Effect of Washing on the Distribution of APAP, CAF, COL, and FLU[a]

test chemical [C_{Nom}]	incubation time [h]	total C_{Medium} [µmol/L medium]	total C_{PBS1} [µmol/L PBS]	total C_{PBS2} [µmol/L PBS]	total C_{Cell} [µmol/L cell]
APAP [60 µmol/L medium]	0	67.8 ± 1.9 (100%)			
	6	50.0 ± 10.3 (74%)	2.6 ± 0.9 (3.8%)	<0.8 (<1.1%)	75.1 ± 33.4 (0.04%)
	24	40.5 ± 2.7 (60%)	2.5 ± 0.8 (3.6%)	<0.8 (<1.1%)	120.1 ± 40.0 (0.12%)
	48	39.1 ± 1.4 (58%)	1.7 ± 0.4 (2.6%)	<0.8 (<1.1%)	38.1 ± 8.0 (0.09%)
CAF [160 µmol/L medium]	0	160.5 ± 5.6 (100%)			
	6	127.0 ± 15.1 (79%)	6.7 ± 0.3 (4.2%)	1.6 ± 0.4 (1.0%)	182.0 ± 91.0 (0.04%)
	24	133.1 ± 12.4 (83%)	4.5 ± 0.7 (2.8%)	<0.9 (<0.5%)	161.1 ± 19.3 (0.03%)
	48	125.9 ± 15.6 (78%)	5.1 ± 0.5 (3.2%)	<09 (<0.5%)	40.9 ± 7.1 (0.03%)
COL [10 µmol/L medium]	0	6.5 ± 0.4 (65%)			
	6	4.2 ± 0.5 (80%)	0.3 ± 0.1 (4.5%)	<0.1 (< 2.3%)	9.1 ± 3.6 (0.05%)
	24	5.2 ± 0.7 (80%)	0.1 ± 0.1 (0.5%)	<0.1 (< 2.3%)	10.1 ± 1.4 (0.10%)
	48	4.9 ± 0.2 (74%)	0.3 ± 0.1 (4.3%)	<0.1 (< 2.3%)	4.3 ± 0.4 (0.11%)
FLU [10 µmol/L medium]	0	9.0 ± 0.3 (100%)			
	6	7.7 ± 1.4 (86%)	0.5 ± 0.1 (5.1%)	1.6 (0.4) (1.5%)	250.4 ± 16.7 (0.93%)
	24	6.4 ± 1.2 (72%)	0.3 ± 0.1 (3.8%)	<0.1 (0.3%)	333.1 ± 12.0 (2.5%)
	48	5.3 ± 0.5 (59%)	0.3 ± 0.1 (3.8%)	<0.1 (0.3%)	344.5 ± 2.0 (6.4%)

[a]Total concentrations in the culture medium (Total C_{Medium}) in PBS collected after the first and second washing steps (C_{PBS1} and C_{PBS2}) and in cells (C_{Cell}) after 6, 24, and 48 h incubation with APAP, CAF, COL, and FLU. Data are represented as mean in µmol/L ± SD, n = 3. Values in brackets are the mass balance percentages given as mean ± SD in % (total C_{Cell} value is without conversion to cell volume).

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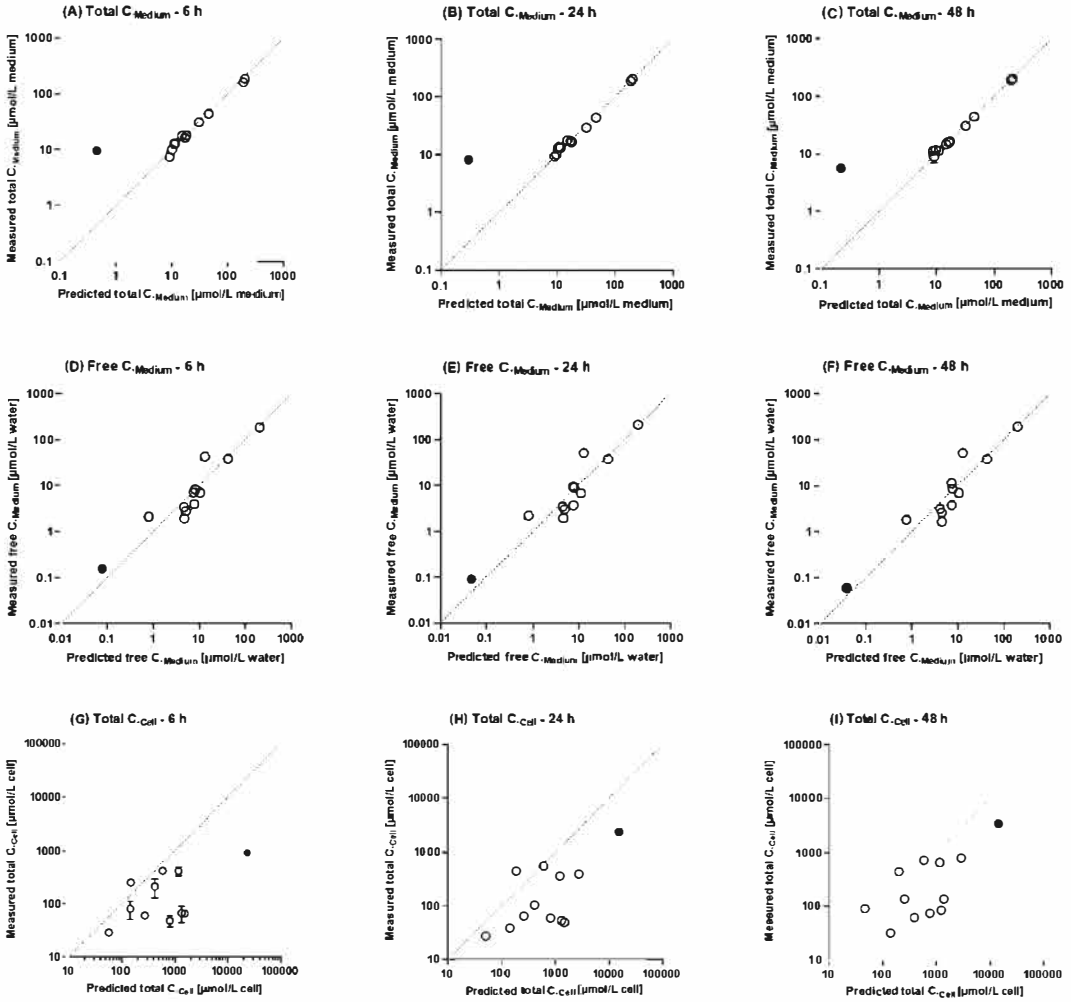

Figure 5. Comparison of predicted and measured values of C_{Cell} and total and free C_{Medium} for all test chemicals after 6, 24, and 48 h of incubation. The test chemicals are denoted by white circles, the black circles indicate the most lipophilic chemical of the set of substances, TAM. The line of identity is denoted by the dotted line. Detailed information on the data is presented in Supporting Information S1, Table S20. Data are represented as mean in µmol/L (SD if $n = 3$ or mean difference between individual values if $n = 2$).

maximum underprediction was for WAR, which was underpredicted by 3.3-fold at all three timepoints. The highest difference between predicted and measured values was the total C_{Cell}, which was mainly overpredicted by up to 26-, 31.4-, and 15.2-fold at 6, 24, and 48 h (Figure 5G–I). The only two chemicals that were correctly predicted with 2-fold of the measured values at all three timepoints were COL and FEN. Most of the total C_{Cell} values were overpredicted, especially those for FLU, MT, TAM, and TRE (by up to 31.4-, 25.5-, 26.0-, and 17-fold, respectively). Despite these differences, the median fold overprediction for all 12 chemicals was still only 3.0-, 4.1-, and 4.1-fold of the measured values at 6, 24, and 48 h, respectively.

DISCUSSION

The use of *in vitro* dosimetry in the *in vitro* testing should be carefully considered and remains a challenge for the development of robust approaches to QIVIVE.[12,14,33] Typically, C_{Nom} is used to extrapolate the blood and tissue concentrations, even though it does not reflect the actual *in vitro* effect concentration.[9,15,48] The reason for this is that methods to experimentally measure concentrations in cells, cell membranes, or other cell compartments are limited or very technically demanding, especially for high-throughput assays.[49] Total or free C_{Medium} or the concentration in the cytosol are closer to the biologically effective concentration and therefore better values for QIVIVE purposes.[10,35,50] In the current study, we measured the concentrations of test chemicals in the cells

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and cytosol, as well as the free and total concentrations in the medium.

Characterization of Balb/c 3T3 Cells. As with any assay, it is important to characterize the cells under the conditions of the assay since different cell sources and media can impact the phenotype of the cells.[51] We selected Balb/c 3T3 cells for this work since they are routinely used in toxicity assays. The determination of the actual cell volume of Balb/c 3T3 cells was not experimentally performed. For the sake of simplicity, the volume of Balb/c 3T3 cells was derived based on the assumption that cultured cells take a spherical shape and a diameter of 16.0−17.6 μm as experimentally determined. The V_{Cell} ranged between 2.0 and 2.9 μL, which is in general agreement with data from Gülden et al.[18] with a V_{Cell} of 1.8 ± 0.7 μL/10^6 cells. Balb/c 3T3 cells contained 0.5 ± 0.2 mg protein/10^6 cells, which is also in line with values of 0.5 and 0.4 mg protein/10^6 cells reported by Gülden et al.[18] and Kramer et al.[12] Genes coding for transporters were detected in Balb/c 3T3 cells; however, the highest expression of transporters was for Slc7a5, which was 255 TPM, which is not high according to Wagner et al.[52,53] The uptake of test chemicals in Balb/c 3T3 cells can be concluded to be largely a diffusion-limited process, with active, transport protein-mediated uptake of minor relevance. In addition, xenobiotic-metabolizing enzymes, e.g., CYP enzymes, are reported to be expressed in negligible levels in Balb/c 3T3 cells.[12,54−56] This was also reflected in the current dataset since there was negligible depletion of the parent chemicals over time. Therefore, these cells represent a suitable cell model for understanding general mechanisms concerning diffusion biokinetics and for the validation of *in silico* models based on this mechanism. Their obvious limitation is that the results cannot be extrapolated to other cell types with a higher transporter function or to chemicals that involve transporter-mediated uptake.

Experimental Design and Sample Preparation Considerations. *Cell Disruption and Cell-Associated Versus Cytosolic Concentrations.* Several methods have been described to prepare samples for the measurement of cell-associated chemical concentrations. These include using detergents,[46,47] freezing and thawing cycles, ultrasonication,[13,41] and liquid homogenization.[42] One of the consequences of each method is the resulting sample may or may not contain plasma membranes together with chemicals that may have bound to the outside of the cells. In this case, the true intracellular concentration is not measured—just the "cell-associated" concentration. Therefore, we compared two methods to disrupt cells in the current study, namely, freeze−thaw cycles to derive C_{Cell} values (including plasma membranes and cytosol) and treatment with digitonin to derive $C_{Cytosol}$ values. Digitonin permeabilizes the cell plasma membranes to release the cytosol into the medium without releasing plasma membranes and associated chemicals. Both methods yielded comparable results, indicating that none of chemicals tested associated with the plasma membrane and that $C_{Cytosol}$ values were a good representation of intracellular concentrations. Although the method involving lysis with digitonin is practically less demanding compared to freeze−thaw cycles, there was more variability in the measurements of $C_{Cytosol}$ of experiments (% CV values were 14−29% for C_{Cell} values and 13−73% for $C_{Cytosol}$ values), indicating less robust results.

Although it is possible to measure total and free C_{Cell}, this was not conducted in this study due to the technically challenging issues with handling low volumes yielded from cell culture preparations.[20,57] While others could demonstrate the measurement of free C_{Cell} in HEK293 cells and primary human hepatocytes,[58,59] this may be an exceptional case. For many purposes, C_{Cell} may be a sufficient proxy and refinements by, e.g., using free $C_{Cytosol}$ may only yield improvements within the experimental error of measuring the concentrations. The current study indicates that two methods provide comparable concentrations: (i) trypsinization and a following disruption of cells by thawing and freezing cycles[12] and (ii) lysis with a digitonin solution.[46] This needs to be verified by further studies, including controls with buffer, and addressing the possible wash out effect.

Impact of PBS Wash on Cell Distribution. A technical concern relating to the washing procedure is that it may contribute to the removal of chemicals from the cells, i.e., diffusing back into the wash medium, thus, resulting in artificially lower C_{Cell} values. To address this, the concentrations of four chemicals removed in the PBS washes were measured in a follow up experiment. There was no link between the percentage of chemical removed in the first wash with their lipophilicity.

The amounts of compounds in the second wash were (with only two exceptions in the wash after 6 h for CAF and FLU) below the LOQ and significantly lower than the first wash. However, the calculated amounts of the compound at the LOQ still exceed the recovered amounts of the compound in the cells for APAP, COL, and CAF and account for about 5−12% of the recovered amounts of FLU in the cells. Although these data were originally generated to prove that chemicals in the cells do not diffuse back into the PBS during washing, this statement cannot be supported based on the current data.

Concentrations in Culture Medium. C_{Nom} of the test chemicals were generally in accordance with the measured total C_{Medium} at t_0, indicating that the preparation of the solutions was in accordance with the target concentrations and that nonspecific binding to the tubes did not occur. The total C_{Medium} remained constant over 48 h of incubation for 11 of the 12 test chemicals. The exception to this was TAM, the C_{Medium} of which decreased over time. This was attributed to the cellular uptake of TAM and accumulation into lysosomes.[61]

One factor affecting the effect concentration resulting in a biological effect is protein binding, as demonstrated for 9 of the 12 chemicals tested in this study. When extrapolating to no-effect levels in the *in vitro* assays, chemicals exhibiting low binding to proteins would not need a correction of the total C_{Medium} by f_u since the total C_{Medium} and free C_{Medium} are similar.[10,25] The more lipophilic test chemicals exhibiting higher binding to proteins, resulting in the free C_{Medium} being lower than total C_{Medium}, may require a correction factor before correlating with an *in vitro* effect. This reduction of free C_{Medium} in the *in vitro* test systems has also been described by Henneberger et al.[5] and Huchthausen et al.[25] While human plasma contains 60−80 g protein/L, of which 50−60% is albumin and is similar to that in newborn calf serum (71.5 g protein/L proteins; with 39.5 g/L albumin),[60,62] in this study, the medium contained only 10% serum (which is typical for many cell cultures); hence, protein concentrations were lower in cell culture media compared to the human serum *in vivo*. Therefore, when performing the correction for protein binding and then extrapolating to *in vivo* concentrations, the

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physiological concentrations of proteins in human plasma and the *in vitro* incubation should be considered.

Factors Impacting Intracellular Concentrations of Chemicals in Balb/c 3T3 Cells. The kinetics of the distribution of chemicals will depend on several properties. Lipinski et al. defined the "Rule of 5" postulating that molecules with the following criteria can pass the cell membrane by diffusion: a molecular weight of <500 g/mol, $\log P_{ow} < 5$, five H-bond donors, and ten H-bond acceptors, e.g., oxygen and nitrogen.[62] All of the test chemicals were of a molecular weight near to or lower than 500 g/mol and most had a $\log P_{ow} < 5$. These data showed that hydrophilic chemicals (CAF and APAP) did enter the cells but did not accumulate, while lipophilic chemicals accumulated, with the extent correlated with the $\log P_{ow}$. This correlation between the $\log P_{ow}$ and cellular uptake has also been reported by others.[12,57,63−65] In addition, lipophilic chemicals preferentially distributed to the cells, with C_{Cell}/C_{Medium} ratios between 9 and 598 for chemicals with $\log P_{ow}$ values at or greater than 2.85.

The mass balance model assumes instantaneous equilibrium of the test chemicals and, indeed, many drugs pass membranes in seconds to minutes.[21,66] However, due to the technical difficulties of measuring the distribution in multiple wells, such short incubations were not possible in the current study. The timepoints chosen were relevant to the assays in which the cells are used. Most accumulation of chemicals occurred in the first 6 h (although this may have occurred in the first few minutes of incubation) but C_{Cell}/C_{Medium} ratios continued to increase until 48 h, indicating additional slower accumulation after this time.

The passage through the lipid layer and the negatively charged cell membrane also facilitates the movement of cationic molecules.[12,21,37,57] Most of the test chemicals were uncharged molecules at a pH 7.4, except GEN, KET, TAM, and WAR which were partly ionized. Due to the negatively charged nature of GEN and WAR, their diffusion through the negatively charged membrane barrier would be impeded and might result in lower C_{Cell}.[10,67]

The free C_{Medium} values could be expected to be linked to a lower cellular uptake of chemicals, as binding the proteins in the medium may prevent this. McManus et al.[41] reported that the use of serum-free medium resulted in higher cellular concentrations in prostate cancer cells than the serum-containing medium. However, our results do not support this hypothesis since chemicals with high C_{Cell}/C_{Medium} ratios were moderately or highly bound (f_u < 35%). The impact of protein may therefore also depend on the affinity of the interaction, with covalently bound chemicals exhibiting lower cellular uptake.

In addition to the properties described above, a chemical's affinity to cellular targets can enhance its uptake into cells, e.g., lysosomal trapping.[57,68] This was observed in this study for TAM and confirmed by other groups.[17,57] TAM is a positively charged molecule at pH 7.4; it is lipophilic and of rather small molecular size. These characteristics tend to facilitate adsorption of TAM to the cell membrane of Balb/c 3T3 cells.[29,37,63] COL also appeared to accumulate more than expected based on its $\log P_{ow}$, which may be due to it binding to tubulin, where it blocks the polymerization of microtubules and suppresses the cell division and proliferation.[21,69]

Prediction Capacity of the Mass Balance Model. The mass balance model predicted the total C_{Medium} within 30% of the measured values for all but one of the test chemicals. The exception was TAM, for which total C_{Medium} was underpredicted by ~25-fold. The reason for this was attributed to the uptake of this positively charged molecule into the cells and accumulation into the lysosomes. Despite this, the model was able to predict the free C_{Medium} of TAM at each time point. The free C_{Medium} of the remaining chemicals were also relatively well predicted by the model. Notably, values for GEN, KET, and WAR were over- or underpredicted by factors of up to 2.9-, 2.5-, and 3.3-fold, respectively. These test chemicals are ionized and lipophilic molecules. In cell culture media with pH 7.4, KET is positively charged and GEN and WAR are negatively charged. Positively charged molecules are known to have a strong affinity to α-glycoproteins and negatively charged molecules to albumin.[70,71] This may contribute to the difference between the predicted and measured values, since the model parameterization was calibrated with neutral molecules. The prediction of the partitioning of chemicals into cells was based on a model predicting binding to liposomes and serum albumin and the ionization of the test chemicals was not considered. This may account for the poorer prediction of C_{Cell} values by the current model. Moreover, binding to serum albumin may not be predictive of binding to other proteins, such as microfilaments, microtubules, and intermediate filaments.[14] Future efforts will aim to refine the model for charged molecules, as well as chemicals that bind to microfilaments, e.g., COL. It is hoped that datasets such as the one presented here will enable such refinements to be conducted.

Although the mass balance model is relatively easy to use and predicts the biokinetics of neutral chemicals relatively well, it does, however, have significant limitations that experimental models also face, i.e., it does not reflect xenobiotic metabolism or active transport. Cell types proficient in xenobiotic metabolism and transport-mediated uptake and efflux, e.g., hepatocytes, will require appropriate, dynamic models. Likewise, concentrations of volatile, ionizing, and spontaneously degrading test chemicals will not be accurately predicted and will require additional refinements to account for these common attributes of test chemicals.

CONCLUSIONS

This study compared measured biokinetics data in Balb/c 3T3 cells with predicted values using a refined *in silico* mass balance model. While the number of chemicals in the study is limited, this is the first time, to our knowledge, that a study combining *in vitro* and *in silico* biokinetics techniques has been published. These data provide information on cell preparation techniques with a well-established and toxicologically relevant cell line, using accurate analytical methods. It is hoped that these experimental data can be used by others for the validation of similar mass balance models. The mass balance model combined relevant, albeit known, QSARs to result in a version that could accurately predict total C_{Medium} and free C_{Medium} for nonvolatile, mostly neutral chemicals with a $\log P_{ow}$ between −1 and 6.6. Predictions were of chemicals with predominantly diffusion-based uptake into cells with low xenobiotic-metabolizing and low active transport capacity. Comparisons of C_{Nom} with free C_{Medium} and $C_{Cytosol}$ already demonstrated the large differences between them and that nominal concentrations may not always be the most relevant when comparing to a bioactivity in the same cells. These measured and predicted values allow the extrapolation of (a) free C_{Medium} to an unbound concentration in human blood; (b) total C_{Medium}

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to the total concentration in human blood; and (c) the total C_{Cell} as surrogate to tissue concentrations in humans *in vivo*. Future studies will aim to expand the set of test chemicals to increase the confidence in the experimental method and improve the accuracy of the *in silico* model. Likewise, the methods should be expanded to be applicable to ionized chemicals and to cells with metabolizing capacities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.2c00128.

