Bench Marks!

Chemical Concentrations in Cell Culture Compartments (C5) – Free Concentrations

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Abstract

In biological systems (cell culture media, cells, body fluids), drugs/toxicants are usually not freely dissolved but partially bound to biomolecules; only a fraction of the chemical is free/unbound (f_u). To predict pharmacological effects and toxicity, it is important that the f_u of the drug is known. As the differences between free and nominal concentrations are determined by test system parameters (e.g., the protein and lipid content, and the type of surface material), comparison of nominal concentrations between two different new approach methods (NAM) may lead to faulty conclusions. The same problem exists when *in vitro* concentrations are compared to those in human subjects. Therefore, the respective f_u of a chemical in a test system needs to be determined for *in vitro*-to-*in vivo* extrapolations (IVIVE). Besides direct measurements, prediction models can help to obtain f_u. Here we describe a simplified approach to approximate f_u and provide background information on the underlying assumptions. Comparative predictions and measurements of f_u of various drugs are shown to exemplify the approach. Basic input data, like protein and lipid concentrations, are also provided. Beyond such test systems data, the only required chemical-specific inputs are the lipophilicity of the candidate drug and its ionization state, as determined by the dissociation constants of its acidic or basic groups. This overview is intended to be used by any lab scientist without specific toxicokinetics training to obtain an estimate of f_u in a given cell culture medium.

1 Concentrations then and now

Many textbooks on pharmacology and toxicology start with Paracelsus' 500-year-old wisdom that only the dose makes the poison. This insight introduced the concept of quantification of chemicals. Concentrations result from the normalization of a chemical's amount to a volume. They are the fundamental input measure for all laws of pharmacology and toxicology that deal with reversible macromolecular interactions. Notably, time is an important additional parameter for non-reversible reactions, e.g., tissue damage. The wisdom on the fundamental role of concentrations (or, in general, normalized amounts) is not immediately obvious in one of the fundamental axioms of toxicology: "Risk is a function of hazard and exposure". To most toxicologists, it is clear that hazard is a function of the concentration of the compound (or of its normalized dose). This implies that the level of hazard can be above or below a certain threshold value and that the concentration (or normalized dose) corresponding to this threshold value may be defined as the threshold concentration for the onset of a hazard. Exposure to a chemical (dose) leads to an internal exposure, which is the concentration reached in the various body compartments over time.

Especially in the field of *in vitro* toxicology, the concepts of hazard and exposure are not strictly separate as suggested by

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Paracelsus' rule; they rather "collapse upon one another" or can be seen as aspects of a comprehensive systems description. In *in vitro* tests, or so-called new approach methodologies (NAM), exposure is the concentration of the test compound, and hazard is the concentration-dependent function describing adverse effects (or biomarkers thereof; Blaauboer et al., 2012) in an *in vitro* system. Thus, concentrations and concentration-dependent effects are at the heart of *in vitro* toxicology. It is important to understand that concentrations are volume-normalized doses. This means that they are not doses (see Box S1[1] for definitions). In most (> 99%) cases, *in vitro* toxicology deals with concentration-response relationships, while dose-response considerations only rarely have a rational and sound scientific basis (Kisitu et al., 2019). For this reason, understanding chemical concentrations in cell culture compartments is essential.

The role of concentrations versus doses can be illustrated using the example that transferring a cytotoxicity test from a 6-well-format to a 384-well-format reduces the dose in each well about 16-fold while the concentration remains constant. Cells will usually react to the concentration of a test compound, not the dose. The few exceptions where the absolute amount of a chemical in a culture well plays a major role are mostly explained by irreversible reactions, like the covalent binding of a chemical to a cell target (mercury ions) or oxidative destruction of cell targets / chemical receptors (hydrogen peroxide). In these cases, the targets are eliminated and, therefore, the number of toxicant molecules relative to the number of receptors in the dish (drug/target molar ratio) plays a role.

2 Compartmentalized concentrations

A concentration, i.e., a certain number of molecules per unit volume, seems like a simple concept, but efforts required to understand it often have been underestimated. One reason for the complexity is the compartmentalization of concentrations in space, time and "microspace". What do these three aspects mean?

2.1 Local concentrations

The total concentration of a chemical is not the same within the different fluid volumes in a human (or an animal): concentrations in blood, interstitial fluid, intracellular aqueous space and extracellular fluids (bile, gastric juice, pancreatic secretions, primary urine, etc.) may differ. Moreover, they may differ between the intracellular spaces of the brain, the liver and muscles, or between mother and fetus. Even the free concentrations may differ: membrane-bound active transport proteins that move compounds against concentration gradients and non-aqueous spaces that dissolve chemicals, like lipid droplets and cell membranes, are among the many reasons for these differences. Moreover, aqueous spaces with large pH differences (lysosomes and mitochondria in cells or liquid spaces in the digestive and renal systems) affect the distribution of chemical species. As it is always the unbound local concentration close to a target that determines its pharmacological or toxicological effects, the understanding of local concentrations is essential background information for mechanistic toxicology and rational pharmacology. The complexities of spatially heterogeneous concentrations are in part also relevant to NAM, where concentrations may differ between cells and medium, and within different cell types. This is particularly relevant for organoids and microphysiological systems (Alépée et al., 2014; Gordon et al., 2015; Marx et al., 2016, 2020; Pamies et al., 2017, 2018; Groothuis et al., 2015; Kramer et al., 2015; Punt et al., 2020).

2.2 Time-dependent changes in concentrations

After oral dosing/exposure, the drug/toxicant concentration increases in plasma until a peak is reached (C_{max}). After this peak time (t_{max}), the concentration falls. In simple cases, this behavior may be described by the Bateman function (i.e., a combination of two exponential functions for absorption and elimination). However, the drug may distribute through different compartments and be eliminated from different compartments (e.g., liver and kidney), and this may result in more complex time courses. Thus, referring to an "*in vivo* concentration" is not trivial, and in most cases, there is not one single *in vivo* concentration.

For most *in vitro* systems, the situation is less complex, and often the effective concentration of a test compound may be constant over 24 h. The reason is that a compound will redistribute and rapidly achieve steady-state equilibrium in such relatively simple systems. If the system does not have elimination mechanisms, steady state can be held over long time periods. However, also in NAM, particularly the more advanced and complex models, there may be relevant xenobiotic metabolism (elimination) and transporter mediated distribution, resulting in a transport-time profile. In such cases, concentrations change with time (usually decreasing).

2.3 Concentrations at a macromolecular level

It is obvious that the distribution of concentrations between medium and cells can be complex. This article nonetheless focusses on the medium alone, and consideration of cell cultures is planned as a follow-up article. However, even within a given cell culture medium (without cells), the definition of concentration is not as simple as it may appear. The concentrations given in most publications and databases are usually not based on analytical data; they are rather the result of theoretical reasoning. The underlying assumption for this theoretical definition of concentrations is that the amount of compound added dissolves completely and homogeneously, and thus knowledge of amount (number of molecules) and volume allows the calculation of the *nominal concentration* (C_{nom}). However, the underlying assumptions may be wrong, as part of the chemical may evaporate, be absorbed into or adsorbed to the plastic, or be degraded (Fig. 1A). In the absence of the above three processes, the C_{nom} still may differ from the free concentration (concentration of unbound drug; C_u) due to binding/adsorption to dissolved lipid or protein in the cell culture medium. Thus, in each tiny volume unit (e.g., at the femtoliter (fL) level), some of

