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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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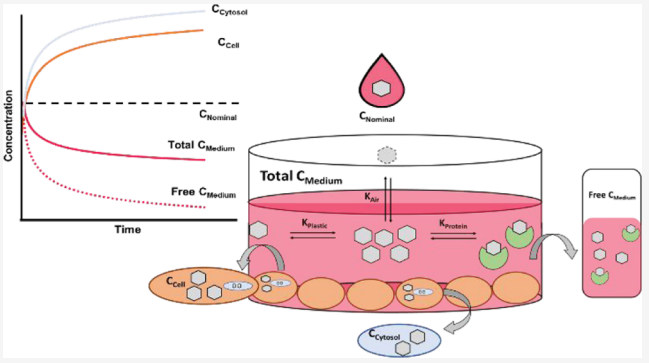
ABSTRACT: Nominal concentrations (C_{Nom}) in cell culture media are routinely used to define concentration−effect relationships in the *in vitro* toxicology. The actual concentration in the medium (C_{Medium}) can be affected by adsorption processes, evaporation, or degradation of chemicals. Therefore, 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after incubation with Balb/c 3T3 cells for up to 48 h. Measured values were compared to predictions using an as yet unpublished *in silico* mass balance model that combined relevant equations from similar models published by others.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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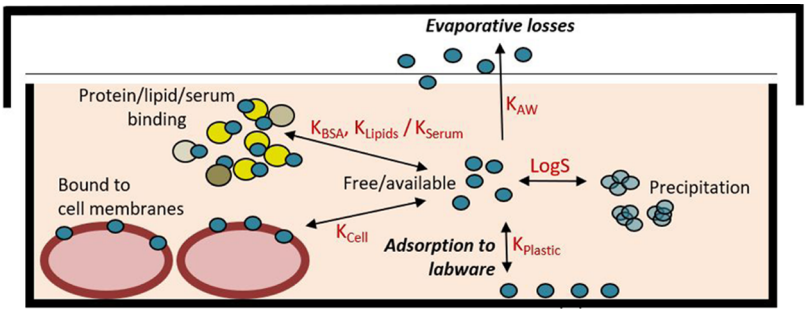

Figure 1. Partitioning within the test system used to describe the mass balance model. Schematic representation of an *in vitro* system and an example cell type, including the processes influencing the concentration of a substance and partitioning within the test system (Adapted with permission from 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).