Detailed information on test chemicals, analytical methods, data summaries, and predicted values using the mass balance model (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

APAP;acetaminophen; $A_{Plastic}$;area of culture vessel; BCA;bicinchoninic acid; BPA;bisphenol A; BPA-d_{16};bisphenol A d_{16}; C_{Cell};total concentration in Balb/c 3T3 cells; $C_{Cytosol}$;total concentration in the cytosol of Balb/c 3T3 cells; C_{Lysate};total concentration of a test chemical in Balb/c 3T3 cell lysate; C_{Medium};concentration of a test chemical in the culture medium; C_{Nom};nominal concentration of a test chemical; C_{PBS};concentration in PBS; CAF;caffeine; COL;colchicine; *d*;diameter; DMEM;Dulbecco's modified Eagle's medium; DMSO;dimethyl sulfoxide; DZP-d_5;diazepam-d_5; EDTA;ethylenediaminetetraacetic acid; F_{Air};fraction in air; F_{Cells};total fraction of a chemical in cells; F_{free};fraction of chemical free in the aqueous media phase; F_{Media};total fraction of a chemical in medium; $F_{Plastic}$;fraction of a chemical bound to plastic; F_{precip};precipitated fraction of a chemical in the test system; FEN;fenarimol; FLU;flutamide; f_u;fraction unbound of a test chemical; GEN;genistein; h hour(s) ISTD;internal standard; K_{Air};distribution coefficient between air and water; K_{Cell};distribution coefficient between cells and water; K_{Lipid};distribution coefficient between lipid and water; $K_{Protein}$;distribution coefficient between proteins and water; K_{Serum};distribution coefficient between serum matrix (lipid, protein) and water; KET;ketoconazole; Log K_{aw};water−air partition coefficient; Log P_{ow};octanol−water partition coefficient; MT;methyltestosterone; MTT;3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; n_{Cell};cell number; PBS;phosphate buffered saline; RED;rapid equilibrium dialysis; RT;room temperature; Slc;solute carrier; $S_{total,max}$;maximum solubility of the test chemical in the test system; TAM;tamoxifen; TAM-$^{13}C_2$;tamoxifen-$^{13}C_2$; TES-d_3;testosterone-d_3; TPM;transcripts per million; TRE;trenbolone; V_{Cell};volume of Balb/c 3T3 cells; V_{Medium};volume of medium; V_{Serum};volume of serum; $V_{Serum\ lipids}$;volume of lipids in serum; $V_{Serum\ proteins}$;volume of proteins in serum; V_{Water};volume of water; WAR;warfarin

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3.1.3 Supporting Information (manuscript no. 2)

Supporting Information 1

Towards realistic dosimetry *in vitro*: Determining effective concentrations of test substances in cell culture and their prediction by an *in silico* mass balance model

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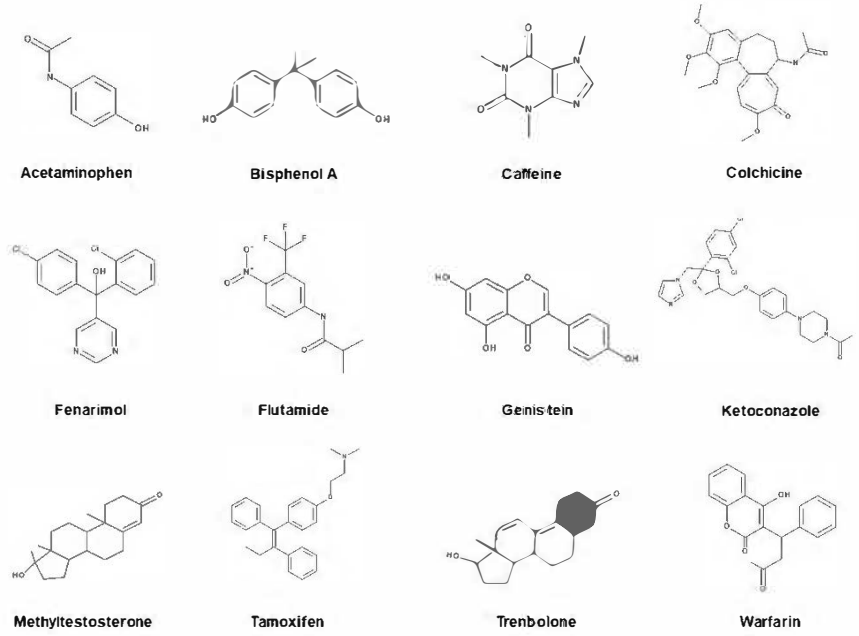
Supplementary Materials S1: Details on (test) chemicals and materials

Figure S1: Test chemical structures



S4

S5

Chemicals

Test chemicals and internal standards (ISTD) were purchased from Sigma Aldrich (Steinheim, Germany) in the highest purity available: Acetaminophen (CAS-No. 103-90-2, ≥ 99.0 %), Bisphenol A (CAS-No. 80-05-7, ≥ 99.0 %), Bisphenol A-d_{16} (BPA-d_{16}, CAS-No. 96210-87-6, ≥ 99.0 %), Caffeine (CAS-No. 58-08-2, ≥99.0 %), Colchicine (CAS-No. 64-86-8, ≥ 95.0 %), Diazepam-d_5 (DZP-d_5, CAS-No. 65854-76-4, 98 %), Fenarimol (CAS-No. 60168-88-9, > 98.0 %), Flutamide (CAS-No. 13311-84-7), Genistein (CAS-No. 446-72-0, ≥ 99.0 %), Ketoconazole (CAS-No. 65277-42-1, 99.0-101.0 %), 17α-Methyltestosterone (CAS-No. 58-18-4, 99.0 %), Tamoxifen (CAS-No. 10540-29-1, ≥ 98.0 %), Tamoxifen-$^{13}C_2$ (TAM-$^{13}C_2$, 93.0 %), Testosterone-d_3 (TES-d_3, CAS-No. 77546-39-5), Trenbolone (CAS-No. 10161-33-8, ≥ 93.0 %), Warfarin (CAS-No. 81-81-2, > 98.0 %). Ammonium acetate (CAS-No. 631-61-8, ≥ 99.0 %), Acetonitrile (CAS-No. 75-05-8, product no. 34851, ≥ 99.9 %), and water (CAS-No. 7732-18-5, product-no. 270733) were purchased from Sigma Aldrich (Steinheim, Germany). Dimethyl sulfoxide (CAS-No. 67-68-5, 99.5 %) and formic acid (CAS-No. 64-18-6, 98 - 100 %) were obtained from AppliChem GmbH (Steinheim, Germany) and Bernd Kraft (Duisburg, Germany). Methanol (CAS-no. 67-56-1, product no. 1060072500, > 99.9 %) was supplied from Merck (Darmstadt, Germany). Dulbecco's modified eagle's medium (DMEM,

product-no. P04-0401), its supplements L-Glutamine (Product-no. P04-80050), Penicillin/ Streptomycin (Product-no. P06-07050), newborn calf serum (NCS, product-no. P30-0401) and cell culture-related reagents (0.05 % Trypsin-0.02, % Ethylenediaminetetraacetic acid (EDTA), product-no. P10-023100, Phosphate buffer saline (PBS, product no. P04-3650)) were obtained from PanBiotech (Aidenbach, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT, CAS-No. 298-93-1, 98 %) from Sigma Aldrich (Steinheim, Germany) was used for the cytotoxicity assay. RNeasy® Mini Kit for the purification of total RNA from animal cells was purchased from QIAGEN (Hilden, Germany). DNAse- and RNAse-free Ethanol was obtained from VWR (CAS-No. 64-17-5, ≥ 99.7 %, Darmstadt, Germany).

Materials

24- and 96- well plates (Product no. 92024 and 92048), Petri dishes (Product no. 93100, 60 cm^2) and cell culture flasks (Product no. 90151, 150 cm^2) from TTP AG (Trasadingen, Switzerland) were used for cell culture experiments. Materials for the RED, e.g. RED base plate; sealing tape and RED device inserts with a membrane molecular weight cut-off of 8 kDa (Product no.

89811, 15036, 89809) as well as the Bicinchoninic acid (BCA) protein assay kit (Product no. 23225) were purchased from Thermo Fisher Scientific (Schwerte, Germany).

Table S1: Balb/c 3T3 cell number and volume

Experimentally determined Balb/c 3T3 cell number and volume (A) 6 h , (B) 24 h and (C) 48 h after incubation with the test chemicals. After incubation, cells were lyzed in a solution containing Triton-X (0.5 % in PBS) to derive the protein content. Obtained protein content of treated Balb/c 3T3 cells was normalized against untreated Balb/c 3T3 cells to determine the cell number. Experimentally determined diameter were taken for the calculation of the respective cell volume (2.9 µL / 10^6 cells). Data are represented as mean (SD if n=3 or mean difference between individual values, if n=2*).

(A) 6 h incubation

Test chemical	Cell number	Cell volume [µL]
APAP*	2.3 x 10^6 ($0.1 x 10^6)	6.8 (0.3)
BPA	2.5 x 10^6 ($0.2 x 10^6)	7.6 (0.3)
CAF*	3.3 x 10^6 ($0.6 x 10^6)	9.8 (1.9)
COL*	3.6 x 10^6 ($0.1 x 10^6)	10.6 (0.4)
FEN*	2.8 x 10^6 ($0.3 x 10^6)	8.4 (0.9)
FLU*	3.0 x 10^6 ($0.4 x 10^6)	8.8 (1.2)
GEN	3.0 x 10^6 ($0.3 x 10^6)	8.5 (1.0)
KET*	2.5 x 10^6 ($0.1 x 10^6)	7.5 (0.1)
MT	2.7 x 10^6 ($0.5 x 10^6)	7.6 (1.4)
TAM*	2.7 x 10^6 ($0.4 x 10^6)	8.0 (1.2)
TRE	2.4 x 10^6 ($0.1 x 10^6)	6.6 (0.8)
WAR*	2.7 x 10^6 ($0.1 x 10^6)	8.0 (0.2)

(B) 24 h incubation

Test chemical	Cell number	Cell volume [µL]
APAP*	3.8 x 10^6 ($0.3 x 10^6)	9.2 (0.7)
BPA	5.9 x 10^6 ($0.2 x 10^6)	14.0 (0.4)
CAF*	4.8 x 10^6 ($0.4 x 10^6)	11.5 (1.0)
COL*	2.7 x 10^6 ($0.2 x 10^6)	6.4 (1.0)
FEN*	4.5 x 10^6 ($0.2 x 10^6)	10.7 (0.5)
FLU*	4.8 x 10^6 ($0.5 x 10^6)	11.5 (1.2)

GEN	5.8 x 10^6 (0.9 x 10^6)	13.8 (2.1)
KET*	4.3 x 10^6 (0.1 x 10^6)	10.2 (0.1)
MT	4.3 x 10^6 (0.6 x 10^6)	10.4 (1.4)
TAM*	4.5 x 10^6 (0.4 x 10^6)	10.8 (1.0)
TRE	4.1 x 10^6 (0.8 x 10^6)	9.8 (1.8)
WAR*	5.3 x 10^6 (0.1 x 10^6)	12.7 (0.2)

(C) 48 h incubation

Test chemical	Cell number	Cell volume [µL]
APAP*	8.6 x 10^6 (0.5 x 10^6)	18.4 (1.1)
BPA	8.9 x 10^6 (1.9 x 10^6)	19.0 (4.0)
CAF*	7.9 x 10^6 (0.3 x 10^6)	16.9 (0.6)
COL*	2.4 x 10^6 (0.1 x 10^6)	5.2 (0.2)
FEN*	8.1 x 10^6 (0.6 x 10^6)	17.3 (1.3)
FLU*	8.1 x 10^6 (0.7 x 10^6)	17.3 (1.6)
GEN	7.0 x 10^6 (0.9 x 10^6)	15.0 (1.9)
KET*	6.8 x 10^6 (0.3 x 10^6)	14.5 (0.6)
MT	7.0 x 10^6 (1.2 x 10^6)	14.9 (2.5)
TAM*	6.6 x 10^6 (0.5 x 10^6)	14.1 (1.0)
TRE	6.6 x 10^6 (0.7 x 10^6)	14.1 (1.5)
WAR*	8.3 x 10^6 (1.1 x 10^6)	17.7 (2.3)

Table S2. Cytotoxicity concentration curves

Balb/c 3T3 cells were incubated for 48 h at five nominal test concentrations [µM] represented on the top, the values in the bottom display the cell viability [%]. Test concentrations represent nominal concentrations and are given in µM. Experiments were conducted in triplicates. Cell viability data are expressed as mean (SD).

Test Chemical	Concentration [µM] and Cell Viability [%, SD]				
APAP	3	30	60	150	300
	101.2 (5.3)	102.6 (3.5)	99.7 (3.7)	89.4 (8.6)	53.6 (17.2)
BPA	1.8	18	36	90	180
	105.7 (1.5)	107.2 (1.6)	100.6 (7.6)	70.1 (15.2)	46.3 (16.6)
CAF	8	80	160	400	800
	101.8 (1.4)	100.3 (2.3)	98.0 (3.8)	98.3 (6.0)	96.4 (8.3)
COL	0.1	1	2	5	10
	17.7 (2.7)	13.9 (1.5)	13.7 (1.9)	13.6 (2.1)	14.6 (1.6)
FEN	0.1	1	2	5	10
	103.9 (7.5)	100.9 (6.3)	98.0 (5.5)	93.9 (0.9)	89.6 (1.8)
FLU	0.5	5	10	25	50
	96.9 (7.3)	94.7 (6.5)	92.9 (4.5)	82.9 (4.3)	76.1 (3.7)
GEN	1.8	18	36	90	180
	103.7 (1.6)	78.0 (6.2)	39.7 (8.2)	29.0 (3.5)	24.9 (9.2)
KET	1.5	15	30	75	150
	101.7 (10.4)	77.3 (8.7)	46.7 (14.2)	20.7 (12.7)	15.0 (9.6)
MT	1.4	14	28	70	140
	99.5 (3.4)	84.8 (9.8)	78.5 (5.8)	54.5 (10.6)	36.2 (2.8)
TAM	0.3	2.5	5	12.5	25
	111.0 (2.3)	116.5 (0.4)	115.5 (3.9)	100.5 (16.0)	94.1 (36.7)
TRE	1.2	11.5	23	57.5	115

	98.3 (5.5)	85.2 (1.0)	75.3 (4.8)	42.7 (1.9)	20.3 (3.5)
WAR	10	100	200	500	1000
	104.2 (2.4)	98.2 (6.2)	91.9 (8.5)	69.2 (9.9)	51.6 (15.4)

Table S3: Total Cmed of incubation medium prior to adding to the cells compared to C_{Nom}.

Data are represented as mean in µM (SD if n=3 or mean difference between individual values, if n=2*).

Test chemical	Total C_{med}	Difference
[C_{Nom}]	[µM]	[%]
APAP* [60 µM]	47.3 (1.2)	-23.7
BPA [36 µM]	34.9 (1.2)	-3.1
CAF* [160 µM]	203.2 (6.3)	23.8
COL [10 µM]	9.3 (1.4)	-7.8
FEN* [20 µM]	18.8 (0.6)	-6.5
FLU* [10 µM]	12.1 (1.5)	19.3
GEN* [18 µM]	15.6 (1.4)	-14.2

KET* [15 µM]	11.8 (1.1)	-24
MT [14 µM]	11.0 (0.7)	-24.4
TAM* [12.5 µM]	12.7 (0.5)	1.6
TRE [23 µM]	17.8 (1.5)	-25.6
WAR* [200 µM]	198.0 (7.0)	-1

Table S4: f_u and recoveries of the test chemicals in culture medium determined with RED.

Test chemical	f_u [%]	Recovery [%]
APAP	88.3 (1.0)	80.6 (7.0)
BPA	22.3 (5.8)	88.3 (8.8)
CAF	105.6 (12.3)	94.3 (14.4)
COL	108.6 (1.7)	90.7 (3.6)
FEN	17.9 (2.8)	110.7 (6.0)
FLU	20.3 (2.5)	98.2 (8.3)
GEN	11.1 (0.8)	98.6 (2.7)
KET	16.5 (2.0)	104.6 (6.7)
MT	34.5 (1.5)	90.8 (6.4)
TAM	1.1 (0.8)	88.3 (8.8)
TRE	51.9 (2.9)	104.2 (1.7)
WAR	46.2 (15.6)	94.2 (9.5)

RED was performed for 6 h at 37°C, data are shown as mean (SD). Experiments were performed in triplicates.

Supplementary Materials S2: HPLC-MS Methods

The system consisted of a Q Exactive Focus mass spectrometer coupled with an Ultimate 3000 UHPLC. The pump flow rate was 0.250 mL/min and depending on the analyte, the separation was performed either on an Ascentis Express C18 (2.7 µm, 100 * 2.1 mm, Sigma Aldrich, Steinheim, Germany), XbridgeTM C18 (2.5 µm, 50 * 2.1 mm, Waters GmbH, Eschborn, Germany) or YMC-Pack Pro C18 RS (5.0 µm, 150 * 4.6 mm, YMC, Dinslaken, Germany). Tables S5 – S9 describe the HPLC methods applied for the test chemicals are described. For all test chemicals except BPA (Table S10), the same mass spectrometry tune file (Table S10) was used. Table S12 shows the ion fragmentations of the test chemicals and internal standards.

Solutions of 10 mM, 150 µM, 100 µM, 10 µM and 1 µM of the test chemicals were prepared in acetonitrile or methanol (BPA and BPA-d_{16}). The performance of the analytical methods was evaluated taking the guidelines EMA, 2009 and ICH, 2014 into account. The results are described in table S13 – S18. Matrix and carry over effects were not observed in any matrix or were negligible. Beside the selectivity and carry-over effects, following parameters were checked:

a) Linearity

b) Limit of detection (LOD) and limit of quantification (LOQ)

c) Accuracy

$$\text{Accuracy [\%]} = \frac{C^{\text{Calculated}}}{C^{\text{Nom}}} \times 100\ \% \quad (1)$$

d) Precision

$$\text{RSD [\%]} = \frac{\text{SD}}{\text{Mean}} * 100\ \% \quad (2)$$

e) Recovery

$$\text{Recovery [\%]} = \frac{\text{Peak area of the extracted sample (A)}}{\text{Peak area of the spiked blank extract (B)}} \times 100\ \% \quad (3)$$

Table S5: HPLC method for APAP, CAF, FLU, GEN, WAR & DZP-d_5.

HPLC Method 1		
Pre-column	Phenomenex Security Guard™ Ultra Catridges, C18 column 3.0 mm	
Analytical column	Ascentis Express C18 column (2.7 µm, 100 * 2.1 mm)	
Column temperature	25°C	
Mobile phase A	950 mL water + 50 mL acetonitrile + 0.1 mL formic acid	
Mobile phase B	950 mL acetonitrile + 50 mL water + 0.1 mL formic acid	
Gradient	0.000 min	0 % B
	5.000 min	70 % B
	7.500 min	70 % B
	7.501 min	0 % B
	10.000 min	0 % B
Flow rate	0.250 mL/min	
Injection volume	10 µL/ 5 µL (GEN)	
Run time	10 min	

Table S6: HPLC method for FEN, MT, TAM & TRE, DZP-d_5, TES-d_3 and $^{13}C_2$-TAM.

HPLC Method 2		
Pre-column	Phenomenex Security Guard™ Ultra Catridges, C18 column 3.0 mm	
Analytical column	Ascentis Express C18 column (2.7 µm, 100 * 2.1 mm)	
Column temperature	25°C	
Mobile phase A	950 mL water + 50 mL acetonitrile + 0.1 mL formic acid	
Mobile phase B	950 mL acetonitrile + 50 mL water + 0.1 mL formic acid	
Gradient	0.000 min	0 % B
	4.000 min	100 % B
	11.000 min	100 % B
	11.001 min	0 % B

	15.000 min	0 % B
Flow rate	0.250 mL/min	
Injection volume	5 µL	
Run time	15 min	

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Table S7: HPLC method for BPA and BPA-d_{16}.

HPLC Method 3		
Pre-column	Phenomenex Security Guard™ Ultra Catridges, C18 column 3.0 mm	
Analytical column	Xbridge™ C18 (2.5 µm, 50 * 2.1 mm)	
Column temperature	35°C	
Mobile phase A	2 mM ammonium acetate + 0.1 mL formic acid	
Mobile phase B	1000 mL methanol + 0.1 mL formic acid	
Gradient	0.000 min	5 % B
	3.000 min	85 % B
	5.000 min	85 % B
	5.001 min	5 % B
	8.000 min	5 % B
Flow rate	0.250 mL/min	
Injection volume	5 µL	
Run time	8 min	

Table S8: HPLC method for KET and DZP-d_5.

HPLC Method 4		
Pre-column	Phenomenex Security Guard™ Ultra Catridges, C18 column 3.0 mm	
Analytical column	Ascentis Express C18 column (2.7 µm, 100 * 2.1 mm)	
Column temperature	25°C	
Mobile phase A	950 mL water + 50 mL acetonitrile + 0.1 mL formic acid	
Mobile phase B	950 mL acetonitrile + 50 mL water + 0.1 mL formic acid	
Gradient	0.000 min	50 % B
	3.000 min	100 % B
	3.001 min	50 % B
	5.000 min	50 % B

Flow rate	0.250 mL/min
Injection volume	5 µL
Run time	5 min

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Table S9: HPLC method for COL and DZP-d_5.

HPLC Method 5	
Pre-column	Phenomenex Security Guard™ Ultra Catridges, C18 column 3.0 mm
Analytical column	YMC-Pack Pro C18 RS (5.0 µm, 150 * 4.6 mm)
Column temperature	25°C
Mobile phase A	950 mL water + 50 mL acetonitrile + 0.1 mL formic acid
Mobile phase B	950 mL acetonitrile + 50 mL water + 0.1 mL formic acid
Isocratic	0.000 - 10.000 min 50 % B
Flow rate	0.250 mL/min
Injection volume	5 µL
Run time	11 min

Table S10: General tune file for parallel reaction monitoring for all compounds except BPA and BPA-d_{16}.

Parameter	Conditions
Spray voltage	3500 (+) resp. 2900 V (-)
Capillary temperature	320 C
Probe heater temperature	350 (+) resp. 300 C (-)
Sheath gas	35 (+) resp. 21 (-)
Auxiliary gas	10 (+) resp. 7 (-)
S-Lens RF level	50
Resolution	35,000
AGC target	5e5
Spectrum data type	Centroid

Table S11: Mass spectrometer tune file for BPA and BPA-d_{16}.

Parameter	Conditions
Spray voltage	4000 V

Capillary temperature	250 C
Probe heater temperature	250 C
Sheath gas	30
Auxiliary gas	8
S-Lens RF level	50
Resolution	35,000
AGC target	5e5
Spectrum data type	Centroid

S22

Table S12: Ion fragments of the test chemicals. The first fragment represents the quantifier, the second fragment the qualifier.