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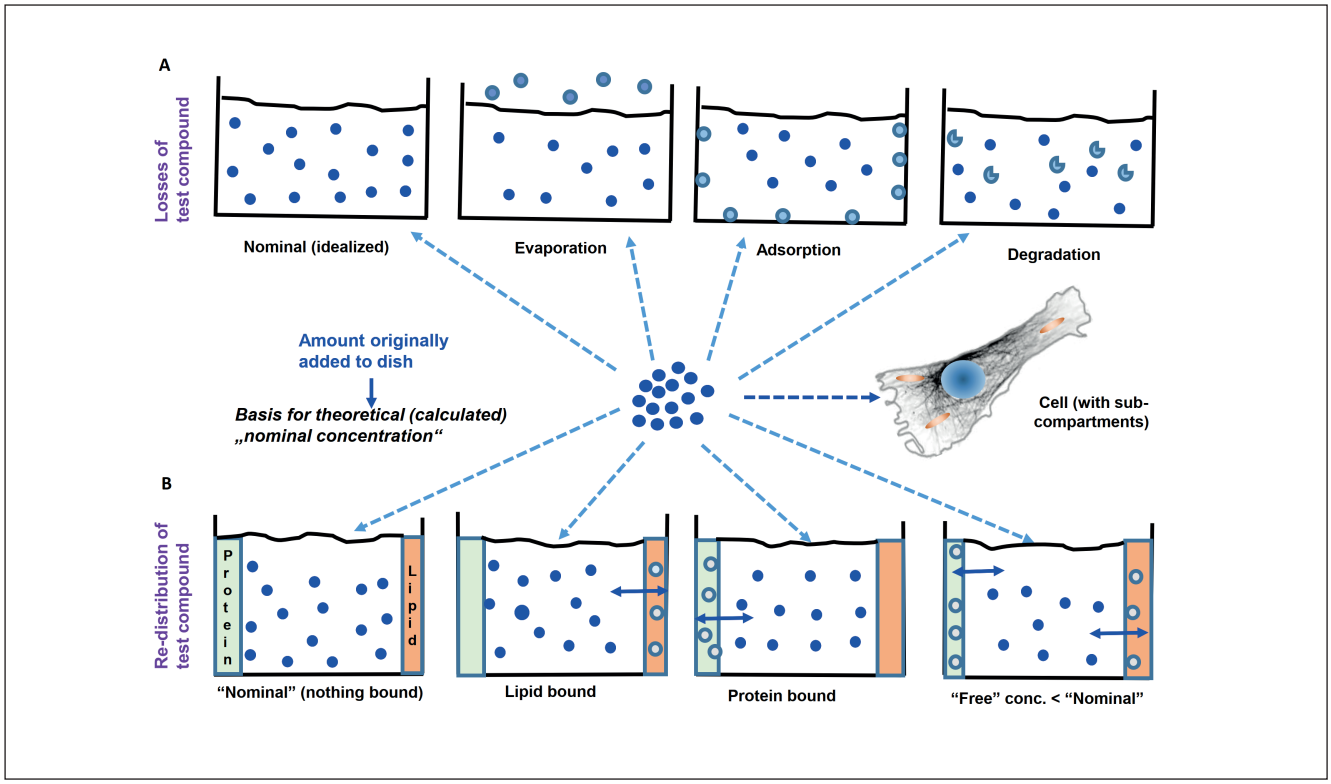

Fig. 1: Major processes affecting the fate of a test chemical in a cell culture dish

The processes include those associated with the loss of the compound (evaporation, adsorption and degradation), which substantially eliminate any interactions between the intact study chemical and the cells in the dish. The other processes (lipid and protein binding) arise from the equilibration and ultimate redistribution of the study chemical into the different phases within the medium. It is assumed that only the truly dissolved fraction of a chemical interacts with cells/targets. However, in a dynamic, real-life biological system, the interaction of a chemical with lipids and proteins may sometimes only slow (but not permanently prevent) the interactions with cells.

the chemical compound is truly dissolved and the rest is bound to biomolecules. The unbound fraction (f_u) determines the free concentration (C_u) of the chemical: $C_u = C_{nom} \times f_u$ (Fig. 1B). The fraction of the nominal chemical concentration (C_{nom}) that is bound is not readily available for target interaction or other phenomena that depend on the free concentration (e.g., for diffusion, osmosis, etc.). It should be noted, however, that the free concentration in cell culture media is not necessarily equal to the free intracellular or target site concentrations (Fisher et al., 2019; Doskey et al., 2015).

2.4 Comparison of concentrations and extrapolation to dose

Why is it so important to look into concentrations in much detail? There are several reasons: An important argument in biomedical research is that we draw many conclusions from the assumption that nominal concentrations are the “real” concentrations, although there are countless examples (Tab. 1) of drug and toxicant concentrations that are affected by all processes visualized in Figure 1.

For toxicology, one important argument is that it is necessary to compare and extrapolate concentrations between systems (Box S1[1]). The factors that modify the free concentration are different between the systems. To understand this issue, one may approach it through three levels of complexity:

i. In the simplest case, one may want to compare results between different NAM. In each system, there may be a different loss of test compound due to adsorption (and/or degradation/distribution). Besides, the systems may have different concentrations of albumin and lipids in their medium, so that the free concentration in the systems might strongly differ at a given (equal) nominal concentration (Krebs et al., 2020).
ii. The next level of complexity is the comparison of a NAM with human (or animal) plasma concentrations (or possibly other relevant compartments in a human). Each of these systems contains different amounts of lipid and protein/albumin, i.e., the biomolecules reducing the f_u of the chemical (Tab. 2). Thus, at similar nominal concentrations, the effective free concentrations may differ a lot. For several drugs, the f_u in plasma may be as low as 1-3% (Tab. 3), for some even lower (Umehara and Cemenisch, 2012; Wishart et al., 2006). *Vice versa*, the same free concentrations in plasma and a NAM may relate to very different total concentrations. A simple comparison of nominal (or total) concentrations in many cases therefore seems to lead to erroneous results. This does not take into account that the plasma

concentration often is not constant but undergoes great changes over time. In pharmacology, often the highest recorded plasma concentration following compound administration can be determined and used as the benchmark for subsequent comparisons. In toxicology, there is often limited knowledge on plasma concentrations, and tissue concentrations are known in far fewer than 1% of all cases.

iii. The third level of complexity refers to the approaches used for *in vitro*-to-*in vivo* extrapolation (IVIVE) or reverse dosimetry. In this process, the effective concentration from a NAM (e.g., the threshold of toxicity, also called the point of departure (PoD)) is translated to a dose or a certain *in vivo* exposure situation. This process is the *sine qua* non of the applicability of *in vitro* toxicology (Leist et al., 2014; Paini et al., 2019; Wam-

Tab. 1: Examples for deviations of nominal concentrations and "relevant/actual" concentrations