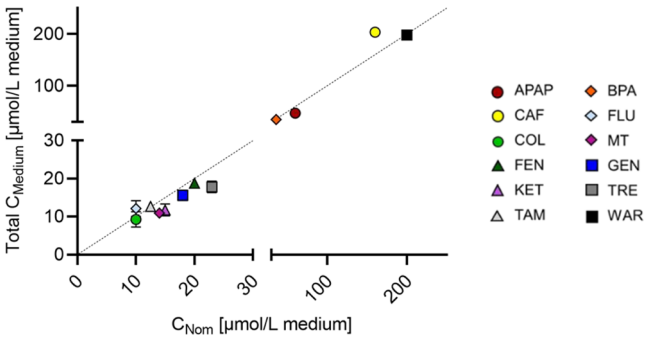

Figure 2. Measured initial total C_{Medium} of test chemicals before addition to Balb/c 3T3 cells (t = 0) compared to C_{Nom}. Each icon denotes one test chemical where circles represent hydrophilic (log P_{ow} −0.07−1.30), rhombus and squares represent moderate lipophilic (log P_{ow} 2.59−3.36), and 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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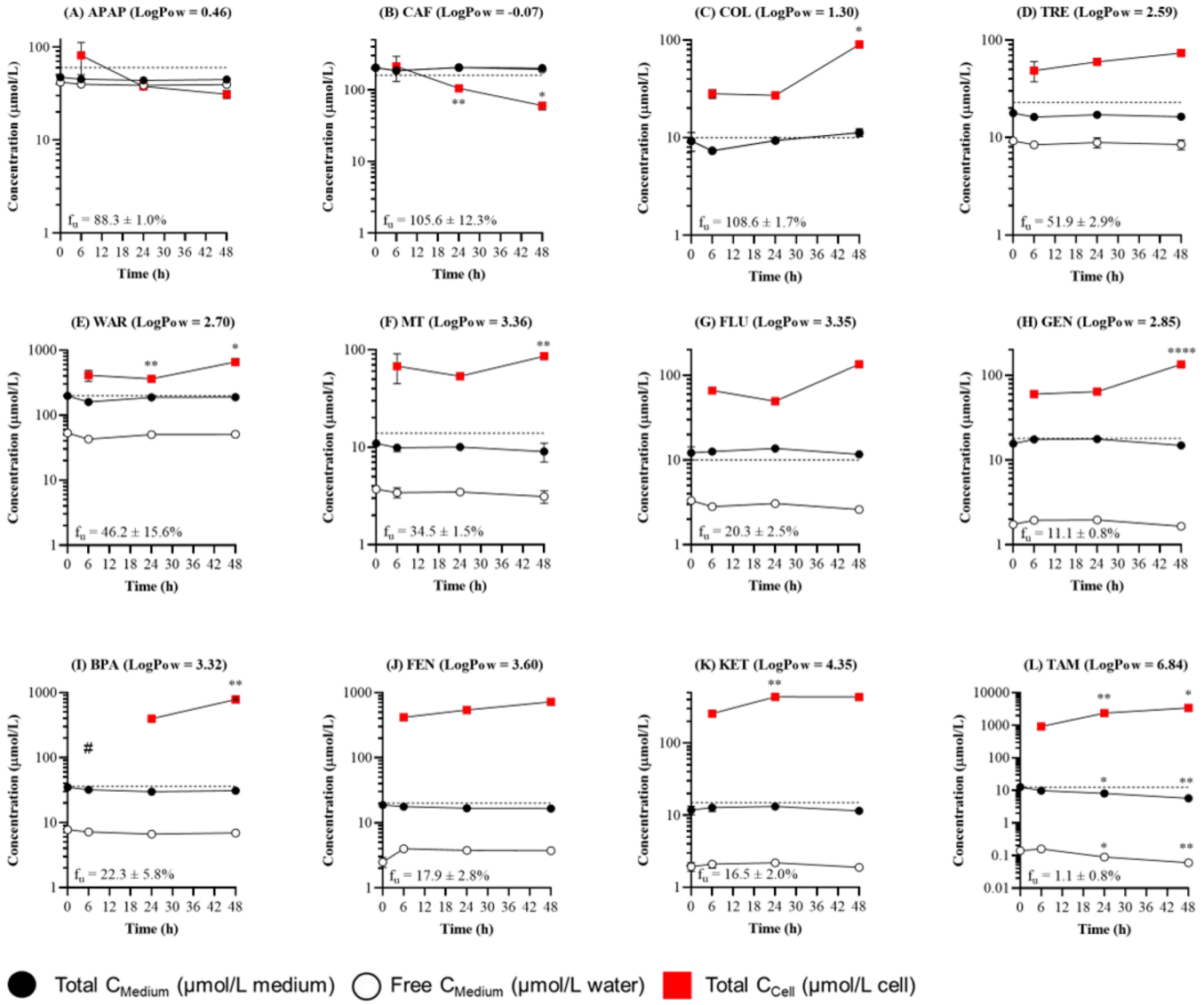

Figure 3. Measured values of f_u, C_{Cell}, and total and free C_{Medium} for all chemicals. The C_{Nom} is denoted by the dotted line, total C_{Medium} by black circles, free C_{Medium} by white circles, and the C_{Cell} by red squares. Data are represented as mean in µmol/L (SD if $n = 3$ or mean difference 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^{-6}% [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.

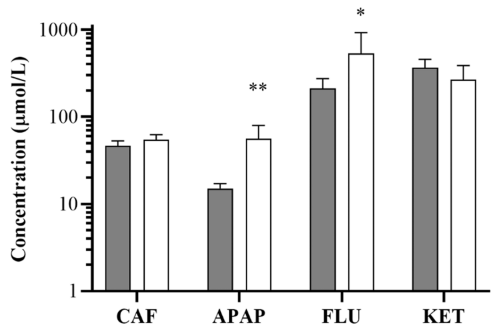

Figure 4. Comparison of C_{Cell} and $C_{Cytosol}$ for APAP, CAF, FLU, and KET. The bars show the total C_{Cell} (gray bars) and the $C_{Cytosol}$ (white bars) after 48 h of incubation. Data are represented as mean in μmol/L (SD of $n = 3$ experiments with triplicates; Welch t test, $p < 0.05$).