Test chemical	Ion mode	Precursor and fragment mass [m/z]	Collision energy [V]
APAP	ESI+	152.07 → 110.06/ 93.03	20/25
BPA	ESI-	227.1078 → 133.06/ 211.08	27/30
BPA d_{16}	ESI+	244.2160 → 142.12	36
CAF	ESI+	195.09 → 138.07/ 110.07	25/ 25
COL	ESI+	400.18 → 310.12/ 358.16	30/ 25
DZP-d_5	ESI+	290.11 → 154.04	33
FEN	ESI+	331.03 → 268.05/ 138.99	30/ 45
FLU	ESI-	275.07 → 202.01/ 205.02	30/ 25
GEN	ESI+	271.06 → 153.01/ 91.06	35/ 45
KET	ESI+	531.16 → 489.15/ 255.01	47/ 60
MT	ESI+	303.23 → 97.07/ 109.07	38/ 35
TAM	ESI+	372.23 → 72.08/ 129.07	44/ 36
TAM-$^{13}C_2$	ESI+	373.2316 → 74.09	33
TES-d_3	ESI+	291.23 → 109.07	32
TRE	ESI+	271.69 → 199.11/107.05	35/ 42
WAR	ESI+	307.10 → 161.02/ 250.06	26/ 32

Table S13: Evaluated parameters in culture medium (a). The parameters in table S9 were monitored taking the guidelines from EMA 2009 and ICH, 2014 into account.

Test chemical	Retention time [min]	Linearity range [µM]	r^2	LOD [µM]	LOQ [µM]	ISTD [µM]
APAP	1.080	0.25 – 2.5	0.999	0.029	0.099	DZP-d_5 173.0
BPA	4.557	5 - 50	0.994	0.121	0.403	BPA-d_{16} 40.9
CAF	1.069	0.25 – 2.5	0.999	0.068	0.226	DZP-d_5 86.5
COL	6.953	1.0 – 10	0.997	0.009	0.030	DZP-d_5 173.0
FEN	5.241	0.1 – 1.0	0.994	0.006	0.018	DZP-d_5 34.5
FLU	7.088	0.1 – 1.0	0.998	0.002	0.005	DZP-d_5 34.59
GEN	5.603	0.1 – 1.0	0.997	0.004	0.014	DZP-d_5 34.5
KET	0.926	0.1 – 1.0	0.997	0.007	0.022	DZP-d_5 34.5
MT	5.032	0.1 – 1.0	0.990	0.016	0.053	TES-d_3 34.3
TAM	4.534	0.25 – 2.5	0.996	0.004	0.012	TAM-$^{13}C_2$ 200

TRE	4.538	0.1 – 1.0	0.998	0.017	0.055	TES-d_5 34.3
WAR	6.757	0.1 – 1.0	0.994	0.002	0.007	DZP-d_5 34.5

S25

Table S14: Evaluated parameters in culture medium (b). The parameters in table S10 were monitored taking the guidelines from EMA 2009 and ICH, 2014 into account. Two concentrations of quality control samples (mid and high concentrations) were proven for accuracy and precision. Precision, accuracy and recovery were determined in five replicates shown as mean ± SD.

Test chemical	C_{Nom} of quality controls [µM]	Accuracy [%]	Precision [%]	Recovery [%]
APAP	1.0	120.4 ± 5.3	4.4	95.6 ± 10.7
	2.0	97.6 ± 3.8	3.9	
BPA	20.0	96.7 ± 3.8	4.0	97.5 ± 3.1
	40.0	98.7 ± 1.1	1.1	
CAF	0.8	106.9 ± 6.3	5.9	104.5 ± 8.3
	1.5	103.1 ± 7.7	7.5	
COL	4.0	100.2 ± 12.2	12.1	96.9 ± 11.8
	8.0	94.3 ± 2.6	2.8	
FEN	0.4	104.6 ± 4.3	4.1	104.5 ± 8.3
	0.8	101.7 ± 9.1	8.9	
FLU	0.4	115.2 ± 2.0	1.7	102.8 ± 8.1
	0.8	107.3 ± 8.5	7.9	
GEN	0.4	94.7 ± 11.3	11.9	95.0 ± 4.1
	0.8	105.8 ± 8.1	7.6	
KET	0.3	113.8 ± 6.0	5.3	105.6 ± 8.0
	0.6	104.2 ± 2.7	2.6	
MT	0.3	101.9 ± 0.9	0.9	99.3 ± 4.8
	0.6	99.0 ± 2.0	2.0	
TAM	0.9	103.6 ± 4.1	4.0	100.3 ± 6.2

	1.8	98.9 ± 5.9	5.9	
TRE	0.4	107.8 ± 3.0	2.8	110.2 ± 12.7
	0.8	111.0 ± 3.2	2.9	
WAR	0.4	92.3 ± 1.9	2.0	106.8 ± 14.0
	0.8	101.8 ± 2.9	2.8	

S27

Table S15: Evaluated parameters in Balb/c 3T3 cell lysate (a). The parameters in table S11 were monitored taking the guidelines from EMA 2009 and ICH, 2014 into account.

Test chemical	Retention time [min]	Linearity range [µM]	r^2	LOD [µM]	LOQ [µM]	ISTD [µM]
APAP	1.080	0.1 – 1.0	0.994	0.007	0.021	DZP-d_5 34.5
BPA	4.554	1.0 – 10	0.991	0.280	0.936	BPA-d_{16} 400
CAF	1.069	0.1 – 1.0	0.998	0.003	0.011	DZP-d_5 34.5
COL	7.120	0.05 – 0.5	0.990	0.007	0.023	DZP-d_5 34.5
FEN	5.241	0.05 – 0.5	0.991	0.002	0.007	DZP-d_5 34.5
FLU	7.088	0.025 – 0.25	0.999	0.006	0.020	DZP-d_5 6.9
GEN	5.603	0.025 – 0.25	0.999	0.003	0.009	DZP-d_5 6.9
KET	0.930	0.1 – 1.0	0.990	0.001	0.004	DZP-d_5 34.5
MT	5.017	0.05 – 0.5	0.990	0.009	0.031	TES-d_3 34.3
TAM	4.565	0.05 – 0.5	0.990	0.002	0.007	TAM-$^{13}C_2$ 200

TRE	4.554	0.05 – 0.5	0.993	0.008	0.026	TES-d_5 34.3
WAR	6.777	0.01 – 0.1	0.996	0.001	0.003	DZP-d_5 6.9

S29

Table S16: Evaluated parameters in Balb/c 3T3 cell lysate (b). The parameters in table S12 were monitored taking the guidelines from EMA 2009 and ICH, 2014 into account. Two concentrations of quality control samples (mid and high concentrations) were proven for accuracy and precision. Precision, accuracy and recovery were determined in five replicates shown as mean ± SD.

Test chemical	C_{Nom} of quality controls [µM]	Precision [%]	Accuracy [%]	Recovery [%]
APAP	0.30	109.4 ± 6.2	5.6	105.1 ± 7.5
	0.60	102.9 ± 3.0	2.9	
BPA	4.0	107.0 ± 6.2	5.8	99.5 ± 6.9
	8.0	107.9 ± 2.8	2.6	
CAF	0.30	88.0 ± 2.5	2.9	100.1 ± 6.1
	0.60	90.9 ± 3.5	3.5	
COL	0.3	110.3 ± 2.6	2.6	100.8 ± 2.1
	0.5	96.8 ± 1.6	1.6	
FEN	0.15	102.8 ± 2.3	2.3	110.9 ± 9.5
	0.30	97.7 ± 1.0	1.0	
FLU	0.08	117.8 ± 6.7	5.7	97.2 ± 8.2
	0.15	99.3 ± 6.2	6.2	
GEN	0.08	104.5 ± 2.3	2.2	107.6 ± 15.2
	0.15	98.1 ± 1.4	1.4	
KET	0.3	114.0 ± 8.4	7.3	87.3 ± 15.2
	0.6	107.4 ± 9.2	8.6	
MT	0.15	102.7 ± 1.6	1.6	99.4 ± 6.9
	0.30	96.9 ± 2.5	2.6	
TAM	0.15	113.0 ± 3.1	2.7	101.7 ± 3.4

	0.30	112.5 ± 6.0	5.3	
	0.15	100.9 ± 1.5	1.5	
TRE	0.30	99.7 ± 4.3	4.3	103.1 ± 2.7
	0.03	114.4 ± 4.8	4.2	
WAR	0.06	103.1 ± 5.1	5.0	106.1 ± 6.4

S31

Table S17: Evaluated parameters in Balb/c 3T3 cytosol lysate (a). The parameters in table S13 were monitored taking the guidelines from EMA 2009 and ICH, 2014 into account.

Test chemical	Retention time [min]	Linearity range [µM]	r^2	LOD [µM]	LOQ [µM]	ISTD [µM]
APAP	1.113	0.05 – 0.5	0.994	0.006	0.021	DZP-d_5 34.5
CAF	1.070	1.0 - 10	0.998	0.029	0.099	DZP-d_5 345.0
FLU	7.088	0.05 – 0.5	0.990	0.021	0.073	DZP-d_5 34.5
KET	1.020	0.05 – 1.0	0.990	0.007	0.023	

Table S18: Evaluated parameters in Balb/c 3T3 cytosol lysate (b). The parameters in table S14 were monitored taking the guidelines from EMA 2009 and ICH, 2014 into account. Two concentrations of quality control samples (mid and high concentrations) were proven for accuracy and precision. Precision, accuracy and recovery were determined in five replicates shown as mean ± SD.

Test chemical	C_{Nom} of quality controls [µM]	Precision [%]	Accuracy [%]	Recovery [%]
APAP	0.2	111.3 ± 5.7	5.2	99.0 ± 4.9
	0.4	115.7 ± 19.9	17.2	
CAF	4.0	97.6 ± 1.1	1.2	83.7 ± 1.4
	8.0	126.3 ± 1.2	0.8	
FLU	0.2	114.1 ± 5.4	4.7	99.8 ± 1.8
	0.4	103.2 ± 5.1	4.9	
KET	0.4	116.7 ± 13.2	11.3	112.1 ± 10.4

0.8	115.7 ± 19.9	26.7

S33

Supplementary Materials S3: *In silico* mass balance model equations

The fraction of the initial amount of chemical that is free in the aqueous phase of the medium is calculated as follows:

$$F_{free} = \frac{1}{1 + K_{serum} \times \frac{V_{serum}}{V_{water,\,med}} + K_{cell} \times \frac{V_{cell}}{V_{water,\,cell}} + K_{plastic} \times \frac{A_{plastic}}{V_{water,\,med}} + K_{air} \times \frac{V_{air}}{V_{water,\,med}}} \quad \text{(S1)}$$

Or

$$\frac{1}{1 + K_{protein} \times \frac{V_{serum\,proteins}}{V_{water,\,med}} + K_{lipid} \times \frac{V_{serum\,lipids}}{V_{water,\,med}} + K_{cell} \times \frac{V_{cell}}{V_{water,\,cell}} + K_{plastic} \times \frac{A_{plastic}}{V_{water,\,med}} + K_{air} \times \frac{V_{air}}{V_{water,\,med}}} \quad \text{(S2)}$$

Where:

F_{free}: Fraction of chemical free in the aqueous media phase

K_{serum}: Distribution coefficient between serum matrix (lipid, protein) and water expressed as [L/L serum albumin]

$V_{serum}/V_{water,\,med}$: Volume ratio serum matrix (proteins + lipids) to media water

$K_{protein}$: Distribution coefficient between proteins and water [expressed as L/L]

$V_{serum\,proteins}/V_{water,\,med}$: Volume ratio serum proteins to media water

K_{lipid}: Distribution coefficient between lipid and water [expressed as L/L]

$V_{serum\ lipids}/V_{water,\ med}$: Volume ratio serum lipids to media water

K_{cell}: Distribution coefficient between cells and water expressed as [L/L cells]

$V_{cell}/V_{water,\ med}$: Volume ratio cells to media water

$K_{plastic}$: Distribution coefficient between plastic and water [expressed as m^3/m^2]

$A_{plastic}/V_{water,\ med}$: Ratio between exposed area of plastic [m^2] and media water volume [m^3]

K_{air}: Distribution coefficient between air and media water [L/L]

$V_{air}/V_{water,\ med}$Volume ratio between headspace in well and media water

From the free fraction in the water phase, the fraction of chemical present in the other assay compartments are calculated as follows:

$$F_{serum} = K_{serum} \times \frac{V_{serum}}{V_{water,med}} \times F_{free} \quad \text{(S3)}$$

- Total fraction in medium (free and bound)

$$F_{media} = F_{serum} + F_{free} \quad \text{(S4)}$$

- Fraction bound to plastic

$$F_{plastic} = K_{plastic} \times \frac{A_{plastic}}{V_{water,\,med}} \times F_{free}$$

(S5)

- Fraction in air

$$F_{air} = K_{air} \times \frac{V_{air}}{V_{water,\,med}} \times F_{free}$$

(S6)

- Total cellular fraction (free and bound)

$$F_{cells\,-\,total} = K_{cell} \times \frac{V_{cell}}{V_{water,\,cell}} \times F_{free}$$

(S7)

- Fraction of the total bound to cell matrix (proteins + lipids)

$$F_{cells\,-\,bound} = K_{cell\,-\,lipids} \times \frac{V_{cell\,-\,lipids}}{V_{water,\,cell}} \times F_{free} + K_{cell\,-\,proteins} \times \frac{V_{cell\,-\,proteins}}{V_{water,\,cell}} \times F_{free}$$

(S8)

- Considering solubility:

The maximum total amount of chemical soluble in the test system ($S_{total,max}$) is calculated from the input aqueous solubility (S) of the chemical (at pH 7.4) and the free fraction (F_{free}).

$$S_{total,max} = \frac{S}{F_{free}} \quad \text{(S9)}$$

The percent fraction of the total amount of chemical added to the test system that is predicted to precipitate (not being soluble in the test system) is calculated as the ration of the concentration in excess of solubility and the nominal test concentration:

$$\%F_{precip} = \frac{C_{Nom} - S_{total,max}}{C_{Nom}} \times 100\% \quad \text{(S10)}$$

The free fraction $F_{free,corrected}$ can then be corrected to account for limited solubility as follows:

$$\%F_{free,corrected} = F_{free} \times (100 - \%F_{precip}) \quad \text{(S11)}$$

Mass fractions in other assay compartments are corrected accordingly:

The free concentration in the media is calculated from the nominal concentration and the corrected free fraction in media:

$$\text{Free } C_{Medium} = C_{Nom} \times F_{free,corrected} \quad \text{(S12)}$$

Accordingly, the total soluble concentration in the media is calculated as follows:

$$\text{Total } C_{Medium} = C_{Nom} \times F_{media,corrected} \quad \text{(S13)}$$

Input parameters

Partition coefficients

K_{serum}: The distribution coefficient between serum matrix (lipid, protein) and water expressed as [L/L serum albumin] is calculated from the fraction unbound in plasma (fup) based on a mean serum albumin concentration in human plasma of 0.0425 kg/L and the specific volume of albumin (0.733 L/kg).

Alternatively, binding to serum proteins and serum lipids is calculated from QSARs for distribution coefficients between serum albumin and water or lipid and water, respectively (Endo & Goss, 2011).

$K_{protein} = 10^{0.71 \times LogK_{ow} + 0.42} \div 0.733$ for chemicals with $LogK_{ow} < 4.5$, expressed as [L/L protein] (S14)

$K_{protein} = 10^{0.37 \times LogK_{ow} + 2.56} \div 0.733$ for chemicals with $LogK_{ow} > 4.5$, expressed as [L/L protein] (S15)

$K_{lipid} = 10^{LogK_{ow}}$, expressed as [L/L lipid]

(S16)

K_{cell}: The distribution coefficient between cells and water expressed as [L/L cells] is calculated from the volume fractions of cell proteins, cell lipids and cell water and the partition coefficients between protein and water and lipid and water.

$$K_{cell} = K_{protein} \times \frac{V_{cell\text{-}proteins}}{V_{cell}} + K_{lipid} \times \frac{V_{cell\ lipids}}{V_{cell}} + \frac{V_{water\ cell}}{V_{cell}}$$

(S17)

The distribution coefficient between plastic and water [expressed as m^3/m^2] is calculated based on the QSAR derived by Kramer, 2010:

$$K_{plastic} = 10^{0.97 \times LogK_{ow} - 6.94}$$

(S18)

K_{air}: Distribution coefficient between air and media water [L/L], predicted from ADMET Predictor (SimulationsPlus).

System volumes

V_{water}: Volume of media water is calculated from the total volume of media in the assay and the volume of serum matrix.

V_{serum}: Volume serum matrix (proteins + lipids) is calculated based on 10% serum added to the media, the total protein concentration [g/L] and lipid (cholesterol + triglycerides) concentration

[g/L] in the newborn calf serum used to supplement the media as stated in the certificate of analysis, a specific volume of protein of 0.733 L/kg, and a specific volume of lipids of 1L/kg.

V_{cell}: The total volume of cells in the well is calculated from the measured number of cells per well and the total volume per cell.

$A_{plastic}$: The area of plastic [m^2] exposed to the media is calculated from the dimensions of the well and the volume of media (filling height) in the well. It is assumed that the bottom of the well is covered with cells and therefore not in direct contact with the media.

V_{air}: The volume of headspace in the well is calculated from the dimensions of the well and the volume of media in the well.

Figure S2: Gene expression of membrane transporters in Balb/c 3T3 cells

mRNA levels of membrane transporters in Balb/c 3T3 cells. mRNA sequencing was performed to determine the expression levels of membrane transporters in Balb/c 3T3 cells focusing on transporters from the uptake solute carrier (Slc, white bars) and efflux ATP binding cassette (ABC, grey bars) transporter families.

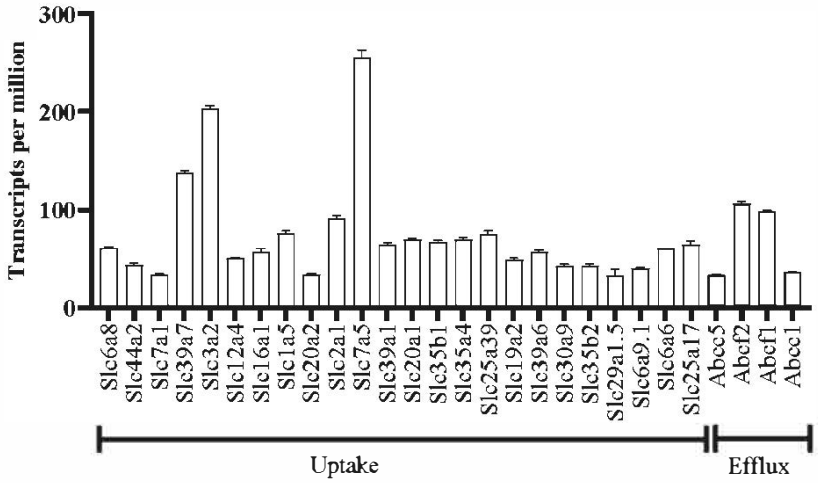

Table S19: Total C_{Medium} of incubation medium prior to adding to the cells compared to C_{Nom}.

Data are represented as mean in µM (SD if n=3 or mean difference between individual values, if n=2*).

Test chemical [C_{Nom}]	Total C_{med} [µM]	Difference [%]
APAP* [60 µM]	47.3 (1.2)	-23.7
BPA [36 µM]	34.9 (1.2)	-3.1
CAF* [160 µM]	203.2 (6.3)	23.8
COL [10 µM]	9.3 (1.4)	-7.8
FEN* [20 µM]	18.8 (0.6)	-6.5
FLU* [10 µM]	12.1 (1.5)	19.3
GEN* [18 µM]	15.6 (1.4)	-14.2
KET* [15 µM]	11.8 (1.1)	-24
MT [14 µM]	11.0 (0.7)	-24.4
TAM* [12.5 µM]	12.7 (0.5)	1.6
TRE [23 µM]	17.8 (1.5)	-25.6
WAR* [200 µM]	198.0 (7.0)	-1

Table S20: Predicted and measured values for total C_{Medium}, free C_{Medium} and total C_{Cell} after 6, 24 and 48 h of incubation.