Reason	Compound	Comments / Literature
Adsorption to plastic or culture plates	Polychlorinated biphenyls (PCBs)	Up to 30% can stick to plastic [a]
	Paclitaxel, verapamil, digoxin	10-75% adsorbed onto conventional polystyrene, plastic microplates, polypropylene microplates or glass vials [b]
	Fluoranthene	60-70% bound to polystyrene plastic in the absence of FBS [c]
Accumulation in cells	Cyclosporine A, methylmercury	100-fold and higher accumulation [d,e]
Degradation/ activation (hydrolysis, photo-oxidation)	Cisplatin	7-fold decrease in cytotoxic potency on storage in culture medium at room temperature [f]
	Dacarbazine	*In vitro* activation by light and hepatic microsomes increases cytotoxic potential [g]
	Retinoic acid	Rapid degradation in serum-free medium or serum-containing medium maintained in the dark and manipulated under yellow light [h]
Degradation (autoxidation)	Dopamine	Autoxidation in culture medium to generate hydrogen peroxide and quinones/semi-quinones. Catalase and reduced glutathione offer protection from the cytotoxicity of degradation products [i]
	H_2O_2	Degraded by exposure to light or through interaction with transitional metal ions [j]
	Ascorbate	Unstable in common culture medium and, just like other polyphenolic compounds, it degrades to hydrogen peroxide in culture medium [k]
Evaporation	Methanol, formaldehyde	Many organic solvents [l,m]
	Phenanthrene	An 8-fold decrease in cytotoxic potency due to evaporation [n]
	N,N-dimethylaniline	6% evaporated from culture medium [o]
Adduct formation	Aldehydes	Aldehydes form adducts with DNA and proteins.[p] Reactive aldehydes are subject to Michael addition reactions with side chains of lysine, cysteine and histidine residues (protein carbonylation) [q]
	Mercurials	Covalent interaction of mercurial compounds with cysteine residues [r]
Deprotonation and protonation	Valproic acid [x]	With a pKa of 4.8, valproic acid will exist in the deprotonated form at physiological pH [s]
	Arginine	The guanidinium side chain of arginine is protonated even at physiological pH and pH values as high as 10 [t,u]
Hydration	Arsenic oxides	Arsenous acid, arsenite ions and arsenic acid have been reported in water to which arsenic oxides have been added [v]
	Metal oxide nanoparticles (ZnO, CuO, CoO, Mn_2O_3, Co_3O_4, Ni_2O_3, and Cr_2O_3)	Growth inhibitory effects of metal oxide nanoparticles in human and *E. coli* cells have been reported to increase as the hydration enthalpies of their oxides become less negative [w]

[a] Nyffeler et al., 2017; [b] Fukazawa et al., 2010; [c] Schirmer et al., 1997; [d] Zimmer et al., 2011; [e] Wilmes et al., 2012; [f] Schuldes et al., 1997; [g] Metelmanna and Von Hoff, 1983; [h] Sharow et al., 2012; [i] Clement et al., 2002; [j] Halliwell et al., 2000; [k] Halliwell, 2003; [l] Yin et al., 2001; [m] Gostner et al., 2016; [n] Halling-Serensen et al.,1996; [o] Zhang et al., 2006; [p] Wang and He, 2018; [q] Grimsrud et al., 2008; [r] Bläser et al., 1992; [s] Manallack, 2007; [t] Fitch et al., 2015; [u] Xu et al., 2017; [v] NRC, 1977; [w] Kaweeteerawat et al., 2015; [x] Compounds like VPA may change the medium pH and thus affect their own ionized fraction.

baugh et al., 2018). The steps are the following: The concentration of a PoD in a NAM needs to be translated to a plasma concentration (conversion based on the calculated free fractions). Then, the plasma concentration has to be converted to a dose leading to this concentration. One (simplified) approach to this is to use a drug distribution model (accounting for the different local concentrations in the body) to calculate an internal dose (total amount of drug in the body). Then, one may use a model of drug uptake (bioavailability) and elimination to translate the internal dose to the external dose (e.g., by oral intake) that would lead to the internal dose (Rostami-Hodjegan, 2012; Zhang et al., 2018; Bell et al., 2018; Jaroch et al., 2018; Brinkmann et al., 2017; Coecke et al., 2013). Finally, one may compare such an external dose to exposure scenarios, e.g., uptake

Tab. 2: Typical concentrations of albumin and lipid

Medium	Average serum albumin [mg/ml]	Albumin [μM]	Protein fraction [P_f x 1000]	Lipid / (average TG) [mg/ml]	Average cholesterol [mg/ml]	Average lipid fraction [L_f x 1000]
Human serum	40[a,b]	600[a]	30 (3%)	6.1[d,r]		6.0(0.6%)[a,z]
				1.5[d,e]		1.70(0.17%)[s]
	47.3[c]	710[c]	34 (3.4%)		1.85[d,f]	1.80 (0.18%)[w]
Calf serum (fetal)	23[g]	345	17 (1.7%)	2.3[h,i,l,r]		2.4 (0.24%)[z]
					0.35[g,i]	0.95 (0.095%)[s]
				0.87[i]		0.30 (0.03%)[w]
Rat serum	29[j]	440[j]	21 (2.1%)	3.1[q,r]		3.20 (0.32%)[z]
					1.01[n,o,p,q]	1.50 (0.15%)[s]
	31[j,k,l,m]	466	23 (2.3%)	1.45[n,o,p,q]		0.98 (0.98%)[w]
RPMI + 10% FCS	2.3	30	2 (0.2%)	0.3[t]	0.033[g]	0.30 (0.03%)[z]
				0.087[t]		0.095 (0.0095%)[s]
						0.0033(0.0003%)[w]
UKN4[v] (or UKN2) (Neuritox/cMINC assays)	0.39[w] (0.37[w])	5.8[x] (5.6[x])	0.28 (0.028%)	0.0029[r]		0.0032 (0.0003%)[z]
						0.0032 (0.0003%)[s]
UKN5[v] (PeriTox assay)	3.3[w]	50[x]	2.4 (0.24%)	0.025[r]		0.027 (0.0027%)[z]
						0.027 (0.0027%)[s]

TG, triglycerides; PSV, partial specific volume; FCS, fetal calf serum; L_f, lipid volume fraction; P_f, protein-albumin volume fraction.
Albumin and lipid values (mg/mL) were collected as average values for rats older than 3 weeks. For cases where total medium lipid was given, the volume fraction was calculated using PSV_{lipids} (the average value for all lipid forms combined). The lipid concentration in FCS (3 mg/mL) was assumed to be 50% of the human serum concentration (6 mg/mL), a similar ratio of the protein content (600 μM:300 μM). Medium containing 10% calf serum (FCS) is assumed to contain 2.3 mg/ml of albumin.
Human serum_albumin (35-50[a,b]), human serum_TG (0.7-2.6[e], 1.24[d]), human serum _cholesterol (1.8[f],1.89[d]).
Calf serum_cholesterol (0.3[g], 0.32-0.4[i]), calf serum_lipid (3[h,r], 1.6[i,r]), calf serum_TG (0.75-0.99[i]) (Calf-serum can be very variable: An alternative is to use serum-free cell culture (Gstraunthaler et al. 2003; van der Valk et al., 2018)).
Rat serum_albumin (29[j],32[k],26[l],36[m]), rat serum_TG (1.5[n,] ,2.0[o],1.5[p], 0.8[q]), rat serum_cholesterol (1.5-2.2[n], 0.9[o], 1.14[p], 0.2[q]).
PSV = PSV_{TG} (trioleate, 1.093 mL/g)[u], PSV_{choles} (0.968 mL/g)[u], $PSV_{choles.esters}$ (1.044 mL/g)[u], PSVphospholipids (0.97 mL/g)[u], PSV_{lipids} (average of all lipid PSV, 1.02 mL/g), $PSV_{albumin}$ (0.73 mL/g)[u]
P_f (protein-albumin volume fraction) =[P (mg/mL)]x PSV_{alb}/1000
L_f (Lipid volume fraction) = [L (mg/mL)] x $PSV_{TG/choles/lipid}$/1000
Cholesterol molecular weight = 386.654 g/mol
[a] Gülden et al., 2003; [b]Anderson and Anderson, 2002; [c] Fischer et al., 2017; [d] Patterson et al., 1988; [e] Pownall et al.,1999; [f] Phillips, 1960; [g] Lindl, 2002; [h] suggested value for calculations (scaled vs related protein count of human serum and FCS); [i] Gülden et al., 2002; [j] Schreiber et al., 1971; [k] Morgan and Peters, 1971; [l] Zaias et al., 2009; [m] Rose and Klemcke, 2015; [n] Ikeda et al., 1993; [o] Anderson et al., 1994; [p] Yaqoob et al., 1995; [q] Miura et al., 1989; [r] specified in literature/ source as total lipid in medium; [s] volume fraction of triglycerides, value derived from plausible assumptions including the consideration of TG molecular weights; [t] value extrapolated based on assumed composition of FCS, i.e., 10% of the value reported for FCS; [u] Redgrave and Calson, 1979; [v] Krebs et al., 2020; [w] volume fraction of cholesterol, total protein in medium; [x] assuming albumin molecular weight of 66500 g/mol: this value is used in calculating the albumin concentration in the medium (in μM) for column 3; [z] Volume fraction of total lipid.