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%)	<0.9 (<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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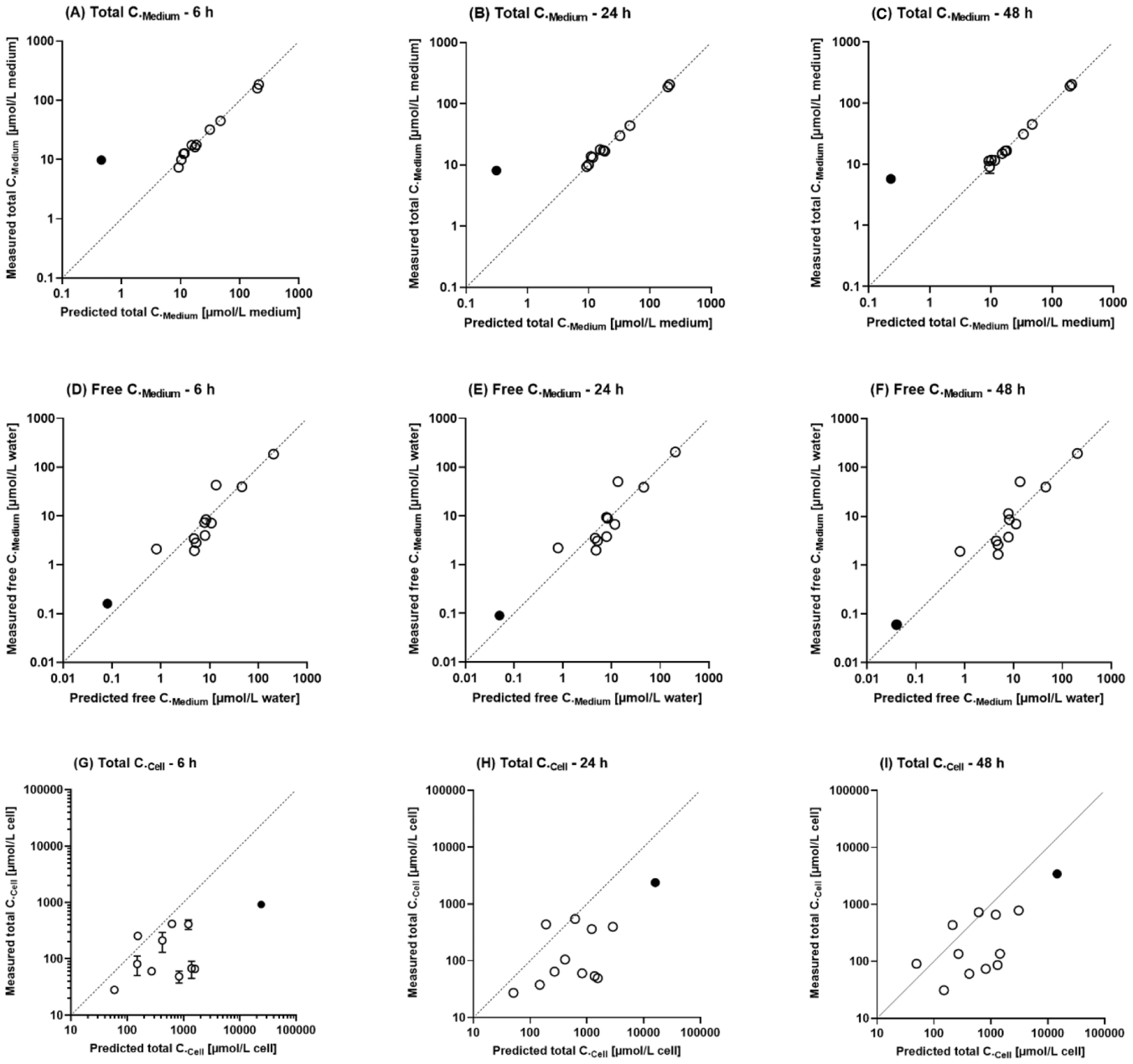

Figure 5. Comparison of predicted and measured values of C_{Cell} and total and free C_{Medium} for all test chemicals after 6, 24, and 48 h of incubation. The test chemicals are denoted by white circles, the black circles indicate the most lipophilic chemical of the set of substances, TAM. The line 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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