Data are represented as mean in µM (SD if n=3 or mean difference between individual values, if n=2*). Values in red font are 2-fold under- or over-predicted ("Pred") compared to the measured values

	Total C_{Medium} 6 h				Free C_{Medium} 6 h				C_{Cell} 6 h				Cell to medium ratios 6 h
Chemical	Predicted	Measured Mean	SD	Pred:Measured	Chemical	Predicted	Measured Mean	SD	Pred:Measured	Chemical	Predicted	Measured Mean	SD	Pred:Measured	Chemical	C_{Cell}:C_{Medium} ratio
APAP	47.22	45.02	2.07	1.0	APAP	45.82	39.76	1.83	1.2	APAP	149.89	81.26	30.92	1.8	APAP	1.8
BPA	31.1	32.13	3.93	1.0	BPA	10.85	7.17	0.88	1.5	BPA	2411.95				BPA	NA
CAF	209.81	185.35	3.85	1.1	CAF	203.93	185.35	3.85	1.1	CAF	418.97	211.73	81.37	2.0	CAF	1.1
COL	9.23	7.34	0.56	1.3	COL	7.87	7.34	0.56	1.1	COL	59.04	28.19	3.06	2.1	COL	3.8
FEN	18.48	17.75	0.1	1.0	FEN	8.03	3.99	0.02	2.0	FEN	623.72	417.07	11.2	1.5	FEN	23.5
FLU	11.22	12.59	0.4	0.9	FLU	5.28	2.81	0.09	1.9	FLU	1592.5	66.32	2.12	24.0	FLU	5.3
GEN	15.47	17.55	0.28	0.9	GEN	4.86	1.94	0.03	2.5	GEN	270.26	60.16	3.05	4.5	GEN	3.4
KET	11.69	12.74	1.5	0.9	KET	0.81	2.1	0.25	0.4	KET	153.92	255.11	2.38	0.6	KET	20.0
MT	10.28	9.89	0.84	1.0	MT	4.78	3.42	0.4	1.4	MT	1387.04	67.89	22.91	20.4	MT	6.9
TAM	0.46	9.8	0.77	0.05	TAM	0.08	0.16	0.01	0.5	TAM	23903.3	919.03	110.72	26.0	TAM	93.8
TRE	17.48	16.24	1.26	1.1	TRE	8.42	8.42	0.3	1.0	TRE	830.63	48.78	11.4	17.0	TRE	3.0
WAR	197.51	159.46	13.45	1.2	WAR	13.61	42.82	3.61	0.3	WAR	1212.42	410.77	78.76	3.0	WAR	2.6
			Median	1.0				Median	1.1				Median	3.0		
			Min	0.0				Min	0.3				Min	0.6		
			Max	1.3				Max	2.5				Max	26.0		

Total C_{Medium} 24 h

Chemical	Predicted	Measured Mean	Measured SD	Pred:Measured
APAP	47.19	43.84	2.05	1.1
BPA	32.33	29.92	1.39	1.1
CAF	209.73	204.42	9.83	1.0
C●L	9.23	9.36	0.43	1.0
FEN	18.34	16.71	0.1	1.1
FLU	10.93	13.7	0.5	0.8
GEN	15.36	17.68	0.07	0.9
KET	11.61	13.23	1.39	0.9
MT	9.93	10.07	0.85	1.0
TAM	0.31	8.05	0.55	0.04
TRE	17.28	17.14	1.47	1.0
WAR	197.08	187.6	4.5	1.1
		Median		1.0
		Min		0.04
		Max		1.1

Free C_{Medium} 24 h

Chemical	Predicted	Measured Mean	Measured SD	Pred:Measured
APAP	45.78	38.71	1.81	1.2
BPA	11.71	6.68	0.31	1.8
CAF	203.85	204.42	6.95	1.0
C●L	7.87	9.36	0.43	0.8
FEN	7.96	3.76	0.12	2.1
FLU	5.14	3.06	0.11	1.7
GEN	4.82	1.96	0.01	2.5
KET	0.8	2.19	0.23	0.4
MT	4.61	3.48	0.12	1.3
TAM	0.05	0.09	0.01	0.6
TRE	8.32	8.89	1.06	0.9
WAR	13.58	50.37	1.21	0.3
			Median	1.1
			Min	0.3
			Max	2.5

C_{Cell} 24 h

Chemical	Predicted	Measured Mean	Measured SD	Pred:Measured
APAP	147.86	37.72	2.05	3.9
BPA	2868.68	396.48	1.39	7.2
CAF	412.47	105.17	9.83	3.9
C●L	50.79	27.22	0.43	1.9
FEN	620.25	542.61	0.1	1.1
FLU	1553.45	49.46	0.5	31.4
GEN	268.82	64.12	0.07	4.2
KET	190.43	436.49	1.39	0.4
MT	1368.36	53.58	0.85	25.5
TAM	16039.7	2367.96	0.55	6.8
TRE	822.08	59.99	1.47	13.7
WAR	1217.97	361.05	4.5	3.4
			Median	4.1
			Min	0.4
			Max	31.4

Cell to medium ratios 24 h

Chemical	C_{Cell}:C_{Medium} ratio
APAP	0.9
BPA	13.3
CAF	0.5
C●L	2.9
FEN	32.5
FLU	3.6
GEN	3.6
KET	33.0
MT	5.3
TAM	294.2
TRE	3.5
WAR	1.9

Total C_{Medium} 48 h

Chemical	Predicted	Measured Mean	Measured SD	Pred:Measured
APAP	47.09	44.79	3.17	1.1
BPA	33.79	31.02	2.26	1.1
CAF	209.46	201.55	7.84	1.0
C●L	9.22	11.3	1.05	0.8
FEN	18.03	16.57	2.05	1.1
FLU	10.16	11.67	0.87	0.9
GEN	15.32	14.88	0.94	1.0
KET	11.54	11.51	0.2	1.0
MT	9.5	9.04	1.98	1.1
TAM	0.23	5.73	0.83	0.04
TRE	17	16.36	1.33	1.0
WAR	196.73	189.34	9.33	1.0
			Median	1.0
			Min	0.0
			Max	1.1

Free C_{Medium} 48 h

Chemical	Predicted	Measured Mean	Measured SD	Pred:Measured
APAP	45.7	39.55	2.8	1.2
BPA	11.21	6.93	0.5	1.6
CAF	203.59	193.71	7.84	1.1
C●L	7.86	11.3	1.05	0.7
FEN	7.83	3.73	0.46	2.1
FLU	4.78	2.6	0.19	1.8
GEN	4.81	1.65	0.1	2.9
KET	0.8	1.9	0.03	0.4
MT	4.41	3.12	0.46	1.4
TAM	0.04	0.06	0.01	0.7
TRE	8.19	8.49	0.98	1.0
WAR	13.56	50.84	2.5	0.3
			Median	1.1
			Min	0.3
			Max	2.9

C_{Cell} 48 h

Chemical	Predicted	Measured Mean	Measured SD	Pred:Measured
APAP	149.46	31.07	3.17	4.8
BPA	3076.39	781.37	2.26	3.9
CAF	416.6	60.21	7.84	6.9
C●L	49.14	90.2	1.05	0.5
FEN	609.22	724.02	2.05	0.8
FLU	1443.21	135.39	0.87	10.7
GEN	268.13	134.9	0.94	2.0
KET	211.38	432.65	0.2	0.5
MT	1309.88	85.95	1.98	15.2
TAM	14533.1	3423.4	0.83	4.2
TRE	809.43	73.81	1.33	11.0
WAR	1216.52	658.14	9.33	1.8
			Median	4.1
			Min	0.5
			Max	15.2

Cell to medium ratios 48 h

Chemical	C_{Cell}:C_{Medium} ratio
APAP	0.7
BPA	25.2
CAF	0.3
C●L	8.0
FEN	43.7
FLU	11.6
GEN	9.1
KET	37.6
MT	9.5
TAM	597.5
TRE	4.5
WAR	3.5

S45

3.2 Manuscript Prepared for Submission

3.2.1 Refinement of *In Vitro* to *In Vivo* Extrapolation (IVIVE) of Potential Endocrine Disruptors based on *In Vitro* Dosimetry Approaches

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Abstract

The conversion of *in vitro* effect concentrations to *in vivo* doses, also called *in vitro* to *in vivo* extrapolation (IVIVE), is supported by physiologically based toxicokinetic modelling. However, the fact that *in vitro* assays rely on nominal test concentrations may cause discrepancies due to different factors, e.g. evaporation, metabolism and binding processes within the test system, diminish the actual concentration. Ergo, C_{Nom} may misrepresent the effective concentration exerting toxic effects and lead to over- and underestimation of *in vivo* doses when performing IVIVE.

For this purpose, we performed *in vitro* dosimetry and IVIVE to predict the endocrine potential of seven substances previously published [14]. Total concentrations in culture medium and cells were determined in the yeast estrogen/ androgen screening assays (YES/YAS assay) [86] and steroidogenesis assay [87] with adrenocortical carcinoma cells. Yeast cells were incubated for 48 hours with Acetaminophen, Bisphenol A, Caffeine, Flutamide, Genistein and H295R cells with Fenarimol and Ketoconazole at the lowest observed effect C_{Nom}. C_{Medium} and C_{Cell} were analytically measured by LC-MS. The *in vitro* derived estimated lowest observed effect doses (est. LOELs) were obtained from C_{Medium} and C_{Cell} by reverse dosimetry using a PBTK 8 compartment model. These est. LOELs were compared to the *in vivo* LOELs. Using total C_{Medium} and C_{Plasma}, est. LOELs were comparable (within a factor of 10) to *in vivo* LOELs for four of the seven substances. Whereas for three substances, Genistein, Flutamide and Ketoconazole, est. LOEL and *in vivo* LOEL did not correlate. For Fenarimol, the correlation even increased when free C_{Medium} instead of total C_{Medium} was used. When using C_{Cell} and C_{Tissue}, est. LOEL and *in vivo* LOEL correlated for five of the seven substances with Flutamide and Ketoconazole not correlating. *In vitro* dosimetry can improve and enable the prediction of in vivo effects, however, has its limitations. Further improvement of the existing methodology and model is advised.

Introduction

Future perspectives in toxicology go towards non-animal based hazard and risk assessment and support the practice of alternative test strategies, e.g. *in vitro* and *in silico* approaches, not only because of ethical and economic considerations, but also because cell-based assays provide information about the mechanism of action of substances and the resulting effects and may afford a better understanding of mechanisms of toxicity than animal tests [5, 24, 26]. Their outcomes provide apical endpoints, mechanisms of action on a molecular level are better reflected by cell-based assays and can get a better understanding of mechanisms of toxicity that may lead to adverse outcomes and the corresponding pathway of toxicity [88, 89]. The assessment of concentration-effect relationships of in vitro toxicity tests enables to define such point of departure for extrapolations in risk assessments [89]. However, the selection of the relevant point of departure for extrapolation is crucial and the possible losses occurring in in vitro tests must be taken into account. Relying on nominal concentrations (C_{Nom} as dose metric might not reflect the concentration that exerts toxic effects: Binding to medium supplements and labware, evaporation, degradation and precipitation of test substances impede and may reduce the actual exposure [30, 39, 40, 88, 89]. Cells contribute to the reduction of a substance's concentration by the uptake and/or metabolism of the substances. Consequently, the freely dissolved concentration is reported to reflect the actual exposure within an *in vitro* test system [38, 40, 41]. The selection of the appropriate in *vitro dose* metric is therefore a prerequisite for IVIVE.

The prediction of relevant human exposure based on *in vitro* derived effects remains challenging but enabled by the translation of *in vitro* effect responses to in vivo doses (*In vitro* to *in vivo* extrapolation, IVIVE) using physiologically based toxicokinetic models (PBTK) [13, 17, 18, 89]. Furthermore, the doses to substances that organisms are exposed to, may be calculated based on measured *in vitro* outcomes and realized by reverse dosimetry approaches using PBTK models. Reverse dosimetry was implemented to predict the potential of various substances to address different toxicological endpoints: nephrotoxicity, [19] liver toxicity, [20, 22, 28] cardiotoxicity, [90] developmental toxicity, [24-26] genotoxicity [21] and endocrine disruption [14, 29, 91]. The latter, endocrine disruption, raised attention in the last years and the identification of potential endocrine disruptors is still a matter of interest. Substances that interfere with the endocrine system and potentially disturb the physiological function of endogenous hormones, are known as endocrine disruptors or modulators. A conceptual framework was proposed by the OECD to assess the interference of endocrine active substances in different *in vitro* and *in vivo* test systems: the transcriptional activation of human estrogen/ androgen receptors in yeast cells, tampering in the steroid synthesis in human adrenocortical carcinoma cells (OECD test guideline no. 456) and morphological changes in test animals hampered by hormones and hormone like substances (e.g. Uterotrophic, Hershberger assay, OECD test guideline no. 440, 441) which are recognized test strategies by the regulatory authorities [7, 87, 92, 93].

Fabian and colleagues implemented a generic PBTK model for rats and predicted *in vitro-in silico* derived lowest observed effect level (LOEL) of ten potentially endocrine disruptors. Based on previous *in vitro* studies, lowest observed effect concentrations (LOEC) were extracted, extrapolated and the *in vitro-in silico* derived LOEL compared to literature derived *in vivo* LOEL - comparable results were yielded for 6/10 test substances [14, 74, 86]. Directing to endocrine effects, [29] demonstrated that the

prediction of dose-dependent uterus growth of Bisphenol A and Estradiol is enabled using PBTK models and *in vitro* data, namely the yeast estrogen screen resulting in matching *in vitro* and *in vivo* data [29]. Although successful IVIVE examples were reported, the consideration of biokinetics arise when converting *in vitro* effect concentrations to *in vivo* doses. The estimation of kinetic parameters *in vitro*, e.g. absorption, metabolism, protein binding and clearance, is not the only factor that alter an accurate translation of *in vitro* effect concentrations – challenges derive regarding the dose metric used in *in vitro* toxicology. In the above-described references, nominal test concentrations of the respective *in vitro* assays were applied in the reverse dosimetry approaches. This may end up in over- or underpredictions [33, 94]. To fill this gap, the published studies concerning endocrine disruption were repeated and *in vitro* dosimetry performed with seven substances [14]. Reverse dosimetry was performed with the published eight compartment model using concentrations in culture medium (C_{Medium}) and cells (C_{Cell}) measured in the respective *in vitro* assays and consequently represent the LOEC or the point of departure. The estimated LOELs were compared to the *in vivo* and published *in vitro- in silico* derived LOELs.

Materials & methods

Test substances & materials

Seven test substances from previous studies were selected and tested in YES/YAS and steroidogenesis assay [23, 28]. All substances and internal standards (Bisphenol A-d_{16}, CAS-No. 96210-87-6, ≥ 99.0 %, Diazepam-d_5, CAS-No. 65854-76-4, 98 %) were purchased from Sigma-Aldrich (Steinheim, Germany) in the highest available purity (Table 1). 6-well plates (Product no. 92024) and cell culture flasks (Product no. 90151, 150 cm^2) were obtained from TTP AG (Trasadingen, Switzerland) and 96-well plates from Greiner bio-one (Product no. 655185 Frickenhausen, Germany). Devices for RED experiments (Product no. 89811, 15036, 89809) were purchased from Thermo Fisher Scientific (Schwerte, Germany).
Yeast cells were counted using the yeast viability kit (Product no. F23202), counting slides (Product no. L12008) and the cell counter LUNA-FL™ supplied by BioCat GmbH (Heidelberg, Germany). The Bicinchoninic (BCA) protein assay kit was obtained by Thermo Fisher Scientific (Product no. 23225, Schwerte, Germany).

Table 1: Test substances. All test substances were obtained from Sigma-Aldrich (Steinheim, Germany) at the highest available purity.

Test substance	Abbreviation	Molecular weight [g/mol]	CAS-No.	Purity [%]
Acetaminophen	APAP	151.16	103-90-2	≥ 99.0
Bisphenol A	BPA	228.29	80-05-7	≥ 99.0
Caffeine	CAF	194.19	58-08-2	98.0
Fenarimol	FEN	331.20	60168-88-9	> 98.0
Flutamide	FLU	276.21	13311-84-7	>99.0
Genistein	GEN	270.24	446-72-0	≥ 99.0
Ketoconazole	KET	531.43	65277-42-1	≥ 99.0

Cell culture, incubation with test substances and sample preparation of yeast cells

Yeast cells (*Saccharomyces cerevisiae*) transformed with the human estrogen or androgen receptor (hER or hAR) and expression plasmids carrying an estrogen/ androgen responsive element as well as the reporter gene lac-Z were provided by Prof. Vollmer from Technical University Dresden. The YES/YAS assay is based on modified yeast cells (*Saccharomyces cerevisiae*) genetically modified with the gene encoding for the human estrogen/ androgen receptor coupled to the reporter gene lac-Z. Different supplement listed in table 2 were added to minimal medium (Product no. SO-50900, Pan Biotech, Aidenhausen, Germany) for optimum culture conditions. The concentrations of the stock solutions and the suppliers of the substances in table 2 are described in detail in the supplementary material 1. A pre-culture with cryopreserved yeast cells was prepared. 1 mL of the cells were transferred in 100 mL culture medium and incubated at 32°C in an incubator shaker (85 rpm) for 24 – 72 h. Optical density (OD) was measured and indicated the cell growth. The pre-culture was appropriately diluted at a final OD of 1 which is required to perform the assays. Stock solution (10 mM) of APAP, BPA, CAF, FLU and GEN were prepared in 100 % DMSO and stock solutions of BPA, FLU and GEN were diluted (100x). Prior to cell seeding, 9.9 mL of the cell suspension were spiked with 100 µL of a stock solution to achieve C_{Nom} (see table 3). A volume of 200 µL of the diluted cell suspension was pipetted into 96-well plates and yeast cells were incubated for 0 - 48 h. At the end of exposure, the optical density of the treated yeast cells was spectrometrically measured at 690 nm. The agonistic and antagonistic effects were not determined within the current experiments as effects concentrations were reported in previous studies [27, 28]. The suspension was centrifuged at 500 x g for 5 min and the medium. The cell pellet was washed with 0.5 mL phosphate buffer saline (PBS, product no. P04-3650, Pan Biotech, Aidenbach, Germany) and centrifuged as described aforementioned. The procedure was performed twice. Then, cells were suspended in 200 µL water. Yeast lysate and medium samples were stored at -20°C. The cell samples underwent three thawing and freezing cycles to lyze the membrane of yeast cells. In previous experiments, the lysis by freezing and thawing proved to be the most efficient and simplest method to destroy the membrane of yeast cells (Data not shown). Experiments were performed in triplicates.

Table 2: Composition of the yeast medium. Detailed description of the supplier and concentrations of each component of the yeast medium are described in the supplementary material 1, table S1 and S2.

Component	Volume [mL]
Minimal medium	500
Glucose solution	55.6
L-Asparagine solution	13.9
Vitamin solution	5.6
L-Threonine solution	4.4
Copper sulfate solution	1.4

Cell culture, incubation with test substances and sample preparation of H295R cells

Human adrenocortical carcinoma cells, H295R cells, were obtained from American Type Culture Collection (ATCC CRL-2128, ATCC, USA). Cells were cultured in 150 cm^2 flasks containing Dulbecco's Modified Eagle's Medium supplemented (Product no. 11039054, Gibco™, Schwerte, Germany) with 2.5 % Nu-Serum™ (Product no. 355100, Corning, New York, USA), 1 % ITS+ Premix solution (Product no. 354352, Corning, New York, USA) and 1 % streptomycin/ penicillin (Product no. P06-07100, Pan-Biotech, Aidenbach, Germany). H295R cells were seeded at a density of 0.3 $x10^6$ cells/mL in 6-well plates at 37°C with 5 % CO_2. After 24 h, medium was replaced and cells incubated for 2, 24 and 48 h with FEN and KET at C_{Nom} (see table 3). After treatment, the culture medium was collected, the cell layer washed twice with 3 mL PBS and treated with 0.5 mL Trypsin/EDTA (Product no. P10-023100, Pan-Biotech, Aidenbach, Germany) for 5 min at 37°C. H295R cells were then collected in 0.2 mL ultrapure water. As previously described, both matrix samples were stored at -20°C until analysis and the suspension of H295R cells in ultrapure water frozen and thawn for at least three cycles. Experiments were performed in triplicates.

Table 3: Nominal concentrations of the test substances for the applied test systems.

Test substance	C_{Nom} [µM]	Effect	Test system/ Cell line
APAP	1000	-	YES
BPA	10	estrogenic	YES
CAF	100	-	YES
FEN	10	estrogenic/androgenic	Steroidogenesis
FLU	10	antiandrogenic	YAS
GEN	1	estrogenic	YES
KET	0.1	androgenic	Steroidogenesis

Determination of protein bound fraction

In *in vitro* assays, protein binding is deemed to bet the limiting factor the substance's concentration in the *in vitro* test system and therefore estimated in culture medium to derive the free concentration of test substances in culture medium [45, 68]. Yeast medium does not contain proteins in the culture medium and the concentration in medium therefore considered as freely available concentration of the test substance [29]. The fraction unbound (f_u) was measured for FEN and KET which were tested in the steroidogenesis assay via rapid equilibrium dialysis (RED) [95]. According to the manufacturer (Thermo Fisher Scientific, Schwere, Germany), culture medium was spiked with FEN and KET at C_{Nom} of 5 µM containing 1 %DMSO. The spiked matrix (500 µL) was incubated for 6 h at 37°C in the sample chamber and dialyzed against the buffer chamber that contained 300 µL PBS. A volume of 20 µL of each compartment was collected and stored at -20°C until analysis. The f_u derived from the ratio of measured concentrations in PBS (C_{PBS}) and culture medium (C_{Medium}) using equation (1). Free C_{Medium} is derived by multiplying the f_u with the total C_{Medium} (2).

$$f_u\,[\%] = \frac{C_{PBS}}{C_{Medium}} \times 100\ \% \quad (1)$$

$$\text{Free } C_{Medium} = f_u \times C_{Medium} \quad (2)$$

Cell number estimates and intracellular concentration (C_{Cell})

Optical density (Yeast cells)

The optical density, OD, of treated yeast cells was measured in order to normalize against the OD of untreated cells. First, untreated yeast cells were counted using a yeast viability kit. The cell suspension was diluted (100x) with the yeast dilution buffer. The diluted suspension (17 µL) was mixed with1 µL Fluorescin signal enhancer 1, 1 µL Fluorescein diacetate and 1 µL Propidium iodide staining solution and incubated for 10 min at room temperature. Viable cells are exposed to Fluorescin diacetate and convert the membrane-permeable substance to the green fluorescent Fluorescin while the dye reagent fluoresce red in nonviable cells. 10 µL of the stained sample were loaded on the counting slides. The slide was inserted into the LUNA-FL™, the yeast cell counting program was selected and cells counted (Logos Biosystems, Villeneuve d'Ascq, France). The absorbance was measured at 494 nm. This number was set in relation to the corresponding OD. Furthermore, information about the cell diameters was documented. Detailed cell numbers of treated and untreated yeast cells are represented in table S7 in the supplementary material.

Bicinchoninic assay (H295R cells)

H295R cells were seeded in parallel to test substance incubations to determine the cell number via protein assays. The cell number of the sample was previously determined with a Casy Cell Counter (Roche Deutschland Holding GmbH, Mannheim, Germany). Therefore, culture medium was removed and the cell layer treated as aforementioned. A volume of 500 µL Triton-X solution (0.5 % in PBS) was added to the cells and incubated for 45 min at 37°C. Afterwards, the cells were collected and the suspension was centrifuged at 1000 rpm at RT for 5 min. The supernatant was taken and stored at -80°C until analysis. The Bicinchoninic assay (BCA) was conducted to measure the protein content and the cell number (Thermo Fisher Scientific, Schwerte, Germany). Therefore, 25 µL of the samples, standard solution (Calibration standards 20 – 2000 µg/mL bovine serum) and 200 µL of the working solution were transferred into a 96-well plate. Working solutions contained reagent A (Sodium carbonate, sodium bicarbonate, BCA and sodium tartrate) and B (4 % cupric sulfate) from the assay kit. The plate was shaken for 30 s and incubated for 30 min at 37°C. The absorbance was measured at 570 nm. Measuring a diameter of 15.9 ± 0.2 µm and a protein content of 206 ± 62 µg protein in untreated 10^6 H295R cells, the protein content of the samples was referred to these values to calculate the cell volume for each sample. Detailed cell numbers of treated and untreated cells are represented in table S7 in the supplementary material.