Tab. 3: Examples for the prediction of free drug fractions (f_u) in human and rat plasma

Drug	State[a]	pKa_1[b]	pKa_2[b]	logPow[b]	f_u human (%)		f_u rate (%)[g]	
					Predicted[c]	*In vivo*[d]	Predicted[c]	*In vivo*[e]
Propranolol	basic	9.42		3.48	3	4-15	4	15
Quinidine	basic	8.56		2.88	12	12-20	15	30
Verapamil	basic	8.92		3.79	1.4	6-14	0.5	6
Cyclosporine A	neutral			2.92	10	7	13	6
Pravastatin	acidic	4.2		2.18	43	52-57	50	64
Valsartan	diprotic acid	4.35	5.86	1.50[f]	2.6	5	3.3	3
Digoxin	neutral			1.26	87	75	90	61
Furosemide	acid	4.25		2.03	52	2.3-4	58	13
Ciprofloxacin	ampholyte	6.09	8.74	1.55f	79	60-80	82	70

[a] State refers to the form that relates to the physiological charge or the major micro-species of the drug at physiological pH 7.4., e.g., a diprotic acid will have a physiological charge of -2 resulting from ionization of two acidic groups; [b] logPow and pKa values were obtained from PubChem; [c] the predicted free fraction of drug in human and rat plasma based on the biokinetics equation presented here (see supplementary information[1]); [d] taken from Drugbank (Wishart et al., 2006); refers to the free fraction of drug in human plasma; [e] taken from Umehara and Camenisch (2012); refers to the free fraction of drug in rat plasma; [f] logPow predicted using ChemAxon; [g] assumption is that binding is the same across albumin orthologues from rat to human to calf

of a potential toxicant via the daily diet. All large-scale NAM-based projects, e.g., SEURAT1, Tox21 and EU-ToxRisk (Gocht et al., 2015; Berggren et al., 2017; Wetmore et al., 2014; Sipes et al., 2017; Siméon et al., 2020; Escher et al., 2019; Krebs et al., 2020; Daneshian et al., 2016; Busquet and Hartung, 2017; Judson et al., 2014; Cote et al., 2016; Kavlock et al., 2018; Graepel et al., 2019) have incorporated an IVIVE procedure to convert free concentrations from NAM to a corresponding external dose. Very sophisticated software has been developed for such purposes. Software like the SimCyp PBPK simulator (Jamei et al., 2013) allow for multiple compartments and incorporate a large body of background knowledge on physiology, e.g., tissue volume, blood flow, enzyme/transporter expression. The httk R package has been developed in the R programming language (www.r-project.org/) as open source software for high throughput applications (Pearce et al., 2017).

3 Free concentrations

As outlined in the introduction, the understanding of free concentrations is an essential basis of mechanistic, predictive toxicology and an absolute requirement of systems toxicology models (Leist et al., 2012, 2014). This does not apply only to *in vitro* toxicology. The interpretation and extrapolation of animal data also requires an understanding of the underlying concentrations. Unfortunately, the measurement of free concentrations is a difficult task for researchers who do not do this routinely. It requires a lot of resources, specialized equipment and experience, and it is also linked to large uncertainties. Some measurement systems are available for *in vitro* systems. They function well for some matrices, but only limited experimental data are available from rapid equilibrium dialysis (Ferguson et al., 2019; Buscher et al., 2014) or miniaturized solid-phase extraction (SPE) (Kramer et al., 2012; Neale et al., 2018). Alternatively, affinity chromatography has been applied (Groothuis et al., 2019). As an indirect approach, it is also possible to measure an activity that depends on the free concentration (e.g., receptor activation, enzyme inhibition or cell death induction) and then to extrapolate free concentrations from nominal concentrations in the same assay (Gülden et al., 2003, 2005).

An alternative approach is the prediction of free concentrations or the respective free fraction of a chemical using a mathematical model. This has required a lot of background thought, experimental verifications, and fine adjustments, as described in the extensive background literature (Gülden et al., 2005; Armitage et al., 2014; Kramer et al., 2012, 2015; Fisher et al., 2019). The overall outcome is an equation (see Eq. 1 and Eq. S12 + S13[1]) that delivers useful predictions (Tab. 3, 4). Working with this equation is a relatively simple procedure accessible to any experimental scientist with very basic school mathematics training. In the context of the work of Fisher et al. (2019), it also has been implemented in the Simcyp In vitro Analysis (SIVA) toolkit (Cetera UK Ltd, Sheffield, UK). As the issue of concentrations is of utmost importance to the field, it should not be handled only by a small group of specialists. We rather suggest here that the basic knowledge and its application should be routine for all those involved in experimental planning and data evaluation. For this reason, the approach has been detailed below for non-specialists. Moreover, potential problems and pitfalls are highlighted at the end of this article.

4 Predicting the free compound fraction (f_u) *in vitro*

The free concentration (C_f), also called the unbound concentration ($C_u = C_f$), can be derived from the nominal concentration (C_{nom}) by multiplying C_{nom} by the fraction unbound (f_u), i.e., the

Tab. 4: Comparing predicted to measured free fractions (f_u) *in vitro*

Compound	Measured f_u	Predicted f_u
Isoniazid	100.0 [b]	99.9 [c]
Amphetamine	90.3 [b]	95.0 [c]
Amitriptyline	34.0 [b]	1.5 [c]
Diazepam	41.0 [b]	45.0 [c]
Tramadol	87.3 [b]	92.0 [c]
β-Estradiol	15.7 [b]	14.5 [c]
Testosterone	35.2 [b]	31.0 [c]
Phenanthrene	1.5 [b]	2.5 [c]
Bisphenol A	25.0 [b]	31.0 [c]
Pyrene	0.2 [b]	1.4 [c]
BAC6	80.0 [b]	99.9 [c]
BAC8	47.0 [b]	99.3 [c]
BAC10	30.4 [b]	93.0 [c]
BAC12	40.1 [b]	54.0 [c]
BAC14	12.4 [b]	9.3 [c]
BAC16	7.6 [b]	1.4 [c]
BAC18	4.6 [b]	0.6 [c]
Tributyltin chloride	0.6 [a]	1.0 [d]
2,3-Dinitrotoluene	0.9 [a]	1.0 [d]
4-Nonylphenol	0.1 [a]	0.01 [d]
p,p'-DDT	< 0.1 [a]	< 0.1 [d]
Dieldrin	0.3 [a]	0.2 [d]
p,p'-DDE	< 0.1 [a]	< 0.1 [d]
2,4,5-Trichlorophenol	0.3 [a]	0.4 [d]
Paraquat	1.0 [a]	1.0 [d]
4-Chlorophenol	0.9 [a]	0.9 [d]
2,4-Dichlorophenol	0.8 [a]	0.7 [d]
Phenol	1.0 [a]	1.0 [d]
Nicotine	1.0 [a]	1.0 [d]
Isopropyl alcohol	1.0 [a]	1.0 [d]
Ethanol	1.0 [a]	1.0 [d]
Methanol	1.0 [a]	1.0 [d]
Ethylene glycol	1.0 [a]	1.0 [d]

[a] Gülden and Seibert, 2005; Gülden et al., 2003; [b] Groothuis et al., 2019; measured at 60 μM albumin, using affinity chromatography; [c] predicted free fraction of compound using Eq. 1 with an *in vitro* system with 60 μM albumin, but without lipids; [d] predicted free fraction of compound using Eq. 1 with an *in vitro* system with 5% FCS (18 μM or 1.2 mg/mL albumin and 0.3 mg/mL lipids); BAC, benzalkonium derivatives

fraction of Cnom that is not bound to lipid or protein. This has been derived in earlier excellent publications (Gülden et al., 2002, 2003; Kramer et al., 2012, 2015; Armitage et al., 2014; Fischer et al., 2018; Fisher et al., 2019).