A generic diameter (d) of both cell lines was calculated with the above-described methods and normalized against 10^7 (Yeast cells) and 10^6 (H295R), respectively. The cell volume was derived with equation 3 assuming a spherical shape of the cells and adding the cell number (n_{Cell}).

$$V_{Cell}\,[\mu L] = \left(\frac{d}{2}\right)^3 \times \pi \times \frac{4}{3} \times n_{Cell} \qquad (3)$$

Yeast and H295R cells were collected in 200 µL water (V_{Water}) and the concentration was consequently measured in cell lysate (C_{Lysate}). To refer to the intracellular concentration, C_{Cell}, C_{Lysate} was corrected by V_{Cell} (Equation 4).

$$C_{Cell}\ [\mu M] = \frac{C_{Lysate}\ [\mu M] \times V_{Water}\ [L]}{V_{Cell}\ [\mu L]} \quad (4)$$

Sample preparation and analysis

Cell lysates and medium were analyzed on a high performance liquid chromatography (HPLC, Ultimate 3000, Thermo Fisher Scientific, Schwerte, Germany) coupled with tandem mass spectrometry (MS, Q Exactive Focus, Thermo Fisher Scientific, Schwerte, Germany). The flow rate was 0.25 mL/min and Phenomenex Security Guard™ Ultra Catridges (C18 column 3.0 mm) was used as a pre column. Details on the analytical methods, e.g. HPLC and MS conditions, have been published [96]. Criteria evaluated in accordance with EMA and ICH were applied to evaluate the analytical methods and can be found in the supplementary material, tables S3 – S6. Conditions about the generic mass spectrometry tune files and fragmentations to perform a parallel reaction monitoring are given in tables 4 and 5. In table 6, the HPLC methods are summarized.

The sample preparation consisted of a liquid-liquid extraction with Acetonitrile (4°C) in a proportion of 1:5 (v/v). Medium, cell lysate and RED samples underwent the equal sample preparation, except the cell disruption. Internal standards were prepared in Acetonitrile. 10 µL of DZP-d_5 (0.5 µM) respectively BPA-d_{16} (0.5 µM) and 400 µL Acetonitrile were added to 100 µL of the sample and centrifuged at 4000 x g, at room temperature for 20 min. The supernatant was taken for analysis and diluted if necessary. Calibration standards underwent the same procedure: appropriate amounts of the stock solutions of the test substances were added to the corresponding matrix (Culture medium, cell lysate, RED matrices).

PBTK model

A published PBTK model comprising eight compartments was set up and applied to describe the kinetic behavior and distribution of test substances in male and female rats [14]. In order to compare *in vivo* LOEL based on C_{Nom} and *in vitro* dosimetry approach, physiological and substance input parameters were extracted from the referenced publication. Maximal concentrations in plasma, ovaries and testes (C_{Plasma}, $C_{Ovaries}$, C_{Testes}) were extracted and used for the reverse dosimetry approach. Concentrations in ovaries and testes were considered as relevant tissues as they are correlated to the sites of action for the targets. Table 7 contains input parameters taken from [23].

Table 7: Input parameters for PBTK modelling [14]. Intrinsic clearance (*) was investigated in different test systems and therefore provides different units: hepatocytes (H, µL/10^6 hepatocytes), microsomes (M, µL/min/ mg microsomal protein) and S9 mix (S9, µL/ mg S9 protein).

Test substance	$logP_{ow}$	f_u [%]	Intrinsic clearance* (Test system)	Permeability [10^{-6} cm/ s]
APAP	0.51	79.0	15 (H)	15
BPA	3.81	2.8	361 (M)	19
CAF	0.24	65	1.4 (H)	12
FEN	3.13	3.2	1 (S9)	16
FLU	2.55	5.7	4.6 (S9)	8.4
GEN	3.04	2.7	505 (M)	5.8
KET	4.30	0.4	55 (H)	9.1

Reverse dosimetry

The IVIVE approach and the required parameters are briefly described in figure 1. Using equation 5, the lowest observed effect concentration (LOEL) was calculated where *in vitro* LOEC correspond to measured total/ free C_{Medium} or total C_{Cell} resulting in an estimated LOEL. Maximum concentrations in C_{Plasma}, $C_{Ovaries}$ and C_{Testes} from the PBTK model where the dose was set at 50 mg/kg bodyweight (bw). Finally, estimated LOEL were compared to *in vivo* LOEL derived from literature.

$$\text{Estimated LOEL [mg/kg bw]} = \frac{\textit{In vitro}\ \text{LOEC [µM]} \times \text{Dose} \left[\frac{\text{mg}}{\text{kg}}\ \text{bw}\right]}{C_{\text{Pasma or Tissue [µM]}}} \quad (5)$$

Data analysis

Data derived from the HPLC-MS/MS analysis were analyzed with Xcalibur, and Chromeleon 7.2. Final analysis was performed with the software Microsoft Excel, GraphPad Prism version 9.4.1. The differential equations of the PBTK model were published, applied in Matlab and calculations performed within an internal app called TK estimator.

Figure 1: Schematic representation of IVIVE based on measured in *vitro* effect concentrations and PBTK modelling.

Results

YES/YAS assay: Measured concentrations in the culture medium and cells

Interactions with the estrogen/ androgen receptor and effects are investigated with the yeast estrogen/ androgen screening assay and conducted with hER and hAR transfected yeast cells [86]. Here, the assay was assessed with five test substances. Total concentrations were analytically measured in medium (Total C_{Medium}) and yeast cells (Total C_{Cell}) which were disrupted prior analysis. Results are summarized in the table 9. C_{Nom} and total C_{Medium} measured before the incubation of cells are described in table 8.

APAP, BPA, CAF and GEN were screened in the YES assay (Table 8). Regarding C_{Nom}, differences in contrast to the total C_{Medium} at pre-incubation were less than 15 % were shown for 3/5 substances, CAF and GEN varied by 19.3 and 74 %. Constant total C_{Medium} were observed for the substances APAP and GEN. Concentration of CAF has shown an increase after 12 h of incubation, but overall, stays stable over time. In contrast to the initial test concentration (9.64 ± 0.92 µM) C_{Medium} decreased after 48 h when exposing the yeast cells with BPA (7.08 ± 0.48 µM). FLU was tested in the YAS assay revealed stable concentrations in culture medium (cf. table 9).

The estimation of C_{Cell} requires the diameter and cell number of the samples – the latter is given in the supplementary material (Table S7). Measurements with LUNA-FL™ revealed diameters of 7.37 ± 1.25 µm and 7.33 ± 1.30 µm in hAR and hER transfected yeast cells and resulting volumes of 0.21 µL. After 48 h, $1.17 \times 10^7 \pm 2.95 \times 10^6$ (hER) and $9.77 \times 10^6 \pm 1.51 \times 10^6$ cells (hAR) were counted in three independent experiments. According to OD measurements, 10^7 cells are equivalent to an OD of 1.17 ± 0.30 (hER) and 0.98 ± 0.15 (hAR) and are comparable to literature derived data where an OD of 1 corresponds to 10^7 yeast cells [97, 98]. Concentrations in cells were determined in yeast cell lysate after freezing and thawing cycles to destroy the membrane of the yeast cells. By calculating the cell volume with the correspondent cell number of the samples, the lysate concentration was correlated to the actual C_{Cell} as described in "*Material & methods*". Until 24 h of exposure, C_{Cell} was in all cases lower than the limit of quantification. High variations were found in yeast cells exposed to the substances at 24 h were found which were more steadied after 48 h. Concentrations of APAP and BPA decreased over time and reduced significantly while concentrations of FLU and GEN slightly reduced. On comparing C_{Medium} against C_{Cell}, results varied by factors of 37 – 74 (BPA, FLU, GEN) where C_{Cell} was higher. Lower concentrations were observed when incubating yeast cells with APAP and equivalent concentrations found between C_{Medium} and C_{Cell} of CAF.

Table 8: Total C_{Medium} (pre-incubation) of yeast and H295R cells compared to C_{Nom}. The test substances APAP, BPA, CAF and GEN were screened in the YES-, FLU in the YAS- assay, FEN and KET in the steroidogenesis assay. Data are represented as mean in µM (SD, n=3).

Test substance [C_{Nom}]	Total C_{Medium} [µM]
APAP [100 µM]	96.59 (3.42)
BPA [10 µM]	9.64 (0.92)
CAF [100 µM]	119.92 (11.64)
FEN [10 µM]	11.93 (1.22)
FLU [10 µM]	10.45 (0.37)
GEN [1 µM]	1.74 (0.10)
KET [0.1 µM]	0.14 (0.01)

Table 9: Experimentally determined total C_{Medium} and C_{Cell} in the YES/YAS assay after 3, 6, 12, 24 and 48 h of exposure. Prior analysis, the cell lysate samples were thawn and frozen (Three cycles). The measured concentration in the cell lysate was related to the cell volume previously determined via the cell number of the samples and the diameter of the yeast cells. Concentrations measured within an incubation period of 0 – 12 h respectively 0 – 24 h (CAF) were below the LOQ of the analytical method and therefore not summarized in the table (n.d., not detectable). Data are represented as mean (SD, n=3).

Test substance [C_{Nom}]	Incubation time [h]	Total C_{Medium} [µM]	Total C_{Cell} [µM]
APAP [100 µM]	3	95.99 (5.21)	n.d.
	6	100.97 (13.60)	n.d.
	12	96.76 (12.49)	n.d.
	24	96.07 (13.83)	434.87 (122.02)
	48	94.44 (10.79)	62.77 (18.95)
BPA [10 µM]	3	8.91 (0.48)	n.d.
	6	9.62 (2.52)	n.d.
	12	12.08 (3.50)	n.d.
	24	8.20 (0.36)	2778.93 (1032.91)
	48	7.08 (0.48)	373.35 (136.92)
CAF [100 µM]	3	125.45 (4.08)	n.d.
	6	162.45 (10.57)	n.d.
	12	127.76 (0.92)	n.d.
	24	120.78 (3.03)	n.d.
	48	108.32 (0.65)	111.06 (26.17)
FLU [10 µM]	3	9.06 (0.79)	n.d.
	6	8.99 (0.87)	n.d.
	12	9.40 (0.47)	n.d.
	24	9.99 (0.63)	4872.05 (3715.90)
	48	10.06 (0.56)	711.2 (330.50)
GEN [1 µM]	3	2.06 (0.40)	n.d.
	6	1.90 (0.41)	n.d.
	12	1.82 (0.28)	n.d.
	24	2.19 (0.36)	253.74 (115.23)
	48	2.20 (0.55)	74.40 (7.74)

Steroidogenesis assay: Measured concentrations in the culture medium and cells

The OECD test guideline no. 456, also steroidogenesis assay, is performed to detect interferences in the metabolic pathway of the hormones 17β-estradiol/ testosterone [87]. Two substances, FEN and KET, were exposed to human adreno-carcinoma cells and as explained in the section before, total concentrations in culture medium and cells were quantified. In addition, the unbound fraction was experimentally determined with RED. For work up, H295R cells underwent freezing and thawing cycles to release the cytosol including the substances that were taken up. The total C_{Medium}, free C_{Medium} and total C_{Cell} are represented in the tables 8 and 10. Further information about the cell numbers and volumes are given in the supplementary material (Table S8).

Table 10 represent the measured concentrations of FEN and KET in culture medium and H295R cells. FEN_{free} and KET_{free} correspond the free C_{Medium} and incorporates the correction by the f_u. Total C_{Medium} differed by 11 and 20 % in comparison to C_{Nom}. Total C_{Medium} of FEN was stable over time while KET concentrations in medium decreased significantly during the exposure and after 48 h, total C_{Medium} of KET was lower than the limit of quantification. RED revealed that the substances bind moderately to proteins in culture medium with resulting free fractions of 69.04 ± 6.40 % (FEN) and 41.31 ± 5.43 % (KET).

Measuring the protein content of each sample via BCA assay enabled the determination of cell numbers and the respective volume of treated H295R cells. The protein content was found at 0.21 ± 0.06 mg protein/ 10^6 H295R cells without treatment. With the experimentally derived diameter (15.95 ± 0.25 µm), a cell volume of 2.12 ± 0.01 µL/ 10^6 cells were calculated (Table S8, supplementary material). C_{Cell} measured in the steroidogenesis assay were shown in table 10. As observed with C_{Medium}, C_{Cell} of FEN was constant over time and KET concentrations reduced until C_{Cell} were below the LOQ. Depending on the incubation time point and test substance, total C_{Medium} differed by factors ranging from 2.9 -18.9 (KET) and 6.3 – 41.1 (FEN). The discrepancies between free C_{Medium} and C_{Cell} were larger.

Table 10: Experimentally determined total and free C_{Medium} in the H295R cells after 2, 24 and 48 h of exposure. The f_u derived by RED experiments, was used a correction factor to calculate the free concentration in medium and is indicated as FEN_{free} and KET_{free}. Prior analysis, the cell lysate samples were thawn and frozen (Three cycles). The measured concentration in the cell lysate was related to the cell volume previously determined via the cell number of the samples and the diameter of H295R cells. Data are represented as mean (SD, n=3).

Test substance [C_{Nom}]	Incubation time [h]	C_{Medium} [µM]	Total C_{Cell} [µM]
FEN (total) [10 µM]	2	11.05 (0.56)	411.24 (277.23)
	24	11.16 (0.54)	61.92 (17.67)
	48	11.21 (0.49)	63.02 (32.81)
FEN (free) [10 µM]	2	7.63 (0.39)	
	24	7.70 (0.38)	
	48	7.74 (0.34)	
KET (total) [0.1 µM]	2	0.10 (0.02)	3.44×10^{-2} (1.09×10^{-2})
	24	0.02 (0.01)	5.27×10^{-3} (3.19×10^{-3})
	48	< LOQ	n.d.
KET (free) [0.1 µM]	2	0.04 (0.01)	
	24	0.01 (0.00)	
	48	< LOQ	

Concentrations in plasma and tissues derived from PBTK modelling

Input parameters for PBTK modelling, e.g. clearance, permeability, are summed up in table 7 and extracted [14]. Using these input data, maximum concentrations in plasma and tissues were estimated and results shown in table 11. C_{Plasma} and the mean of $C_{Ovaries}$ and C_{Testes} was used for further calculations to determine the estimated LOEL.

Table 11: Maximum C_{Plasma}, $C_{Ovaries}$ and C_{Testes} derived from PBTK modelling. The concentrations were predicted in male and female rats assuming a dose of 50 kg/mg bw and are given as mean [µM].

Test substance	C_{Plasma} [µM]	$C_{Ovaries}$ [µM]	C_{Testes} [µM]
APAP	67.36	56.76	55.75
BPA	30.17	110.14	111.09
CAF	183.70	151.54	151.12
FEN	37.26	88.80	89.23
FLU	42.85	73.16	73.16
GEN	13.05	29.75	29.54
KET	12.72	67.29	66.69

Performance of IVIVE using the *in vitro* dosimetry approach

In vitro LOEC, corresponding to experimentally determined total C_{Medium}, free C_{Medium} and C_{Cell}, as well as the PBTK derived concentrations (Table 11) of seven test substances were inserted into equation 5. In table 12 and 13, estimated LOEL are summarized and compared to *in vivo* derived LOEL deriving from *in vivo* assays depending on the described endpoints. As acceptance criteria for acceptable estimated LOEL, the factor between LOELs was set to ≤ 10. In the Uterotrophic assay, substances acting as estrogen agonists are identified by measuring an increase in uterine weight in female rats. The counterpart for androgenic effects is the Hershberger assay detects the androgenic/ antiandrogenic substances in male rats where changes in the weight of five androgen dependent organs are observed [7]. The Pubertal assay was selected for identification of substances that interfere in the endocrine system including the steroidogenesis [14].

Experimental derived *in vitro* data from 48 h of exposure were taken for the analysis and declared as LOECs as C_{Medium} was not considerably varying during incubation time. The exception is KET where the concentration in medium was too low to quantify and therefore, experimental data from the 24 h was included. Regarding C_{Cell}, it was assumed that an equilibrium must be elaborated only after 48 h. Since APAP and CAF are not active within the endocrine system, no limits were set for the test substances.

5/7 test substances were predicted correctly within a factor of 10. Considering C_{Medium} of FLU and GEN and for the calculation of the LOELs, 21- and 16-fold higher predictions were derived and therefore the estimated LOEL overpredicted. As yeast medium has a negligible number of proteins, free C_{Medium} was only determined in the steroidogenesis assay. Using free C_{Medium} of FEN, the estimated LOEL is even closer to the *in vivo* LOEL and the opposite occurred for the test substance KET. Similarities were found when introducing cellular and tissue derived concentrations. APAP and CAF were not reported as endocrine active substances. The differences between estimated LOEL and *in vivo* LOEL of BPA differed by 2-times. Good correlations were found for FEN and GEN where the LOELs differed by less

than 7-fold. Larger deviations, also in comparison to C_{Medium} and C_{Plasma} derived data, were observed regarding FLU (49-times compared to *in vivo* LOEL) and KET (38200-times compared to *in vivo* LOEL). In summary, the estimated LOEL of 5/7 test substances were predicted within the range and defined to be acceptable.

Table 12: Extrapolation of *in vitro* derived LOECs to *in vivo* LOEL of seven test substances based on analytically measured concentrations in medium. *In vitro* LOEC were experimentally determined and represent the total and free C_{Medium} from YES/YAS- and steroidogenesis assay after 48 h of exposure, except KET (24 h). *In vitro* LOEC and PBTK derived concentrations (C_{Plasma}) were used to perform reverse dosimetry and determine the estimated LOEL which were compared to in *vivo* LOELs of the corresponding assay. Data of *in vitro* assays are shown as mean (SD).

Test substance	In *vitro* LOEC [µM]		Estimated LOEL [mg/kg]	In *vivo* LOEL [mg/kg]	Assay
	Total C_{Medium}	Free C_{Medium}			
APAP	94.44 (10.79)		55.38		-
BPA	7.08 (0.48)		172.60	375	Uterotrophic
CAF	108.32 (0.65)		35.10		-
FEN	11.21 (0.49)		469.91	50	Pubertal
		7.74 (0.34)	324.56	50	Pubertal
FLU	10.06 (0.56)		205.91	10	Hershberger
GEN	2.20 (0.55)		312.67	20	Uterotrophic
KET	0.02 (0.01)		15.29	100	Pubertal
		0.01 (0.00)	1.68	100	Pubertal

Table 13: Extrapolation of *in vitro* derived LOECs to *in vivo* LOELs of seven test substances based on analytically measured concentrations in yeast and H295R cells. *In vitro* LOECs were experimentally determined and represent the total C_{Cell} from YES/YAS- and steroidogenesis assay after 48 h of exposure, except KET (24 h). *In vitro* LOECs and PBTK derived concentrations ($C_{Ovaries}$ and C_{Testes}) were used to perform reverse dosimetry and determine the estimated LOEL which were compared to in *vivo* LOELs and the corresponding assay. Data of *in vitro* assays are shown as mean (SD).

Test substance	In *vitro* LOEC [µM] Total C_{Cell}	Estimated LOEL [mg/kg]	In *vivo* LOEL [mg/kg]	Assay
APAP	62.77 (18.95)	55.79		-
BPA	373.35 (136.92)	168.76	375	Uterotrophic
CAF	111.06 (26.17)	36.69		-
FEN	63.02 (32.81)	35.40	50	Pubertal
FLU	711.23 (330.50)	486.06	10	Hershberger
GEN	74.80 (7.74)	126.15	20	Uterotrophic
KET	5.27×10^{-3} (3.19×10^{-3})	2.62×10^{-3}	100	Pubertal

Discussion

Considerations on *in vitro* dose metrics: YES/ YAS- and steroidogenesis assay

The screening for estrogenic respectively androgen receptor binding was assessed with the YES-/YAS assay. Yeast cells transformed with the human estrogen or androgen receptor are exposed to test substances to assess the binding to estrogen or androgen receptors. The cells are cultured as a suspension in a buffer (Table S1, supplementary material) which is free from proteins [29]. Protein as well as plastic binding, evaporation and precipitation in the test system can be neglected and was confirmed by stable total C_{Medium} of the test substances. Total C_{Medium} was constant over time with minor reduction after 48 h observed for the test substances BPA and FLU indicating an uptake by yeast cells

[47, 50]. The increase of CAF concentrations in medium (6 h) might be explained by inhomogeneously soluted substance in incubation media. Measurements revealed that the initial C_{Medium} of GEN were 74 % higher (1.74 ± 0.10 µM) than the actual C_{Nom} (1 µM) at other timepoints. The significance of such measured concentrations is discussed in the next sections. Data from the 48 h exposure were taken for the extrapolation as we assumed that equilibrium was reached at this time point. Concentrations of APAP and CAF were comparable to total C_{Medium} denoting that an equilibrium was reached. In general, C_{Cell} was higher than C_{Medium} varying by factors of 53 (BPA), 71 (FLU) and 74 (GEN) and coincides with the results from previous work [50, 96]. The determination of C_{Cell} faces limitations concerning the analytics. Trends concerning cellular uptake kinetics were unfeasible to set because C_{Cell} were below the LOQ (3 – 12 h of incubation) and have shown high variations due to the low amounts of cells in the beginning of the experiment.

With the steroidogenesis assay, interferences into the biosynthesis of estradiol and testosterone in an adrenocortical carcinoma cell line is studied and already accounted as an OECD test guideline (OECD test guideline no. 456). The total C_{Medium}, C_{Cell} and, in addition, free C_{Medium} were determined. According to the certificate of analysis of the culture medium and supplements and the dilution in culture medium, bovine serum albumin is found at concentrations of 4.5 µM respectively 18.8 µM deriving from the grow supplements Nu-Serum^{TM} and ITS + premix solution. Therefore, binding to proteins must be considered and experimentally derived by RED indicating moderate binding of FEN (69.04 ± 6.40 %) and KET (41.31 ± 5.43 %) to albumin or other proteins from the supplements or medium.

Total and free C_{Medium} of FEN were constant over time while C_{Medium} KET decreased significantly. As reported in the yeast cells, decreasing C_{Medium} is referred to cellular uptake. FEN concentrations in cells reached an equilibrium after 2 h of incubation. In contrast, decreasing C_{Cell} of KET were observed or not quantifiable. Biotic processes, e.g. metabolism, accumulation in cells and the influx/efflux are indicators of decreasing C_{Medium} as well as C_{Cell}. confirmed by the determination of mass balances.