The equation for the prediction of the free compound fraction (f_u) that is used and explained here is given below:

$$f_u = \frac{1}{1+K_{alb} \times f_{alb} + \frac{D_{vow} \times f_{NL}}{1+Y}}$$ (Eq.1, corresponding to Eq. S13[1])

The terms in this equation are as follows: K_{alb}, albumin-water partition coefficient of a compound; f_{alb}, the volume fraction of albumin in the culture medium; f_{NL}, the volume fraction of neutral lipids (NL), also called TG, in the culture (TG are assumed to be representative of all neutral lipids); D_{vow}, the olive oil-water partition coefficient of the non-ionized form of the compound (see supplementary material[1] for the proper calculation and use of this term); Y, the ratio of the concentration of the ionized to the non-ionized drug/compound species in the test system.

The equation looks difficult to apply, as the five terms determining f_u are not readily known. However, following the instructions we provide here (and explained in the supplementary material[1] for those interested in more background), all this information can be derived from readily available material. The only knowledge required is the constitution of the test system and two physicochemical determinants of the drug.

The test system parameters that must be known are the lipid and the protein content of the medium. This should be part of any test method description, as defined, e.g., by the ToxTemp (Krebs et al., 2019). The required chemical descriptors are the protonation/dissociation constant (pK_a) and the lipophilicity descriptor logPow. The latter is the decadic logarithm of the octanol-water partition coefficient, Kow. A high logPow (> 2) indicates hydrophobic drugs (cyclosporine ~ 3, hexamethylbenzene ~ 4.5); a low or negative logPow indicates hydrophilic drugs (e.g., mannitol ~ -3, ascorbic acid ~ -1.5, caffeine ~ 0, doxorubicin ~ 1.3). Such physiological properties can be obtained from databases such as PubChem, Drugbank, EpiSuite or DDBST[2]. These databases also indicate whether the data are experimental or based on computational models.

5 An example of the use of the biokinetics equation

The use of the equation is best illustrated by a practical example. We chose valproic acid (VPA) and amphetamine as drugs, and UKN5 as test method (see details in Krebs et al., 2020). The test system does not use serum supplements, but the medium still contains protein and lipid (Box S2[1]).

From this, we can already determine two of the missing values: f_{alb} and f_{NL}. f_{alb} is the volume fraction of albumin, i.e., the part of the medium volume covered by albumin. F_{NL} is the fraction of the medium taken by neutral lipids. These two fractions are considered here, as proteins and lipids are the dominant drug-binding biomolecules present in cell cultures.

[2] http://www.ddbst.com/prp-estimate.html

The third term, K_{alb}, refers to the distribution of compound between albumin and the aqueous phase, i.e., the albumin-water partition coefficient. There are several experimental methods to determine such equilibrium binding constants (Groothuis et al., 2019). The disadvantage is that an analytical method needs to be available for each drug that is considered. Moreover, methods of phase separation (bound versus unbound drug) are difficult for low-affinity binding processes. Additionally, the definition of "albumin" is not trivial, as it may refer to human or rat albumin, mixed serum proteins or other protein fractions. In view of these problems, it seems justified to use a modelling approach instead of experimental measurements. One frequently used model (Endo and Goss, 2011) assumes that albumin binding can be predicted from logPow. The equation(s) for the conversion of logPow to K_{alb} are given in the supplementary file[1]. Example values for two compounds for the step-by-step derived terms are demonstrated in Box S2[1].

The fourth term, D_{vow}, can be addressed relatively simply. The olive oil – water distribution coefficient is used in the equation, as this partitioning better reflects binding to neutral lipids than the logPow. Conversion equations and example use are worked out in the supplementary material (see Eq. S11[1], Box S2[1]).

The fifth term to be addressed is Y. In some simplifications of the equation, this is missing altogether (Y = 0). If the drug was not VPA (2-propyl-pentanoic acid), but rather 2-propyl-pentanol or trichloroethylene, then Y would be negligible (set to zero). So, why is this correction term required for some drugs? Y takes account of the fact that some fraction of VPA is in the acid form and the other is in the carboxylate (negatively charged) form, and that the ionized and the non-ionized forms bind to lipid in different ways. Here, the simplifying assumption is that only the neutral form binds to neutral lipids, and Y indicates the ratio of the ionized to the non-ionized drug at the given pH (7.4). Notably, the acid form of VPA is non-charged (neutral), while the base form (carboxylate) is negatively charged.

Using the test system information for UKN5 and fitting the five terms (Box S2[1]) into Equation S13[1], we obtain an f_u prediction for VPA of 0.69, i.e., about 70% would be free (available for uptake or target interactions) and 30% would be bound to protein and lipid in the medium. For amphetamine, the f_u prediction is 0.96, i.e., only 4% of the drug would be bound.

6 Protein binding

Culture medium may contain lipids, proteins, sugars, inorganic ions, hormones, vitamins and heparin to mention but a few. Most models of free concentrations consider only the interaction of the test substance with medium lipids and proteins. This is largely justified by the fact that these two classes of biomolecules constitute the largest fraction of potential extracellular binding partners in the test system. In special cases, it needs to be considered that other medium components, like complex carbohydrates, polynucleotides, detergents or artificial substances added to confer certain test system properties (e.g., coating material) may also bind test chemicals. All considerations for cell culture media apply to test media in general, i.e., also buffers for pharmacological test systems or biochemical assays. For standard medium, and for the majority of test compounds, protein takes the most dominant role in adsorption.

Tab. 5: Dependence of the fraction unbound (f_u) *in vitro* on the albumin concentration

Compound	f_u [a]	
	18 μM albumin	600 μM albumin
Hexachlorophene	< 0.01	0.01
Mercuric chloride	0.80	0.10
Thioridazine HCl	0.77	0.09
Potassium cyanide	0.93	0.28
Pentachlorophenol	0.05	< 0.01
Amitriptyline HCl	0.88	0.18
Malathion	0.88	0.23
Lindane	0.54	0.13
Dextropropoxyphene HCl	0.97	0.49
Warfarin	0.90	0.21
2,4-Dichlorophenoxy acetic acid	0.90	0.24
Acetylsalicylic acid	0.95	0.39

[a] free fraction estimated through *in vitro* experimentation by Gülden and Seibert (2005) and Gülden et al. (2003) in the presence of either 18 μM or 600 μM albumin

In human serum, albumin is by far the major protein fraction (50 g/L, Anderson and Anderson, 2002; Zhang et al., 2012), but immunoglobulins are another large fraction. Fibrinogen is an important protein of blood plasma as is, e.g., α1-acid glycoprotein, which binds a number of drugs (e.g., propranolol) (Hill et al., 1989). Some proteins in serum are specialized to bind endogenous hormones (Bartalena, 2004; Hammond, 1995; Bartalena and Piantanida, 2019) or vitamins (Vahlquist et al., 1979), and they may therefore also bind test chemicals that are structurally related to endogenous compounds, e.g., thyroxin or retinol. A further consideration for very detailed studies is that the serum protein composition may change, e.g., by alterations in acute phase proteins or lipoprotein fractions. For example, human, calf and rat serum contain different fractions of proteins and lipids, and even orthologous structures are not identical (see Tab. 2). The need to consider the heterogeneity of constituents applies even more to compositions of artificial cell culture media. They may contain proteins from different species, partial protein lysates or, e.g., transferrin – either fully bound to iron or emptied of iron (holo-transferrin).