Since it was assumed that the test substances diffuse into cells, an equilibrium would have been expected between the free C_{Medium} and total C_{Cell}. Regarding the yielded concentrations in culture medium and cells, comparable concentrations were measured for the test substances APAP and CAF in the YES-assay. Other test substances where the affinity to estrogen and androgen receptors was confirmed have shown higher concentrations in cells in contrast to total/free C_{Medium} in both assays (BPA, FEN, FLU, GEN). This observation may emphasize that, beside physicochemical properties, the affinity to certain cellular targets drives the uptake of substances into cells when an evident mode of action of a substance is understood. The determination of C_{Cell} in both cell types outlined the difficulty of the measurement of intracellular concentrations and the most discussed limitation, the analytical procedure that enables a simple and rapid analysis for further use. Depending on the cell line, difficulties arise concerning the extraction procedure since differences in the membrane structure must be considered. While the membrane of H295R cells consists of a typical phospholipid bilayer structure, the cell membrane of yeasts possesses three layered cell walls containing beta-glucan. Previous experiments have shown that thawing/freezing cycles, lysis with glass beads and extraction with toluene yielded in comparable results (Data not shown). Generally, identified and measured C_{Cell} were expressed as total C_{Cell} meaning that no binding to cellular proteins, e.g. actin, is assumed. A closer estimate to the

biologically effective dose would be either the unbound concentration in cytosol, receptor or enzyme, yet technically not developed to reliably determine such concentrations [30, 69].

Comparison of *in vivo* LOELs

Reverse dosimetry was applied where total/ free C_{Medium} and C_{Cell} and C_{Plasma} and $C_{Ovaries/Testes}$, derived from i*n vitro* assays and PBTK model, were used to calculate the corresponding *in vivo* dose in rats. *In vitro* LOEC implicated the most sensitive endpoint for the data assessment. Since the effects, binding to receptors and enzymes, are reversible, the application of maximum concentrations of the test substances in plasma is an appropriate measure for the extrapolation. *In vivo* LOELs were obtained from *in vivo* assays: the Hershberger, Uterotrophic, and the pubertal assay [99, 100]. Table 14 summarizes the LOELs of the test substances which were estimated based on the *in vitro* dosimetry approach, C_{Nom} and LOELs derived from *in vivo* assays [14]. Predictions were ranked as correct when an accordance within or equal a factor of 10 in comparison to literature derived LOEL were achieved. Previous studies used APAP and CAF as negative controls in the *in vitro* as well as in vivo assays because no significant endocrine effects were reported in literature [74]. On comparing estimated LOEL by the *in vitro-in silico* approach with measured and nominal concentrations, CAF resulted in similar LOEL (35 – 39 mg/kg bw), APAP LOELs varied by a factor of 1.7. However, results indicated that LOEL higher than the estimated LOEL would correspond to doses where adverse outcome may be observed. BPA is a known modulator of the endocrine system [29]. Similar estimated LOEL of BPA were found using total C_{Medium} and C_{Cell}. and in close agreement to the in vivo LOEL (factor 2 – 3). LOEL derived by Fabian et al. was overpredicted (515 mg/ kg bw) [14]. Good correlations were yielded for the fungicide and aromatase inhibitor FEN. Estimated LOEL from the previous study and *in vitro* dosimetry approach were similar when considering nominal (470 mg/ kg bw) and total C_{Medium} (420 mg/ kg bw) varying by factors of 8.4 – 9.5. The correction of the total C_{Medium} by the f_u, yielded to closer estimated LOEL in comparison to the in vivo derived LOEL (50 mg/ kg bw). The closest results (factor 1.4) in comparison to the in vivo doses resulted by incorporating total C_{Cell} of FEN (35 mg/kg bw).
Since the total C_{Medium} of GEN differed by 74 % of the C_{Nom}, an appropriate prediction of the estimated LOEL caused an overestimation due to technical errors (313 mg/ kw bw). The approach using in vitro derived C_{Nom} (143 mg/ kg bw) performed better differing by one order of magnitude to the in vivo LOEL (20 mg/ kg bw). C_{Cell} derived LOEL of GEN (126 mg/ kg bw) correlated better to the *in vivo* LOEL within a factor of 6. Expecting a lower test concentration and less available compound in the test system, we expect that the C_{Cell} and the resulting LOEL might be reduced and therefore closer to the *in vivo* LOEL. Interestingly, the antiandrogen FLU failed in both approaches. Results from *in vitro- in silico* approaches using total C_{Medium} and C_{Nom} were equal (205 mg/ kg bw and 206 mg/ kg bw, factor 21). Larger deviations (486 mg/ kg bw, Factor 49) using cellular concentrations. The antifungal substance KET is known to modulate CYP enzymes and steroid metabolizing enzymes, especially the inhibition of CYP11A1 which is part of steroidogenesis [101-103]. LOEL derived from the *in vitro* dosimetry approach were impaired in contrast to LOEL derived by C_{Nom}. Concentrations in both matrices, medium and cells, decreased and imply that either metabolic processes occur after the cellular uptake or the extraction of KET in the respective matrices was insufficiently.

Beside metabolic clearance, e.g. renal clearance, active transport and enterohepatic circulation was not considered in the PBTK model [104, 105]. However, it might be of interest when substances like APAP, BPA, FLU, GEN and KET are tested and are known modulator of α-glycoprotein, or multidrug resistance-associated proteins [106]. Furthermore, it is assumed that only the parent compound is responsible for the effects, not the metabolites.

Table 14: Comparison of estimated LOELs derived by reverse dosimetry approaches to *in vivo* derived LOEL. Concentrations in medium and cells were measured in the corresponding assays and used for the calculation of LOELs supported by a PBTK model (*In vitro* dosimetry approach). Two values were given for FEN and KET indicating the total/ free C_{Medium}. Fabian et al. proceeded similarly but used nom *in vitro* concentrations to estimate the LOELs. Both estimated LOELs were compared to published LOEL extracted from *in vivo* assays. The respective in vivo assays are described by circles • (Uterotrophic assay), triangles ▲ (Hershberger assay) and squares ▪ (Pubertal assay). Not available *in vivo* data are indicated by n.a.

	LOEL [mg/kg bw] derived from			
Test substance	***In vitro* dosimetry approach based on**		**Fabian et al., 2019 based on**	***In vivo* assays**
	C_{Medium}	**C_{Cell}**	**C_{Nom}**	
APAP	> 55	> 56	> 93	n.a.
BPA•	173	169	515	375
CAF	> 35	> 37	> 39	n.a.
FEN▪	470/ 325	35	420	50
FLU▲	206	486	205	10
GEN•	313	126	143	20
KET▪	15/ 2	3 x 10^{-3}	100	100

Conclusion

Numerous studies implied and clarified the discrepancies between biologically active concentrations and C_{Nom} and push not only of the freely dissolved but also cell concentration as dose metric. According to Mielke, cellular concentrations are a better estimate for IVIVE corresponding to tissue concentrations [94]. When *in vitro* effects based on cell concentration should be linked to in vivo doses, several challenges arise. First, a suitable and relevant counterpart must be defined. C_{Medium} is related to free C_{Plasma} – consequently, C_{Cell} should be linked to concentrations in tissue. However, corresponding *in vivo* data where concentrations of test substances are not reported, especially for the endpoint endocrine disruption. Second, the unbound fraction of a substance exerts the toxic effect and a correction of the cellular concentration by binding to proteins and lipids must be considered but faces experimental challenges and was not applied yet [107, 108]. Furthermore, influx and efflux processes as well as effects by metabolites are not considered.

Both *in vitro* assays reflect a specific mode of action: the interaction with enzymes of the steroidogenesis and binding to human estrogen and androgen receptors. One should keep in mind that the test systems only depict one mechanism while these interactions provoke a signal cascade of key events that lead to adverse outcomes observed in rats. Beside reversible effects, total concentration is a useful metric in a single exposure when (1) the test substance has a low binding affinity to cellular components, (2) low

or no proteins are detectable in culture medium (< 10 %), (3) the metabolic activation by cells is low or (4) not an equilibrium between culture medium and cells is present.

To conclude, 4/7 estimated LOEL agreed with in vivo LOEL within a factor of ≤ 10 when using total C_{Medium} for IVIVE respectively 5/7 estimated LOEL when incorporating C_{Cell}. LOEL of 6/7 test substances were in agreement with *in vivo* LOEL. In both reverse dosimetry approaches, FLU predictions failed. In summary, C_{Nom} may be a useful dose metric when the impact of binding to medium supplements can be neglected proven in the YES/YAS assay. The principle can be inherited to other substances, e.g. structural analogs, to predict the endocrine potential of test substances to evaluate the applicability of the approach.

Abbreviations

APAP, Acetaminophen; BPA, Bisphenol A; BCA, Bicinchoninic acid; CAF, Caffeine; C_{Cell}, Concentration of a test substance in cells; C_{Lysate} , concentration of a test substance in cell lysate, C_{Medium}, concentration of a test substance in culture medium; C_{Nom}, nominal concentration of the test substance; C_{PBS}, Concentration in phosphate buffer saline; COL; Colchicine; d, diameter; DMSO, Dimethyl sulfoxide; FEN, Fenarimol; FLU, Flutamide; f_u, fraction unbound; GEN, Genistein; H, intrinsic clearance determined with hepatocytes; hAR, Human androgen receptor; hER, Human estrogen receptor α; IVIVE, in vitro to in vivo extrapolation; KET, Ketoconazole; LOEC, lowest observed effect concentration; $LogP_{ow}$ octanol-water partition coefficient; M, intrinsic clearance determined with microsomes; MW, molecular weight; n_{Cell}, cell number; OD, optical density; PBTK, Physiologically based pharmacokinetic modelling; PBS, phosphate buffer saline; RED, rapid equilibrium dialysis; S9, intrinsic clearance determined with S9 mix; TAM, Tamoxifen; TRE, Trenbolone; V_{Cell}, Volume of cells; V_{Water}, Volume of water; WAR, Warfarin; YAS, yeast androgen screen; YES, yeast estrogen screen;

3.2.2 Supporting Information (manuscript no. 3)

I. Components of the yeast culture medium

Table S1: Vitamin solution. All substances were purchased from Sigma Aldrich, Steinheim, Germany.

Substance	Amount	Product no.
Biotin solution [20 mg/L]	20 mL	SAB4639
Myo-Inositol	40 mg	SAI7508
D-Pantothenic acid hemicalcium salt	8 mg	SAP5155
Pyridoxine hydrochloride	8 mg	SAP9755
Thiamine hydrochloride	8 mg	SAT4625

Table S2: Concentrations of other solutions for the yeast culture medium. Substance solutions were prepared in ultrapure water and filtered through a 0.2 µm filter. All substances were purchased from Sigma Aldrich, Steinheim, Germany.

Substance	Concentration	Product no.
D-(+)-Glucose	20 % (w/v)	SA49159
L-Asparagine	4 mg/mL	SAA4284
L-Threonine	24 mg/mL	SAT8441
Copper sulfate	5 mg/mL	C8027

II. Determination of analytical parameters for method validation in culture medium and cells of the steroidogenesis and YES/YAS- assays

Table S3: Linearity, LOD and LOQ measured in yeast and steroidogenesis medium. The parameters were monitored in yeast medium and steroidogenesis medium according to the guidelines from EMA 2009 and ICH, 2014.

Test substance	Linearity range [nM]	r^2	LOD [nM]	LOQ [nM]
APAP	500 - 5000	0.993	7.8	26.1
BPA	1000 - 50000	0.999	135.3	446.6
CAF	500 - 5000	0.998	514.0	1713.2
FEN	100 - 1000	0.994	0.6	1.8
FLU	100 - 5000	0.990	3.4	11.2
GEN	100 - 1000	0.994	0.3	1.0
KET	100 - 1000	0.989	0.7	2.2

Table S4: Linearity, LOD and LOQ measured in yeast and H295R cell lysate. The parameters were monitored in yeast and H295R cells according to the guidelines from EMA 2009 and ICH, 2014.

Test substance	Linearity range [nM]	r^2	LOD [nM]	LOQ [nM]
APAP	100 - 1000	0.990	4.2	14.1
BPA	100 - 1000	0.993	59.0	133.1
CAF	100 - 1000	0.996	1.6	5.2
FEN	50 - 500	0.995	0.2	0.6
FLU	25 - 500	0.997	0.7	2.4
GEN	25 - 250	0.992	0.3	1.0
KET	100 - 1000	0.996	1.1	3.8

Table S5: Accuracy, precision and recovery determined in yeast buffer and steroidogenesis medium. The parameters in table S5 were monitored in culture medium of two *in vitro* assays according to the guidelines from EMA 2009 and ICH, 2014. Two concentrations of quality control samples (mid and high concentrations) were proven for accuracy and precision. Precision, accuracy and recovery were determined in five replicates shown as mean ± SD.

Test substance	Quality control [nM]	Accuracy [%]	Precision [%]	Recovery [%]
APAP	2000	10.9	117.2 ± 12.8	96.1 ± 18.4
	4000	2.8	99.8 ± 2.8	
BPA	2000	2.7	92.3 ± 2.5	101.4 ± 8.8
	4000	3.6	82.5 ± 3.0	
CAF	2000	3.8	127.4 ± 4.8	102.3 ± 2.9
	4000	3.6	95.9 ± 0.5	
FEN	400	4.1	104.6 ± 4.3	106.8 ± 14.0
	800	8.9	107.6 ± 10.5	
FLU	2000	5.9	112.3 ± 6.6	88.8 ± 22.3
	4000	19.8	121.1 ± 24.0	
GEN	400	12.2	106.2 ± 12.9	94.9 ± 4.2
	800	7.7	120.1 ± 9.3	
KET	300	5.3	113.8 ± 6.0	105.6 ± 8.0
	600	2.6	104.2 ± 2.7	

Table S6: Accuracy, precision and recovery determined in yeast and H295R cell lysate. The parameters in table S6 were monitored in yeast and H295R cells according to the guidelines from EMA 2009 and ICH, 2014. Two concentrations of quality control samples (mid and high concentrations) were proven for accuracy and precision. Precision, accuracy and recovery were determined in five replicates shown as mean ± SD.

Test substance	Quality control [nM]	Accuracy [%]	Precision [%]	Recovery [%]
APAP	400	2.0	108.0 ± 2.2	101.9 ± 4.4
	800	2.3	95.2 ± 2.2	
BPA	400	15.1	89.3 ± 13.5	79.9 ± 23.9
	800	5.7	97.5 ± 5.6	
CAF	400	1.1	108.0 ± 1.1	109.3 ± 1.7
	800	4.3	79.7 ± 3.3	
FEN	150	2.3	102.8 ± 2.3	97.3 ± 3.1
	300	1.0	97.7 ± 1.0	
FLU	200	17.9	104.1 ± 18.6	95.8 ± 16.2
	400	16.1	107.5 ± 17.3	
GEN	100	5.3	88.6 ± 4.7	93.7 ± 4.3
	200	1.3	97.4 ± 1.3	
KET	300	7.3	114.0 ± 8.4	103.4 ± 6.3
	600	8.6	107.4 ± 9.2	

III. Cell numbers

Table S7: Experimentally determined yeast cell number and volume during incubation with the test substances. The OD of the cell suspension was measured after the incubation of cells with the test substances (24 and 48 h). The OD was normalized against untreated yeast cells to determine the cell volume (µL/10^7 yeast cells). Taking the diameter of yeast cells transfected with human estrogen/androgen receptor (hER/AhR) into account, the calculation of the respective volume of yeast was yielded. Data are represented as mean (SD, n=3).

Test substance	Cell number		Cell volume [µL]	
	24 h	48 h	24h	48 h
Untreated cells (hER)	5.27 x 10^6	1.17 x 10^7	0.09	0.21
	(6.15 x 10^5)	(2.95 x 10^6)	(0.00)	(0.00)
Untreated cells (hAR)	3.23 x 10^6	9.77 x 10^6	0.10	0.21
	(0.97 x 10^5)	(1.51 x 10^6)	(0.00)	(0.00)
APAP	4.24 x 10^6	9.81 x 10^6	0.04	0.20
	(9.57 x 10^5)	(1.00 x 10^6)	(0.01)	(0.02)
BPA	3.77 x 10^6	9.38 x 10^6	0.03	0.19
	(1.16 x 10^6)	(8.57 x 10^5)	(0.01)	(0.02)
CAF	4.16 x 10^6	9.72 x 10^6	0.04	0.20
	(6.37 x 10^5)	(8.86 x 10^5)	(0.01)	(0.02)
FLU	2.58 x 10^6	9.78 x 10^6	0.03	0.20
	(7.48 x 10^5)	(3.13 x 10^6)	(0.01)	(0.06)
GEN	3.96 x 10^6	9.08 x 10^6	0.03	0.19
	(1.18 x 10^6)	(9.91 x 10^5)	(0.01)	(0.02)

Table S8: Experimentally determined H295R cell number and volume after incubation (2, 24 and 48 h) with the FEN and KET. Human adrenocortical carcinoma cells were lyzed in a solution containing Triton-X (0.5 % in PBS) to derive the protein content. The protein content of treated H295R cells was normalized against untreated H295R cells to determine the cell volume (µL/10^6 H295R cells). Experimentally determined diameter were taken for the calculation of the respective volume of H295R cells. Data are represented as mean (SD, n=3).

Test substance	Cell number			Cell volume [µL]		
	2 h	24 h	48 h	2 h	24 h	48 h
Untreated H295R cells	1.78 x 10^6	2.17 x 10^6	1.93 x 10^6	2.43	2.05	2.12
	(7.79 x 10^5)	(3.22 x 10^5)	(4.82 x 10^5)	(0.00)	(0.00)	(0.00)
FEN	1.24 x 10^6	1.40 x 10^6	1.38 x 10^6	3.01	2.86	2.93
	(3.22 x 10^5)	(3.01 x 10^5)	(4.63 x 10^5)	(0.79)	(0.47)	(1.01)
KET	1.18 x 10^6	1.45 x 10^6	1.66 x 10^6	2.87	2.96	3.53
	(2.82 x 10^5)	(3.84 x 10^5)	(2.87 x 10^5)	(0.75)	(0.88)	(0.60)

4 Discussion

A remarkable interest on alternative toxicity tests increased in the last years resulting from the urgent need of high throughput technologies, caution on animal welfare and especially the elucidation of substances modes of action. The practice of the AOP concepts is, rather than with animal models, more concretely addressed with *in vitro* assays. The new concept does not only provide new opportunities to study toxicological endpoints and increase the role of cell-based assays, but also may consider relevant PoD for mechanistic extrapolation of *in vitro* derived effects to external doses for humans [5, 12, 18]. However, implementations of *in vitro* models focused rather on the determination and explanation of modes of action than the exposure assessment or *in vitro* biokinetics [89]. The reflection of *in vitro* effects needs to be addressed deliberating different perspectives, especially with respect of its quantification. In this context, concerns on *in vitro* biokinetics became essential where *in vitro* dose metrics represent one key point since most studies rely on C_{Nom} to define concentration-effect relationships for which an appropriate PoD for the extrapolation to human exposure must be declared. Also, many studies on the *in vitro* and *in vivo* exposure postulated that the practice of free concentration as dose metric leads to more reliable results [40, 41, 109, 110]. Generally, exposure prior testing is commonly assessed in standardized *in vivo* protocols, however, less attention is paid to such assessments prior *in vitro* tests although standardization and harmonization is highly appreciated [111]. With respect to these concerns, the application of *in vitro* tests and QIVIVE may represent a potentiated source of errors, question the accuracy of the use of *in vitro* effect concentrations as PoD and thus strengthen the plea to take *in vitro* biokinetics into consideration.

As a proof of concept, the projects within this work promote the determination of relevant exposure concentrations in *in vitro* assays as well as the evaluation of the applicability of prediction models and their prediction power (manuscript no. 2, chapter 3.1.3). The predominantly binding to proteins was assessed as the key parameter limiting the concentration of substances in an *in vitro* test system and analyzed in detail (manuscript no. 1, chapter 3.1.1). The application of QIVIVE using *in vitro* effect concentrations as PoD was given attention in manuscript no. 3 (chapter 3.2.1) in order to assess endocrine effects on a basis of a published study [14].

Considerations on Suitable Dose Metrics for *In Vitro* Assays

In living organisms, substances are absorbed, circulate in the blood to be distributed in the body and may undergo metabolic processes before the elimination of the substance [38]. In a simplified way, biokinetic processes also occur *in vitro* where the culture medium represents the blood circulation distributing in the test system and expose the cell layer. The administration of substances systemically available to a whole organism is, by definition, represented by doses, e.g. amount of substances per kg body weight while local exposures are mostly related to concentrations [37]. Nevertheless, the concept of using concentrations is only valid for reversible binding to targets, irreversible reactions are described by doses [37, 38]. Target concentrations in cells can be defined by different proxies: substance concentration in cells, on/in membranes, in the cytosol, cellular organelles like lysosomes or nucleus or by the occupation of receptors. Analogous, the availability of the added substance decreases in *in vivo* organisms due to partitioning to macromolecules in the blood or in tissues and thus state the implementation of free concentrations when performing *in vitro* assays which can exert toxic effects and interact with the specific target [37, 38, 64, 112].

The representativeness of C_{Nom} as effect concentration might be ambiguous in specific cases since the concentration of substances can be significantly lower than the theoretically defined measure prior testing [30, 40]. The investigations on effective concentrations have been summarized in manuscript no. 2. Mouse fibroblasts (Balb/c 3T3 cells) have been exposed to 12 different test substances with varying physico-chemical properties at one C_{Nom} and at three timepoints (6, 24 and 48 h). Analytical methods have been validated and established for the analysis of substance concentrations in culture medium and cells. In addition, the impact of washing effects and the preparation of cell samples were investigated for a subset of compounds. The experimental results should serve for the validation of a mass balance model likewise published and discussed in detail in the section below.

Exceptional for the substances APAP and CAF, C_{Cell} was found to be 2 to 274-fold higher than C_{Nom} and total C_{Medium}. Vice versa, free C_{Medium} was below these concentration surrogates due to significant binding to proteins in culture medium, or equal to C_{Nom} (APAP, CAF). Washing steps, with PBS for example, are commonly performed in *in vitro* assays to remove culture medium from the cell layer. After exposure of Balb/c 3T3 cells with APAP, CAF, COL and FLU and collecting all fluids, i.e. culture medium, cell lysate and PBS of two washing steps, 50 – 90 % of substances have been recovered in culture medium, while lower fractions were found in cells (0.04 – 6.4 %). In PBS, concentration of substances amounts of 0.5 – 6.7% of C_{Nom} after the first and below the LOQ (corresponding to 0.3 – 2.3 %) after the second washing step. In order to assess the intracellular concentration of substances, i.e. substances in cytosol, two preparation procedures have been conducted for cell lysis and the results compared: a) Balb/c 3T3 cells were collected in demineralized water after trypsinization and underwent freezing and thawing cycles and b) Balb/c 3T3 were lyzed with a digitonin solution in order to release the cytosolic fraction which was collected and analyzed after sample extraction [113]. The experiment was performed for four test substances leading to comparable results (APAP, CAF, FLU, KET).

From the practical point of view, defining and measuring such concentrations related to these exemplary cellular targets is hardly achievable due to the absent analytical methodologies [69]. In this work however, it was demonstrated that cellular concentration is an accessible metric using simple

techniques, i.e. thawing and freezing of cells and the permeabilization of cell membranes in order to release the cytosol containing substances [113]. The analysis of four exemplary chemicals has furthermore demonstrated that both mentioned methodologies result in comparable measured concentrations concluding that the content of substances in the cytosolic fraction was the one analyzed. A final proof for this conclusion needs to be taken, i.e. subcellular fractionation, western blots for the detection of specific proteins. Such information may allow to determine the exact allocation of the substances in cellular organelles (e.g. nucleus, lysosomes, membranes), binding affinity to possible cellular targets (specific receptors) and/or identify possible mechanisms occurring in cells. In order to expand knowledge on substances behavior and/or to find appropriate PoD, the development of such techniques can be beneficial. In less sophisticated systems, steady state will be achieved at a certain point due to a continuous reallocation and would lead to equal concentrations in all compartments, mainly medium and cells. From the experiments described above, clear statements on the achievement of steady state cannot be set where increasing cellular concentrations were examined over time, i.e. lipophilic substances such as BPA, FEN, TAM. The inclusion of more sampling time points may facilitate a conclusion on this point.

Difficulties arose as many parameters related to cells are tainted with uncertainties. Assumptions are continuously used for specific data on cell characteristics such as volume, weight or cell number. Although a known number of cells is seeded prior testing, the cell proliferation represents a variable parameter and the relation to a certain target remains indefinite because the reference parameter alters over time. These uncertainties may explain the high variation of the yielded results for C_{Cell}. Practically, the counting of exposed cells per well during the actual toxicity test is time-consuming due to the high number of samples. In order to assess i.e. the amount of substances per cell(s), weight substance per cell(s) or any molecular target in a high-throughput manner, faster procedures like imaging techniques or protein content assays can be also performed [114].

Knowing the mechanism of action of a substance may indicate which dose metric surrogate is considered most useful. A shift of the overall equilibrium leading to increasing concentration in cells is attributed to a substance specific affinity to targets in the cells or/ and a specific mechanism of action. The estrogen modulating substance TAM is assigned to accumulate in lysosomes [115]. Consequently, higher concentrations in comparison to the C_{Medium} have been determined (manuscript no. 2). Lipophilicity of substances further contributes to the distribution of substances in cells [116]. Although not stated as lipophilic substance, COL was found at higher levels in cells explained by its affinity to cellular tubulin [117]. Examples where the consideration of intracellular concentration mattered have been discussed in literature as well, e.g. methylmercury chloride sulfhydryl groups of cellular proteins, cyclosporine A mitochondrial to pores or taxol binding to microtubules, however, measuring at these sites of action is technically unachievable and elaborating. Surrogates related to receptors and intracellular target sites are useful when it comes to investigations of cellular mechanisms or provision of data for prediction models and its validation. The next steps closer to the effective target concentration would be the free C_{Cell} and were already faced in previous work for pharmaceutical investigations [118]. These experiments need a high amount of cell mass which is highly time-consuming, or highly diluted cell lysate samples, which brings another level of uncertainty with respect to the cellular concentration estimates. The closest estimate represented in this work is the total cell concentration and a suitable

approximate, however, brings uncertainty in the results obtained showing high variations. Furthermore, investigations of the impact of washing steps needs to be considered and its contribution to measured concentrations in cells.

Significance of *In Vitro* Assay Parameters

Beside the actual dose metric selection and physico-chemical properties of substances, considerations on the assay setup need further attention and the factors contributing to the reduction of the free substance concentration in the tests. For the test substances in this work, the most practical way is to address protein binding as the most abundant factor limiting a substance's availability [41, 68, 111, 112, 119]. Appropriate methodologies to address protein binding have been demonstrated in this work (manuscript no. 1) as well as in other publications [40, 55, 68, 72, 120]. Significant binding to proteins was considered providing three different methodologies investigated on robustness and reliability to estimate f_u. Representative proteins, e.g. hormone binding proteins, alpha glycol proteins and most prominently, albumin, contribute to the decrease of a chemical's freely available concentration in plasma. To obtain protein binding prior testing, RED, UF and UC are suitable methods for polar substances (log P_{ow} < 2), while RED should rather be considered for moderate as well as lipophilic molecules as shown in manuscript no. 1. Accordingly, lipophilicity of test substances may act as a meaningful indicator for lower f_u. The tested domain of the current framework is limited to test substances possessing properties, e.g. logP_{ow} within a range of -1 to 6.8, non-volatile and neutral molecules. Because RED delivered the most suitable and robust results on f_u, the methodology was implemented successfully in manuscript no. 2 to calculate the measured C_{Medium} by the protein bound fraction. The results obtained furthermore correlated well with prediction derived data from the mass balance model within factors of 3 and constituted a simple framework for the analysis of free fractions in culture media. Serum, e.g. fetal bovine serum, is generally supplemented to the culture medium with the purpose of maintaining the function and providing optimized conditions for the growth of cells. Actions towards minimizing the amount of serum applied or the absence of serum have been taken to reduce the partitioning of test substances towards serum proteins. It was however confirmed that macromolecules like albumin may increase the rate of diffusion and accelerate the uptake of substances into cells [46]. Consequently, the overall equilibrium decelerates resulting in a retarded achievement of the steady state because of the absence of serum [30, 46, 121]. Another reason to keep the supplementation of serum is the fact that a comparable situation is found in living organisms where substances bind to macromolecules after uptake and the free amount automatically reduces and is, from a pharmacological point of view, required (e.g. ibuprofen) [94].

Neither the binding to cellular lipids nor the binding to medium derived lipids was in focus in this work. Generally, partitioning to lipids are mostly in both, *in vitro* and *in silico* assays, summarized and attributed to bulk lipids as surrogate. Different categories may enforce the partitioning process towards lipids in culture medium but also in cells, e.g. storage lipids, membrane lipids and sterols. Amongst the substance targets, binding to lipid can be a main driver of cellular uptake (e.g. BPA, KET, TAM) and thus, increased concentrations of test substances in cells as explained in manuscript no. 2, however, not experimentally quantified or proven.

The interaction between highly lipophilic compounds and devices can occur during incubation or/and sample preparation using labware. Appropriate estimates and methods have been described in previous

work [40, 44]. In order to evaluate a diffusion-based model, it was clearly assigned that the cell line Balb/c 3T3 has no relevant influx and efflux transporters, respectively [122, 123]. The metabolic competence of this cell line was further negligible but toxicity effects in metabolic active cells need to be attributed either to the parent compound or its metabolite(s) [123]. However, steady state assumptions are not valid for every *in vitro* assay and cell line, respectively. Degradation as well as dynamic system where metabolically active cells cause the transformation of parent compounds needs consideration, e.g. for hepatic cell lines or more complex *in vitro* systems with multiple cell lines, organoids or organ-on-chips [47].

The factors and processes described in detail are valid for single exposure testing strategies not for repeated exposure experiments. Other requirements need evaluation for repeated exposure scenarios [31, 50]. Furthermore, no considerations on mixtures as test items were taken. The implications of *in vitro* test systems, in particular for mass balance models, are addressed in the next paragraph.

Contemplations on *in vitro* simulation models

Integrated testing strategy for human health risk assessment may include the usage of mechanistic modelling to address *in vitro* toxicokinetics. Validation of mass balance models with experimental data is not always granted however incorporated in this work (manuscript no. 2). Data on total and free C_{Medium} and total C_{Cell} have been generated in a first approach experimentally, in order to validate and compare them with simulated data. To sum up, total C_{Medium} were predicted correctly (11/12 test substances), predictions on free C_{Medium} varied by max. 3-fold and total C_{Cell} by 4-fold. Correlations between simulated and experimentally derived cellular concentration yielded poorer correlations.

Major contributing pathways as discussed in the section above, i.e. losses through cellular uptake, binding to medium constituents or plastic and evaporation, have been considered in published models [40, 45, 52, 64, 66, 67, 124]. Typically, the test system's compartments, i.e. medium and cells, are simplified and subdivided into three subsequent phases: water, protein and lipid phase. With simple assumptions, efforts have been taken to mimic distribution processes and biochemical pathways however, not covering all important pathways occurring in cells, i.e. metabolic transformation of cells or binding to cellular targets. Also, only a part of the biochemical pathways and processes can be depicted with equations to a certain degree. Addressing the appropriate surrogate remains difficult to realize within a mass balance model as well.

Generally, uncertainties may derive from the assumptions and selected parameters made for the prediction model. Despite the accessible parameters on assay setups which are not obligatory to measure, specific data related to cells are hardly obtained in standardized studies. As discussed in the sections above, dynamic and time-weighted processes like cell proliferation over incubation times can be hardly represented and obtained by prediction models. Cell proliferation might de- or accelerate by specific mode of action – such phenomenon was observed for the mitosis inhibiting substance COL (manuscript no. 2). Variations between the experimentally derived total cell volume at the sampling timepoints (6, 24, 48 h) were not detected. Depending on the cell line, the doubling time, growth stage of cells, metabolic competence and activity of xenobiotic-mediated influx or efflux of transporters are differently characterized and play a role for the input parameters used for the prediction model in terms of their complexity. The metabolic capacity and transporter expression in Balb/c 3T3 cells however has

negligible effects on the substances concentration as both characteristics were reported and experimentally proven to be low. Standardization of methodologies to estimate particular parameters are either not available or difficult to realize, especially in a high throughput manner. The partitioning to cells can be either described by experimentally derived data/ based on physico-chemical descriptors, e.g. lipophilicity or by binding to surrogates, e.g. cellular proteins and/or lipids [52, 64, 125]. Similar concepts are feasible to derive the partitioning to plastics [40, 45, 65].

In general, the achievement of a steady state equilibrium is an assumption for *in vitro* systems where the distribution of substances to all phases occurs instantaneously [46]. This concept may fail where binding to targets with high affinity, accumulation, metabolism, evaporation or degradation occur, i.e. irreversible reactions, where time dependent exposure metrics need to be considered.

How discrepancies between the outcomes of different models may appear was investigated in a side project where a publicly available prediction model was established using basic principles from [45] named virtual *in vitro* distribution model (VIVD) in the following [45, 126]. After validation of the VIVD model with published data [52], experimental data of manuscript no. 2 have been used to evaluate the prediction power. On comparing (figure 4), the outcome is similar to the published results in manuscript no. 2. Indeed, predictions on the total C_{Medium} of all 12 substances were predicted correctly within factors of < 1.5 while free concentrations were overpredicted for two respectively four test substances (BPA, GEN, KET, WAR). Improved correlations compared to the published mass balance model in the *in vitro* dosimetry approach were yielded for the prediction of cellular concentrations, however, varies for most of the substances by factors of 2 - 7. The applicability of the model is limited to 12 test substances; however, it is assumed that substances with similar physico-chemical properties ($LogP_{ow}$ -0.07 – 6.84, molecular weight of < 532, g/mol, neutral speciation at pH 7.4, Henry's law constant below 10^{-3} $\frac{atm \times m^3}{mol}$), can be well predicted to comprehend the behavior of substances in cell-based systems. Furthermore, the definition of cellular compartments is in the VIVD model more complex, i.e. subdivision of the cells into a protein, lipid and water phase. The inclusion of cellular organelles, i.e. lysosomes and mitochondria do not trigger the discrepancies between model outputs of neutral molecules. As highlighted in the sections before, an experimental procedure for the determination of concentrations at specific target sites meaning concentration in the organelles, are not established at the current stage within this work. Thus, a comparison or validation of the model with experimental data is not enabled yet. Differences in the results derive from the most likely from the assumptions of the models, i.e. the inclusion of lipids, since both models are driven by $logP_{ow}$. The VIVD model takes partitioning to membrane and storage lipids, i.e. phospholipids while the mass balance model in manuscript no. 2 considers the partitioning to liposomes. Liposomes act as a reservoir for substances and could affect to the shifting of the equilibrium towards cellular compartments leading to higher predicted concentrations in cells. Indeed, C_{Cell} correlated with the predicted concentrations from VIVD model. The prediction model merely simulates the partitioning of the substances in the water phases of the different compartments, i.e. culture medium, cells and headspace. However, the assumption that albumin represents an approximate for cellular proteins still does not mirror the actual situation [127]. To sum up, the selection on the parameter assumption and the following description in form of equations plays an important role in the construction or mirroring of the *in vitro* test system [126].

Prediction models can serve as a useful tool for screening purposes or to improve test design of *in vitro* assays. As demonstrated in this section, prediction models and the set equations work differently depending on the introduced parameters, assumptions and applicability domain. For the sake of validation and practicability, the assessment of *in vitro* concentrations is of importance and may enforce and improve the prediction power of future models as also stated by Chang and colleagues [6]. The methods provided in manuscripts no. 1 and 2 give an exemplary workflow to derive such data.

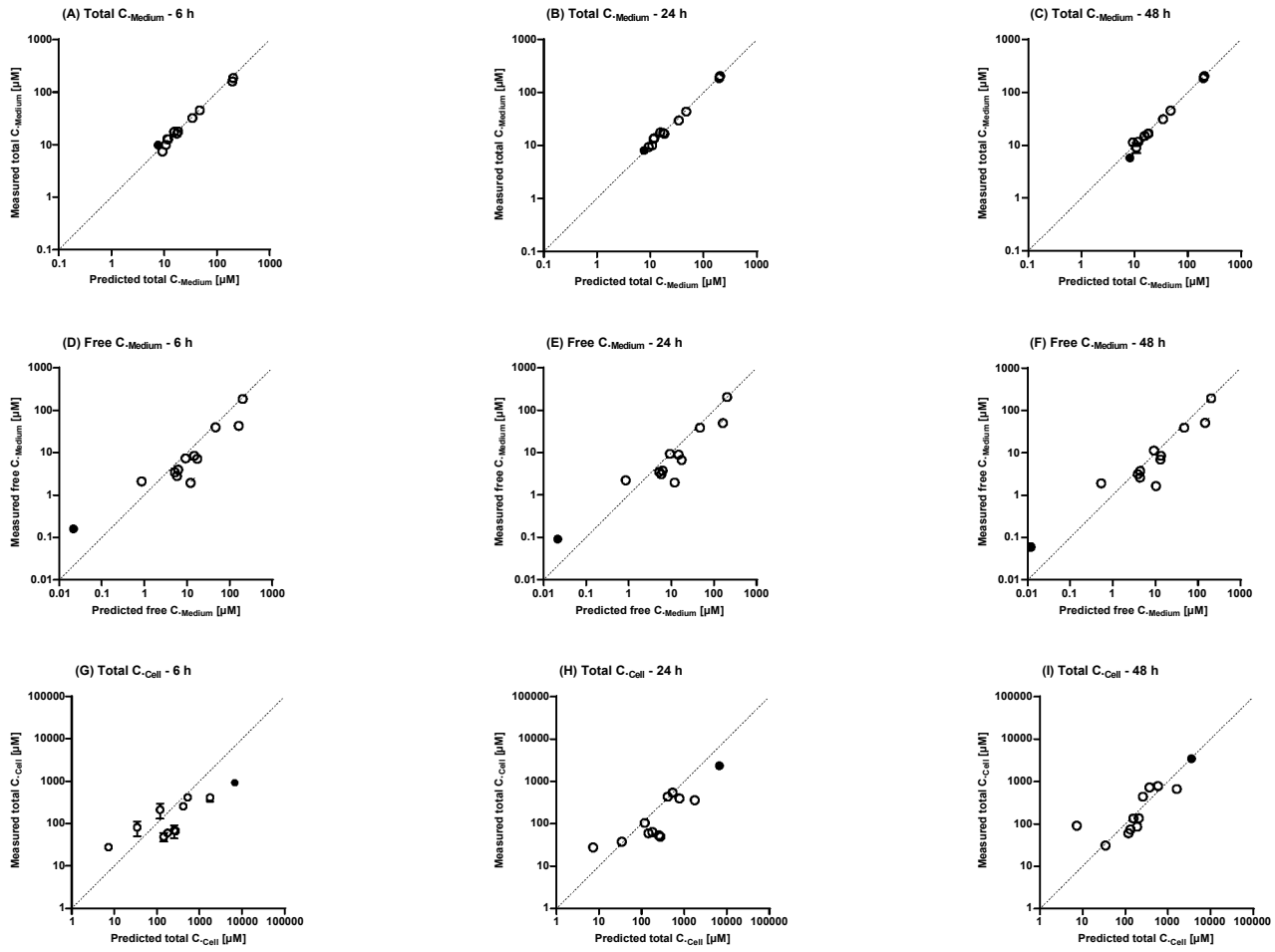

Figure 4: Predicted and measured concentration of twelve test substances after 6, 24 and 48 h of exposure based on the VIVD model [45, 126]**.**The experimental data at different time points of manuscript no. 2 was compared to prediction derived data from VIVD model. Therefore, the total C_{Medium} (row 1, A-C), free C_{Medium} (row 2, D-F) and total C_{Cell} (G-I) after 6 (column 1), 24 (column 2) and 24 h (column 3) of incubation have been included. The dotted line represents the line of identity indicating the closeness of both data sets. Data are represented as mean in µmol/L (SD if n=3 or mean difference between individual values if n=2).

Implications of dose metric selection on QIVIVE

As stated, considering C_{Nom} for the extrapolation to *in vivo* doses may lead to over- or underpredictions. The methodologies and principles described in manuscripts 1 and 2 laid the foundation to refine a previously published case study on the estimation of endocrine disruptive effect doses taking *in vitro* dosimetry into consideration [14]. Therefore, yeast cells transformed with human estrogen/ androgen receptor and adrenocortical carcinoma cells were incubated with the test substances and total/ free C_{Medium} and C_{Cell} analytically determined after different incubation time points (manuscript no. 3). Both metrics were evaluated as PoD for a PBTK model for reverse dosimetry. By getting these effect concentrations in medium to plasma concentrations, the lowest observed effect doses (LOEL) were derived by reverse dosimetry using a PBTK 8 compartment model.

On the level of the performance and findings in the *in vitro* assays, comparable conclusions to manuscript no. 2 have been made in manuscript no. 3. A prolonged time is required to state equilibrium-reached systems when exposing *in vitro* test systems with lipophilic substances. Concentration in yeast cells could not be quantified up to 12 h of incubation due to analytical limitations. C_{Nom} and total C_{Medium} respectively are suitable to describe the overall *in vitro* exposure in test systems where the supplementation of proteins is absent, i.e. YES-/YAS- assay with reported no proteins in the assay medium [128]. Instead, free C_{Medium} is more relevant in the steroidogenesis assay. In proportion to culture medium concentrations, C_{Cell} were found to be equal (e.g. APAP and CAF), up to 70 times higher (BPA, FLU, FEN, GEN) or reduced (KET) over time. Steady state can be concluded to be reached when exposing yeast cells to APAP and CAF. Since BPA, GEN (cellular lipids and estrogen receptor), FLU (androgen receptor) and FEN (enzymes of the steroidogenesis pathway) have an affinity to specific molecular targets in yeast respectively H295R cells, an increase of the concentration in cells was found for these substances as expected. However, the sample preparation does not allow to conclude on the location of substances – it is not clear of which cellular structure the substances have been extracted from. Therefore, more steps need to be included in the sample preparation, e.g. differential centrifugation in order to compartmentalize cellular structures for the analysis of substances' concentration. The results obtained for the substance KET may point out the involvement of the substance in the cellular metabolism or other pathway, e.g. steroidogenesis, as the total amount of the substance in both main compartments, cells and culture medium, decreases over time. Substances without the affinity to the targets in yeast cells, i.e. APAP and CAF, led to equivalent *in vivo* LOELs when introducing cellular or medium derived concentration for the reverse dosimetry approach. Obtaining plasma concentrations for such substances and medium-related (nominal or free) concentrations are sufficient dose metrics for QIVIVE concerning endocrine effects.

On the level of prediction models and simulations, not only the selection of exposure conditions, i.e. considerations in i*n vitro* dose metrics, contributes to successful QIVIVE, but also the selection of appropriate parameters used in the PBTK model. Since standardization and harmonization on obtaining physiological data on animals or specific substance characteristics is not always assured, the selected parameters reflect also a source of uncertainties. Comparably to the prediction models, several descriptors as well as the PBTK model itself rely on assumptions. Such parameters can be derived by experiments, e.g. hepatic clearance, *in vivo* data or by simulations, e.g QSAR or QSPR and are mostly utilized when *in vivo* data is absent [129]. However, the variability of assumptions on models and the

presence of different test systems for parameter characterization impedes a harmonized procedure on generic model development. One example for differences in the availability of test systems are the assays to characterize ADME properties. For example, colon carcinoma cell line and human intestinal epithelial cells as monolayer or in transwell systems, address the absorption of substances which are naturally not occurring in living organisms. Uncertainties generally arise when using cell lines, i.e. differences in primary cell cultures, batches. Furthermore, the individuality and variability between species and the reaction to certain exposure needs further consideration [39]. Regarding the toxicological endpoint endocrine disruption, specific mechanisms are not fully understood or discovered. Thus, a full complement of involved enzymes and pathways, i.e. the tissue and organism complexity, cannot be reflected in the PBTK model. With this concept, it is not postulated whether the actual measured C_{Cell} and therefore, the PoD used for reverse dosimetry, reflects the concentration affecting the estrogen/ androgen receptor or the effective concentration interacting with enzymes of the steroidogenesis. Exposure metric plasma as substance is able to distribute and reach all targets.