To assume that all protein behaves like albumin or that albumin is always the dominant component in all media is thus a rough approximation. However, it often can be clearly shown that albumin content plays a major role in determining the free drug fraction (Tab. 5). In this context, it is important to highlight again that serum has a much higher albumin content than any cell culture medium, and this needs to be taken into account for IVIVE. It is also a good occasion to remind the reader of the biokinetics equation

Tab. 6: Free plasma fractions (in %) for different systems

Compound	logPow	EC50 (μM)[b]	State[a]	f_u		
				Measured *in vitro*[b]	Predicted[c]	Human plasma[d]
Amitriptyline HCl	4.81	226	basic	18	0.16	5
Acetylsalicylic acid	1.18	4630	acid	39	90	> 98
Dextropropoxyphene HCl	4.18	332	basic	49	0.50[e]	24[f]

[a] State refers to the form of drug or the major micro-species of the drug at physiological pH 7.4., e.g., a diprotic acid will have a physiological charge of -2 resulting from ionization of two acidic groups. It would be labelled a di-acid/diprotic acid. A drug like acetylsalicylic acid (monoprotic acid) would have a major species with a charge of -1. It is therefore labelled as an acid; [b] free fraction and EC50 values estimated through *in vitro* experimentation (using cytotoxicity as endpoint) by Gülden and Seibert, (2005) and Gülden et al. (2003) in the presence of 600 μM albumin. Notably the concentrations considered here differ from the known human plasma concentrations (therapeutic C_{max} for these compounds are in the range of 0.18-1.1 μM (0.05-0.3 μg/mL), 111-1110 μM (20-200 μg/mL), 0.15-0.9 μM (0.05-0.3 μg/mL) for amitriptyline, acetylsalicylic acid and dextropropoxyphene, respectively); [c] predicted free fraction using Equation S12[1] and assuming an albumin concentration of 600 μM and lipid of 0.3 mg/ml (much lower than in plasma); [d] free fraction of drug in human plasma (Wishart et al., 2006); amitriptyline binds to α1-acid glycoprotein. If the *in vitro* system accounts only for binding to albumin, then transition to *in vivo* is demanding and may in many cases be done wrongly. This could be corrected in more complex models and with the respective background knowledge; [e] possibly a saturation effect occurred *in vitro* and the prediction model assumed linearity (not given) and binding to albumin only (not true); [f] Giacomini et al. (1978)

discussed above and that it assumes similar protein binding for all forms of a drug (ionized and non-ionized). This assumption may not always be fulfilled. To exemplify this, we have assembled a few examples where the predictions were poor (Tab. 6). In all these cases, the explanations are not trivial.

Here, we have discussed mainly protein binding, as this is in most cases the dominant component of the biokinetics equation. Over this, it should not be forgotten that there are special media or test situations (e.g., considering milk as test medium), in which lipids may take a large role. In this context, hybrid structures of lipids and proteins, like chylomicrons or lipoproteins, are of importance. Some well-known drugs/toxicants such as cyclosporine A and polychlorinated biphenyls (PCBs) are reported to bind and interact with lipoproteins (Wishart et al., 2006; Brown and Lawton, 1984). Here we remind the reader that only the neutral form of a chemical is considered to bind to TG. This reduces the number of test substance species to account for (Caron et al., 2007). In more elaborate models, one may also consider that the ionized fraction is likely to interact with polar lipids.

7 Typical assumptions in *in vitro* biokinetics

The famous statistician G. Box said, "*All models are wrong, some are useful*". This applies also to the biokinetics equation predicting free concentrations. So, when is this model useful? It is when we do not have anything better available and when we understand that the basic assumptions underlying the model are met. Thus, we need to recapitulate briefly some of these basic assumptions (see detailed application in the supporting information[1])

One common assumption of steady state models, as described here, is that a distribution equilibrium between the *in vitro* system phases is reached and that this process is not saturable. The fraction of albumin binding sites occupied by the toxicant is considered negligible compared to the total protein concentration, and the toxicant is assumed to bind to one or more sites with the same binding affinity (Seibert et al., 2002). This also implies that the models do not account for time-dependent processes (N.B.: Dynamic models that take time into account have also been developed). This may be true especially when interactions with cells are considered. It is also important to note that the model assumes that the concentrations achieved at equilibrium are constant throughout the entire period of toxicant exposure (Gülden et al., 2001). These steady state assumptions are not fulfilled in the case of irreversible interactions. They also may not be fulfilled for some competitive, non-covalent interactions when this interaction is coupled to a downstream reaction that proceeds at a slow timescale. Examples are when the unbound drug is transported (e.g., into a cell), is catalytically removed, or binds to a high-affinity partner in another compartment. This would change the total concentration of the compound in the medium over time. Similarly, the free fraction may change when the amount of albumin or lipid changes (e.g., by cellular uptake). In such cases, other concentration measures and more complex (dynamic) models may be more appropriate.

The logPow is considered a major predictor of adsorption to protein and/or lipid. As a rule of thumb, compounds with a logPow > 4 are fully adsorbed (f_u < 5%), and chemicals with logPow < 0 are considered to be mostly free (f_u > 95%). The strongest changes occur in the range of logPow = 2 (Fig. 2B and Tab. S2[1]). In practice, this relationship is not perfect (Fig. 2A), as, e.g., some compounds may bind specifically to certain proteins (different from albumin). A smaller group of compounds shows a significant free fraction although they are very hydrophobic.

Another assumption made here is that there are no major losses of chemical due to plastic adsorption or evaporation. However, these may occur (Fig. 1A), and they may be accounted for in more complex models (Kramer, 2010; Fisher et al., 2019). An alternative is to define conditions under which the simplest model (presented here) would still be applicable. For example, thresholding

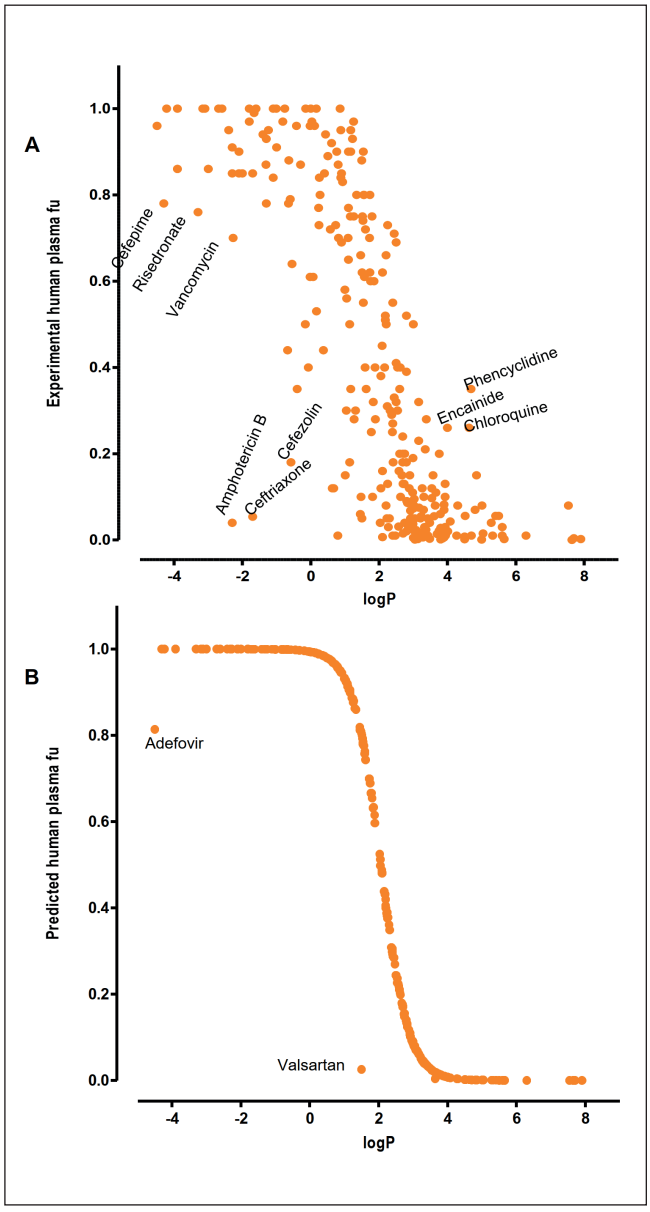

Fig. 2: Measured versus predicted free fractions of drugs
(A) The measured human plasma free fractions of 285 drugs and chemical substances are plotted against the compounds' logPow values. The measured human plasma free fractions are taken from the publications and databases of Varma et al. (2010); Wishart et al. (2006); Houzé et al. (1990), and the US-EPA chemicals dashboard (https://comptox.epa.gov/dashboard/) (see Tab. S2[1]). The measured values represent clinical data, patient data, and box labels of regulators/manufacturers. (B) Variation in the predicted (using Eq. 1, assuming pH 7.4) f_u with a changing logPow. The logPow values were obtained from the EpiSuite database or otherwise predicted using ChemAxon software version 17.1.23.0. In these plots, a few compounds of interest depicting an outlying trend are selected and labelled. In (A), cefepime, risedronate, vancomycin, amphotericin B, ceftriaxone, cefazolin, encainide, chloroquine and phencyclidine are pointed out, while adefovir and valsartan are singled out in (B) (see Tab. S2[1] for more details).

can be done by comparing the logPow to other equilibrium constants such as the air-water partition coefficient (K_{AW}) to determine whether evaporation from culture medium is a significant factor in reducing the nominal amounts of the toxicant under consideration (Fischer et al., 2017). For instance, colchicine has a logPow of 1.3 and a logK_{AW} of -15 (Henry constant of 1.82 x10^{-12} Pa·m^3/mol). It can therefore be considered to undergo no significant evaporation from the culture system.

For most compounds, the water-polymer (cell culture plastic) binding constant is much lower than the corresponding lipid (logPow) and protein binding constants (Armitage et al., 2014), and for most compounds with good solubility in cell cultures, plastic binding plays a minor role. However, important exceptions need to be considered. If there is clear evidence of plastic binding, e.g., experimental data that 30% of total PCB concentration is bound (Nyffeler et al., 2017), then this amount may be subtracted from the total concentration.

Other assumptions include that compound degradation or transformation in any of the *in vitro* system phases is negligible, which may not be true as reported for some compounds (Tab. 1). An example for this consideration is demonstrated in Fisher et al. (2019).

8 Acid-base equilibria and their effect on chemical charges

Many drugs are either weak acids or bases. When dissolved in media of about neutral pH, they are partially dissociated. The protonated form of bases is positively charged (cationic form), while the deprotonated form of acids is negatively charged (anionic form). Of the many proteins present in human plasma, only three account for the major part of drug binding (Zhang et al., 2012). Albumin mainly carries anionic drugs, some cations and neutral drugs, α1-acid glycoprotein carries cationic and neutral drugs, while lipoproteins carry cationic and neutral drugs. An acid is a species that will dissociate into the anionic form and a proton at a pH above its pK_a, while a basic species will accept a proton at a pH below its pK_a value to generate the cationic species (Manallack et al., 2014). Some compounds may be at the same time acids and bases (e.g., amino acids), i.e., at their isoelectric point, they have no charge (neutral), even if the molecule contains proton donor and acceptor groups. Therefore, the presence of ionizable groups and what is defined as physiological pH will drive the behavior of a compound under experimental conditions. Notably, many groups different from carboxylic groups and primary amines also have acid/base characteristics. Common structural groups associated with acidity include sulfonamides, hydroxamates, phenols, some phosphates, sulfates, tetrazoles, hydrazides, imides, thiols, carbamates and alcohols; basic groups comprise aliphatic amines, guanidines and amidines. Other groups such as heterocyclic nitrogen atoms, anilines and basic amides can be acidic or basic (Mallack et al., 2014; Martin, 2005; Gleeson, 2008). Some compounds such as acids with pK_a values around zero and bases with pK_a values below 12 are always ionized. One such example is the guanidinium side chain of arginine (Fitch et al., 2015; Xu et al., 2017).

In complex cases, the prediction of the ionization (protonation) state of a substance can be performed using cheminformatics soft-

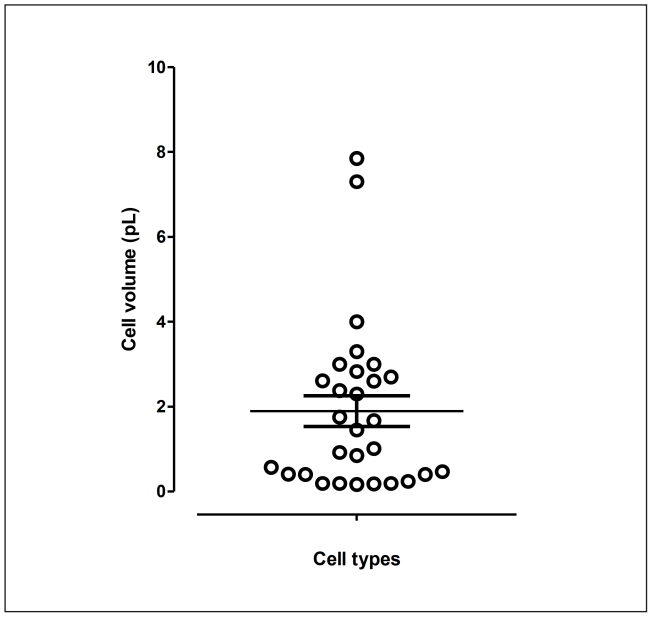

Fig. 3: Overview of typical cell volumes
The volumes of cells are shown (for details also see Tab. S1[1]). Each dot represents a literature value for a cell volume or a cell volume calculated from the cell diameter referenced in the literature. The average cell volume is 1.9 picolitres (pL: 10^{-12} L = 10^{-6} μL), based on the data presented here. This corresponds to a diameter of 15 μm for spherical cells and may be taken as default value in the absence of other data.