As highlighted in *in vitro* testing in the sections before, limited data and information on cellular concentrations and exposure are available and the generation of such data is difficult to obtain. Similarly, tissue concentrations *in vivo* are neither obtained nor the generation of data on tissue concentrations is commonly generated. Nevertheless, the importance of tissue surrogates becomes relevant when specific mode of actions in organs occur or when local effects triggered by substances are known. Exemplary, substances like BPA, FLU but also MT, TRE and TAM (not included in the case study) are expected to accumulate in relevant tissues (or cells) due to their affinity to estrogen/ androgen receptors and their reported mode of action (endocrine disruption). Thus, the findings have been confirmed for the substances BPA, GEN, FEN and FLU. Here, tissue surrogates may become more relevant for IVIVE than for APAP and CAF, where equally distributed concentrations in medium and cells were localized. When high affinity to cellular structures is present, tissue concentrations may play a bigger role for QIVIVE than plasma concentrations. Specific interactions with the CYP system, i.e. steroidogenesis, have been reported for FEN and KET indicating a preference in using cellular obtained concentrations. In this work, the *in vitro* dosimetry concept for QIVIVE does not cover the metabolization or any enzyme-related interaction of substances. Although the overall concentration of the parent compound decreases, the metabolites which result from these reactions or interactions were not analyzed. KET concentrations reduced and thus questions which amount is actually responsible for the endocrine effects. Furthermore, the general steady state assumption which is mostly considered for *in vitro* tests might not be conferrable for this substance and time-dependent dose metrics may describe the actual exposure, i.e. area under the curve or time weighted average [30, 38].

The results obtained in manuscript no. 3 are only relevant for endocrine disruption in two specific *in vitro* test systems, however, can be applied to other toxicological effects. Tissue concentrations can serve as an important surrogate for high binding affinity to specific organs or locally occurring effects within the tissue. However, the appropriate counterpart on extrapolation cellular concentrations remains questionable since doses in tissues could reflect it properly.

Recommendations for future *in vitro* testing

Summing up, following situations have been observed (manuscripts no. 2 and 3) and may represent general findings in *in vitro* assays that needs to be considered:

1) Medium concentration of substances stays stable over incubation time while the cellular concentration adapts until steady state is reached as assessed for example for APAP or TRE in the *in vitro* dosimetry study.
2) A depletion of the medium concentration is observed while cellular concentration increases attributed to a substance's affinity to specific cellular targets as observed for COL and TAM in the *in vitro* dosimetry study.
3) Amongst the physico-chemical properties, considerations on metabolic activities and degradation processes need to be taken when observing decreasing concentrations of substances. Concentrations of KET decreased in both compartments, H295R cells and culture medium, however, it is not stated whether degradation or metabolic processes are involved in this process.

Based on physico-chemical properties, following thoughts need to be integrated to avoid the performance of experiments:

1) Polar substances with low $logP_{ow}$ (< 2) and protein binding ≤ 10 % are likely comparable to the nominal concentration in culture medium. Therefore, nominal concentration is sufficient to describe effect concentrations. Depending on the assay conditions, a correction factor, i.e. protein binding, needs further consideration to characterize the effect concentration.
2) For non-polar substances with high molecular weight, $logP_{ow}$ (> 5) and concomitant with high protein binding (≥ 10 %), the concentration in the compartments of the *in vitro* test system may enormously differ and nominal concentration is not representative describing effect concentrations. The determination of concentrations in medium and cells as well as the additional consideration on protein binding is recommended.
3) Moderate lipophilic substances $logP_{ow}$ (2 - 5) tend to interact at different levels with proteins (10 - 90 % protein binding). Therefore, the impact of binding properties needs consideration case by case.

By means of reducing animal testing for the toxicological assessment of substances, the work presented investigations towards useful dose metrics for *in vitro* assays and exemplary application of *in vitro* dose metrics for QIVIVE.

5 Outlook

In vitro dosimetry was investigated in this work. Studies with known methodologies were performed and evaluated in order to assess the *in vitro* exposure towards twelve exemplary substances and generate experimental data. Therefore, relevant and robust methodologies for the investigation of protein binding, sampling procedures, *in vitro* dosimetry and on the practicability of predictive mass balance models as such have been achieved, e.g. for investigative purposes.

Considerations on the expansion of the data set for protein binding studies and *in vitro* dosimetry is essential, especially for the latter one to reflect the actual dose metrics for *in vitro* toxicity assays and avoid tedious lab work. On combining *in vitro* and *in silico* methods, the prediction power of simple mass balance models was confirmed with experimental data. The current results from the project on *in vitro* dosimetry are transferrable to assess the substance's behavior of neutral, non-volatile substances in diffusion-based test systems. However, risk characterization includes additionally the testing of mixtures composed of different active substances, highly volatile, inorganic or degradable substances and nanoparticles [46]. For this purpose, the present framework needs 1) an expansion of the data set to train the present prediction model with more experimental data and 2) an adaptation with regards to the mass balance model and analytical procedures for substances with deviating physicochemical properties from the twelve test substances. High-throughput screening is limited to experiments with single exposed cell monolayers in plastic well plates where diffusion-based uptake is considered. The complexity of *in vitro* test systems is still growing and the use of spheroids, 3D cell cultures, organ-on-chip, organoids getting more attention and recognition for toxicity testing. Thus, application of different physicochemical properties, dissimilar distribution processes due to multiple compartmented systems as well as repeated dosing scenarios needs consideration [111].

The selection of appropriate POD for the extrapolation of *in vitro* effects in form of QIVIVE has been assessed in the last study namely the extrapolation of an *in vivo* dose based on *in vitro* effect concentrations. Comparable correlations between *in vivo* doses and *in vitro* derived concentrations were yielded. It is still questionable if a proper translation using cellular derived concentration is conceivable to the current *in vivo* dose. Furthermore, the proportionality of the total and free concentration in medium and cells to the BED is uncertain. The validity of the refined reverse dosimetry approach needs further verification, e.g. including additional endocrine modulators and or using the current concept for other toxicological endpoints. Furthermore, uncertainties given through non-standardized estimates of parameters like the hepatic clearance, POD based on highly, saturated doses, poor simulation of transporter mediated uptake, signal cascades and enzyme interaction depicted by both, *in vitro* and *in silico* models, needs further improvement in future works [6, 43].

6 Summary

Potential hazard and risks of substances, pesticides and pharmaceuticals are traditionally addressed with animal experiments, for which numerous standardized, recognized and harmonized methods are available. In accordance with the paradigm of the 21st century concerning toxicological risk assessment and the 3R principle ("Reduction, Replacement, Refinement"), i.e. replacing animal experiments, *in vitro* and *in silico* based methods are increasingly being used to assess concentration-effect relationships. To derive risk assessments from results of *in vitro* methods, relevant human and animal doses should be extrapolated from concentrations in cell-based assays (*in vitro-in vivo* extrapolation, IVIVE). Usually, the effective concentration *in vitro* is based on a pre-defined amount of substance per unit cell culture volume, the so-called nominal concentration. However, factors such as binding to proteins in the cell culture medium, to adsorption to the cell culture vessel, and processes such as volatilization and enzymatic degradation contribute to the reduction of the concentration of a test substance in an *in vitro* test system. This hampers the extrapolation to relevant *in vivo* doses. The actual concentration of test substances in *in vitro* systems was examined in this work. For this purpose, analytical methods were developed, validated and used to determine actual *in vitro* concentrations of twelve test substances. Finally, the obtained effective *in vitro* concentrations were used to assess the endocrine disrupting potential of substances based on *in vitro* study results.

The work on determining actual *in vitro* concentrations was divided into three parts:

(1) Binding to proteins, which depends on the amount of protein in the medium, has a major influence on the unbound concentration of a substance in an *in vitro* test system. Likewise, substances bin to proteins, such as albumin, in blood plasma *in vivo*. Since the free fraction (f_u) of a substance might be responsible for an effect, the fraction bound to proteins of the test substances in human plasma was determined. Applied methods for the investigation of protein binding are the rapid equilibrium dialysis (RED), ultrafiltration (UF) and ultracentrifugation (UC), which were checked for repeatability, accuracy and robustness in this work. In comparison to published literature data, more reliable f_u ten test substances were obtained using RED with recovery values of 70 – 130 %. Physico-chemical properties of the substances, e.g. the octanol-water partition coefficient, are significant when selecting the appropriate separation method. The methods UF and UC are also recommended for polar substances (f_u >70 % and $\log P_{ow}$ < 2) since nonspecific binding to devices are not significant, while the protein binding of lipophilic substances ($\log P_{ow}$ of 3.6 - 6.84) should be rather determined using RED based on comparable derived f_u with literature data and suitable recovery values (68.2 – 118.1 %). Recoveries below 50 % and higher f_u in comparison to published reference data were derived for moderately lipophilic to lipophilic substances when using UC of UF.

(2) Methods for the quantification of test substances in the culture medium and cell lysates and as well as different sample preparations for the cell lysates were developed and the impact of sampling preparation on the measured cellular concentrations evaluated. A diffusion-based model for predicting the concentration in the compartments of the *in vitro* test system was used and the experimental data utilized to evaluate the model. Based on the results obtained from the protein binding studies, analytical concentrations of the test substances at 6, 24 and 48 h of incubation were corrected for the

experimentally determined free fraction. The experiment was carried out with the Balb/c 3T3 cell line assuming that the cellular uptake of substances is based on diffusion due to the low transporter expression in this cell line. With regards to the mass balances and the measured data, minor effects on the concentration of the test substances with little or no protein binding in the culture medium were determined. On the other hand, substances with a defined mechanism of action and particular cellular targets or high octanol-water partition coefficient (TAM) showed deviations from the nominal concentration or analytically measurable concentration in the medium and thus resulted in a shift of the equilibrium towards the cell compartment. Cellular concentrations are up to 2 – 274 times higher than the nominal concentration and thus confirmed the discrepancies between medium and cell compartment. Computational and experimental data on the total and free concentration of the test substances in the culture medium agreed for eleven substances for which accordance below a factor of 2 were observed. In contrast, up to 4-fold higher concentrations were predicted for the Balb/c 3T3 cells. Discrepancies could be explained by the lack of involvement of target compartments such as cell organelles where substance specific mechanisms may be triggered (e.g. lysosomes as target organelle for tamoxifen), metabolic activation, transporter-mediated uptake and the degree of ionization of the molecules in the model. The model can be expanded by including these parameters in future work and needs to be validated again. The simple model can be used as a first screening method to assess the behavior of a substance in diffusion-based test systems.

(3) Finally the approaches from previous work, the investigation on plasma protein binding and *in vitro* dosimetry, were combined with a reverse dosimetry approach and applied to in vitro effect concentrations in YES/YAS and steroidogenesis assay to determine oral doses in rats with regards to endocrine effects. For this purpose, concentrations of seven test substances (APAP, BPA, CAF, FEN, FLU, GEN, KET) were quantified in culture media and yeasts and human H295R adenocarcinoma cells using validated analytical methods. These serve as the POD for a QIVIVE based on physiologically based toxicokinetic model (PBTK model) to calculate an external, oral *in vitro* dose. This extrapolated in vivo does was and to compare the data obtained with published results on *in vivo* doses based on nominal *in vitro* concentrations. Increasing concentrations in cells has been found for for 4/7 test substances (BPA, FEN, FLU, GEN) which are known to have an affinity to the estrogenic/androgenic receptor or interfere with steroidogenesis respectively. An equilibrium between the medium and cell compartments was observed in the negative controls (APAP and CAF) which are known to not induce effects in the YES-/YAS- and steroidogenesis assay. Using cellular concentration, oral doses for 6/7 compounds were correctly calculated within a factor of 10, providing results with higher correlation to *in vivo* data than the estimated LOEL based on total and medium concentrations. However, the study highlights the analytical difficulties that arise when determining cellular concentrations over multiple time points (3, 6 and 12 h). Accordingly, no statements could be made on the actual substance uptake over time in the YES-/YAS-assay (APAP, BPA, CAF, FLU, GEN). Nominal as well as total concentrations of substances can serve as POD if there is no affinity to cellular targets, low protein binding (less than 10%) and no metabolic activation by the cell lines are present. Future studies may include other endocrine disruptors and promote the use of reverse dosimetry in relation to other toxicological endpoints.

7 Zusammenfassung

Potenzielle Gefährdungspotenziale und die Risikobewertung von Substanzen, Pestiziden und Arzneimitteln werden heute, abhängig von der Substanzklasse gemäß den regulatorischen Anforderungen durch Tierversuche adressiert, wofür zahlreiche standardisierte, anerkannte und harmonisierte Methoden verfügbar sind. Entsprechend des Zeitgeists zur toxikologischen Risikobewertung des 21.Jahrhunderts und dem 3R Prinzip („*Reduction, Replacement, Refinement*“), also der Reduzierung von Tierversuchen, finden *in vitro* und *in silico* basierte Methoden vermehrt Anwendung zur Erstellung von Konzentration-Wirkungs-Beziehungen. Im Zuge einer breiteren Anwendung von *in vitro* Methoden und daraus abgeleiteter Risikobewertungen sollen relevante *in vivo* Dosen mit Hilfe von *in vitro* Effekt-Konzentrations-Beziehungen aus zellbasierten Assays abgeleitet werden (*In vitro-in vivo* Extrapolation, IVIVE). Eine theoretisch definierte Menge an Substanz pro Volumeneinheit, die sogenannte nominale Konzentration, wird als Testkonzentration definiert, der gegenüber den Zellen exponiert sind. Dennoch tragen Faktoren, z.B. die Bindung an Proteine, Kunststoffe der Zellkulturgefäße sowie Prozesse wie Verflüchtigung und enzymatischer Abbau zur Reduktion der freien Testkonzentration einer Substanz in einem *in vitro* Testsystem bei, was unter anderem die Extrapolation zu relevanten *in vivo* Dosen beeinträchtigen kann. Die effektive Konzentration von Substanzen im *in vitro* System wurde im Rahmen dieser Arbeit untersucht. Zu diesem Zweck wurden analytische Methoden entwickelt, validiert und genutzt, um *in vitro* Konzentrationen von zwölf Testsubstanzen zu bestimmen. Schließlich wurde das *in vitro* Dosimetrie Konzept angewandt, um das endokrine Potential von Substanzen basierend auf *in vitro* Ergebnissen zu bewerten.

Die Arbeit zur Bestimmung der tatsächlichen *in vitro* Konzentrationen lässt sich in drei Abschnitte gliedern:

(1) Einen großen Einfluss auf die freie Konzentration einer Substanz in einem *in vitro* Testsystem ist die Bindung an Proteinen, die vom Proteingehalt im Kulturmedium abhängt (z.B. fetales Kälberserum). Ebenso binden Substanzen *in vivo* an Proteine wie Albumin im Blutplasma. Da postuliert wird, dass die freie Fraktion (f_u) einer Substanz für einen Effekt verantwortlich ist, wurde der Anteil an gebundener Fraktion an Proteinen der Modelsubstanzen in humanem Plasma bestimmt. Angewandte Methoden zur Untersuchung der Proteinbindung sind die sogenannte *Rapid equilibrium dialysis* (RED), Ultrafiltration (UF) und Ultrazentrifugation (UC), die im Rahmen dieser Arbeit auf Wiederholbarkeit, Genauigkeit und Robustheit geprüft wurden. In Bezug auf den vorhergenannten Parameter und im Vergleich zu publizierten Literaturdaten wurden verlässlichere f_u (basierend auf einer Wiederfindung von 70 – 130 %) für zehn Testsubstanzen mithilfe der RED erzielt. Physikalisch-chemische Eigenschaften der Substanzen, z.B. der Octanol-Wasser-Verteilungskoeffizient, sind bei der Auswahl der Trennmethode von großer Bedeutung. Daher sind die Methoden UF und UC für polare Substanzen (f_u >70 % und $\log P_{ow}$ < 2) ebenfalls zu empfehlen, da nicht spezifische Bindungen an den Geräten kaum auftreten, während die Proteinbindung von lipophilen Substanzen ($\log P_{ow}$ von 3.6-6.84) vielmehr mittels RED ermittelt werden sollte, da die f_u mit den Literaturdaten übereinstimmten und akzeptable Wiederfindungen erreicht wurden (68.2 – 118.1 %). Wiederfindungen unter 50 % und hohe f_u im Vergleich zu Literaturdaten wurden für moderat lipophile und hoch lipophile Substanzen erzielt.

(2) Methoden zur Quantifizierung der Testsubstanzen in Kulturmedium und Zell-Lysaten und unterschiedliche Probenaufarbeitungen der Zell-Lysate wurden entwickelt und die Auswirkung von Aufarbeitungsmethoden auf die gemessenen zellulären Konzentrationen bewertet. Ein Diffusion-basiertes Model zur Vorhersage der Konzentration in den Kompartimenten des *in vitro* Testsystems wurde herangezogen und die experimentellen Daten zur Evaluierung des Modells genutzt. Basierend auf den Ergebnissen der Protein-Bindungsstudien wurden analytische Konzentrationen der Testsubstanzen um die experimentell bestimmte, freie Fraktion korrigiert. Das Experiment wurde mit der Zelllinie Balb/c 3T3 durchgeführt, in der Annahme, dass die Aufnahme der Substanzen in die Zellen auf Diffusion basiert, da die Transporter-Expression in Mausfibroblasten gering ist. Im Hinblick auf die Massenbilanzen und den gemessenen Daten konnten geringe Auswirkungen auf die Konzentration der Testsubstanzen mit geringer bzw. keiner Proteinbindung im Kulturmedium festgestellt werden. Substanzen mit einem definierten Wirkmechanismus, einem bestimmten zellulären Zielmolekül oder hohem Octanol-Wasser-Verteilungskoeffizienten (TAM) hingegen wiesen Abweichungen zur Nominalkonzentration bzw. analytisch messbaren Konzentration im Medium auf, woraus sich eine Verlagerung des Gleichgewichts im System auf das Zellkompartiment ausgerichtet wurde. Zelluläre Konzentrationen sind um bis zu 2 – 274-fach höher im Vergleich zur nominalen Konzentration und konnten somit die bekannte Problematik in Bezug auf *in vitro* Dosis-Metriken aufweisen. Simulierte und experimentelle Daten zur totalen und freien Konzentration der Testsubstanzen im Kulturmedium stimmten für elf Substanzen überein, da die Übereinstimmung der Daten bei weniger als einem Faktor von 2 beträgt. Im Gegensatz dazu wurden um bis zu 4-fach höhere Konzentrationen in den Balb/c 3T3 Zellen vorhergesagt. Unstimmigkeiten könnten durch die fehlende Einbindung von Zielkompartimenten wie Zellorganellen, in denen Wirkmechanismen ausgelöst werden (z.B. Lysosomen als Zielorganell für Tamoxifen), metabolische Aktivierung, Transporter-vermittelte Aufnahme sowie den Ionisationsgrad der Moleküle im Model erklärt werden und könnten im nächsten Schritt um diese erweitert werden. Als erstes Screening Verfahren zur Beurteilung des Verhaltens einer Substanz in Diffusion-basierten Testsystemen kann das simple Model genutzt werden.

(3) Die Ansätze aus den vorherigen Arbeiten, die Untersuchung der Plasma-Proteinbindung und *in vitro* Dosimetrie wurde mit der reversen Dosimetrie kombiniert und auf *in vitro* Effekt-Konzentrationen im YES-/YAS- und Steroidogenese-Assay angewandt, um orale Dosen in Ratten im Hinblick auf endokrine Effekte zu bestimmen. Hierzu wurden Konzentrationen von sieben Testsubstanzen (APAP, BPA, CAF, FEN, FLU, GEN, KET) in Kulturmedien und Hefen und humanen H295R Adenokarzinom-Zellen des YES-/YAS- bzw. Steroidogenese-Assay mit validen analytischen Methoden quantifiziert. Diese dienen als sogenannte „*Point of Departure*“ für ein QIVIVE basierend auf physiologisch basierten toxikokinetisches Model (PBTK-Model) um eine externe, orale Dosis zu berechnen und die erzielten Daten mit publizierten, berechneten *in vivo* Dosen, die mittels nominaler Effektkonzentrationen aus den YES-/YAS- und Steroidogenese-Assay bestimmt wurden, verglichen.
Steigende Konzentrationen an Testsubstanzen wurden in den Zellen für 4/7 Substanzen (BPA, FEN, FLU, GEN) nachgewiesen, wobei bei diesen Substanzen bekannt ist, dass sie eine Affinität zum estrogenen/ androgenen Rezeptor haben bzw. in die Steroidogenese eingreifen. Eine Gleichgewichteinstellung zwischen den Kompartimenten Medium und Zellen wurde bei den

Negativkontrollen (APAP und CAF) beobachtet, die laut Literatur keine Effekte in den besagten *in vitro* Assays induzieren. Unter Anwendung der zellulären Konzentration wurden orale Dosen für 6/7 Substanzen innerhalb eines Faktors von 10 korrekt berechnet und lieferte somit im Vergleich zu den geschätzten LOEL basierend auf analytisch gemessenen Medium-Konzentrationen eine höhere Korrelation zu den *in vivo* Daten. Allerdings unterstrich die Studie die analytischen Schwierigkeiten, die bei der Bestimmung zellulärer Konzentrationen über mehrere Zeitpunkte (3, 6 und 12 h) zustande kommen. Demnach konnten keine Aussagen über die Kinetik der Testsubstanzen im YES-/-YAS Assay (APAP, BPA, CAF, FLU, GEN) über die Zeit getroffen werden. Nominale sowie totale Konzentration von Substanzen können als POD dienen, wenn keine Affinität zu zellulären Bestandteilen, eine geringe Proteinbindung (unter 10 %) und keine metabolische Aktivierung durch die Zelllinien. Künftige Studien könnten weitere endokrin wirksame Substanzen testen sowie die Anwendung der reversen Dosimetrie in Bezug auf andere toxikologische Endpunkte herangezogen werden.

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