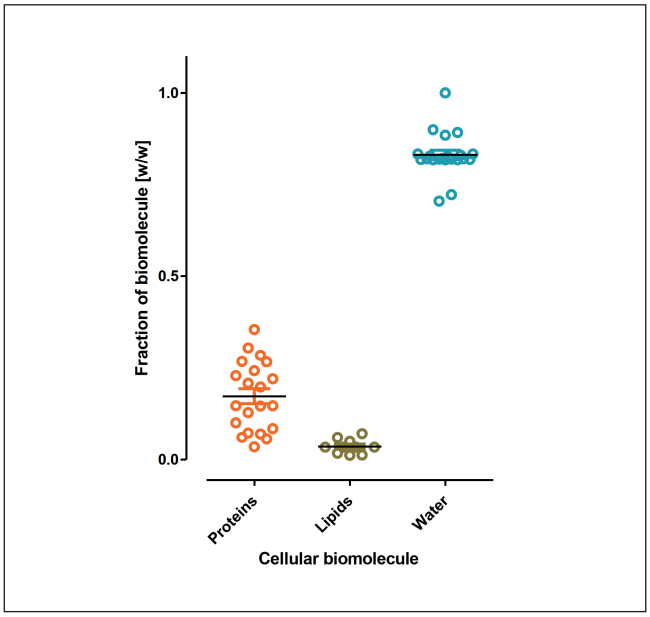

Fig. 4: Overview of typical cell compositions
The plot shows the relative contribution of proteins, lipids and water to cell weight. Each dot represents a value reported in the literature. The average fractions for cell proteins, lipids and water relative to total cell weight are 0.16, 0.03 and 0.82 respectively. (about 0.23 mg lipids per mg protein). These values may be taken as default value in the absence of other data.

ware such as ChemAxon. These take the different pK_a of all ionizable groups into consideration and determine the major form (microspecies) at the defined physiological pH.

9 The contribution of cells

All the above considerations apply to homogeneous medium. They may be directly applied to, e.g., enzymatic assays like the acetylcholinesterase assay (provided the test compound is not metabolized itself). However, in many cases, the medium will be on top of cells, and a major question is whether the basic assumptions are still fulfilled then. We will not deal with predictions of cellular uptake here, but only focus on the medium. Some thoughts are useful to decide whether the above considerations are still valid in medium on top of cells. More details on medium-cell equilibria of test compounds can be found in published reports (Doskey et al., 2015; Fisher et al., 2019), and explanations are being prepared for the next article in this series.

One initial key question is how much the cells contribute to the overall test system (volume). For this, we need to know the volume of a cell, which is on average 1.9 picolitres, i.e., 1.9 µL per one million cells (Fig. 3, Tab. S1[1]). If we consider a well in a 96-well plate, it may contain up to 50,000 cells. These cells together have a volume of 0.1 µL. Compared to the typical medium volume of 100 µL, the cell volume is 0.1%. This suggests that the total cell volume is negligible compared to the volume of the medium.

However, one may go one step further and ask what the contribution of the cells is to culture proteins and lipids. Cells mainly consist of water, but about 20% (15-35%) of their volume is protein (Brown, 1991). On a weight basis, proteins contribute 15-25% (Srivastava and Bernhard, 1986) of the total cell weight (see Fig. 4). This means that 50,000 average-size cells contain about 16 µg protein and 3-5 µg lipid (Fig. 4, Tab. S1[1]). If cells are kept in serum-containing medium (Tab. 2), the cell protein is a small fraction (< 10%) of the total protein in a culture well, and it may therefore be neglected for the calculation of the free drug fraction. This is even more true, if one considers that some of the cell lipid and protein may not be accessible to chemicals.

However, the situation may be different if the medium contains hardly any protein but the cells contain a large fraction of highly hydrophobic, test compound-binding proteins. An important potential pitfall is that some cell proteins may bind a test chemical with high affinity (e.g., microtubules bind taxol or colchicine; glutamate receptors bind domoic acid, etc.). Other phenomena not discussed here, like the membrane potential, could also affect the uptake of compounds from the medium into cells and thus affect the free concentration (Fisher et al., 2019). This is, for instance, known for the toxicant MPP^+ or mitochondrial probes like ethidium bromide (Schildknecht et al., 2015). Also, ion trapping due to pH gradients across cell membranes can lead to large chemical shifts from the medium to the cell. This is used, e.g., to target molecules like chloroquine to acidic cell compartments or to load cells with calcium indicators or viability stains like calcein. In the absence of such ef-

fects, one simplified default approach is to assume free accessibility of cell constituents and to add the amount of cell protein/lipid to the amount determined to be present in the medium.

10 Special cases and novel solutions

All considerations of this article refer mainly to small molecules that are reasonably water-soluble and for which exposure occurs from the liquid phase to cells submerged in medium. Many other situations are possible and are of toxicological and pharmacological relevance. For instance, test compounds such as colloids or aggregates and larger particles (nanoparticles, microplastics, fine dust particles) need special consideration and particular methods of dose/concentration normalization (Lee et al., 2009; Hussain et al., 2005; Espinosa et al., 2018; Heinrich and Braunbeck, 2019; Jeong and Choi, 2019). Another major field is exposure to airborne particles and aerosols, which is often performed with cells cultured at the air-liquid interphase (Upadhyay and Palmberg, 2018; Ji et al., 2019; Thimraj et al., 2019). Here, the dose metrics and standardizations also are a specialty area.

Finally, a large fraction of the small-molecule chemical universe is not water-soluble (low solubility in cell culture media). Such compounds will eventually need to be assessed in NAM, and approaches need to be worked out and standardized. One particular problem of hydrophobic compounds is that they may accumulate in the biological test system (e.g., cells or zebrafish larvae) (Zimmer et al., 2011; Wilmes et al., 2012; Armitage et al., 2017; Bittner et al., 2019; Siméon et al., 2020), and, therefore, concentrations in the medium may change greatly. One emerging approach to this is "passive dosing" (Smith et al., 2010; Butler et al., 2013; Seiler et al., 2014). The underlying principle is that a hydrophobic solid, e.g., polydimethylsiloxane (PDMS), or liquid (e.g., silicone oil) material is loaded with the test compound (e.g., polycyclic aromatic hydrocarbons (Seiler et al., 2014)). A pre-condition is that this matrix can store a large amount of test compound (compared to the amount present in the aqueous medium). Incubations are then carried out in such a way that the culture medium is in continuous contact with the hydrophobic matrix and the test compound can continuously leach out to the test medium and is then continuously present at its aqueous solubility limit (also if it is taken up by the test system). In summary, passive dosing ensures a constant exposure level corresponding to the solubility limit of the test compound in a cell culture medium. In this case, the solubility limit refers to the free fraction. If the medium contains lipid and protein, these may also bind test chemical so that the total concentration is much higher than the free concentration. Passive dosing in medium containing protein/lipid can be very complex and needs more exploration. The main use up to now has been in ecotoxicology testing using water as the medium.

11 Conclusions and outlook

As discussed in the last chapter, the presence of cells can make biokinetic considerations very complex, especially if predictions are made not only for chemical concentrations in the cell culture medium but for cellular compartments. This will be dealt with in follow-up articles. Approaches will be presented to predict the average cellular concentration of chemicals, and the potential subcellular, heterogeneous distribution will be discussed. In such follow-up considerations, metabolism is initially assumed to be absent. Moreover, the cell number, cell surface area and properties are considered constant (Armitage et al., 2014; Fischer et al., 2017; Fisher et al., 2019), and the partitioning of toxicant is assumed to be similar in both living and dying cells. Predictions will become more challenging for compounds that are not easily permeable through the cell membrane in their ionized form but permeable in their neutral form (Fisher et al., 2019). Even more complex situations may arise when active transport is to be considered. Further sophistication steps are considerations of cell growth during the exposure time and metabolism of the test compounds. All these require a move away from steady-state assumptions.

This brief outlook serves to make clear that simplified predictions of free concentrations in medium can be extremely helpful in many cases, but there will be experimental conditions under which intracellular concentrations can be largely different from medium concentrations (Zimmer et al., 2011; Wilmes et al., 2012). This is not just an issue for *in vitro* systems, but also for *in vivo* studies. The question of local concentrations, as compared to plasma concentrations, is highly complex but also very important for understanding drug/toxicant effects.

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