

# The importance of protein binding for the in vitro–in vivo extrapolation (IVIVE)—example of ibuprofen, a highly protein-bound substance

H. Mielke<sup>1</sup>  · E. Di Consiglio<sup>2</sup> · R. Kreutz<sup>3</sup> · F. Partosch<sup>4</sup> · E. Testai<sup>2</sup> · U. Gundert-Remy<sup>1,3</sup>

Received: 1 June 2016 / Accepted: 28 September 2016 / Published online: 21 October 2016  
© Springer-Verlag Berlin Heidelberg 2016

**Abstract** A physiologically based human kinetic model (PBHKM) was used to predict the in vivo ibuprofen dose leading to the same concentration–time profile as measured in cultured human hepatic cells (Truisi et al. in *Toxicol Lett* 233(2):172–186, 2015). We parameterized the PBHKM with data from an in vivo study. Tissue partition coefficients were calculated by an algorithm and also derived from the experimental in vitro data for the liver. The predicted concentration–time profile in plasma was in excellent agreement with human experimental data when the liver partition coefficient was calculated by the algorithm (3.01) demonstrating values in line with findings obtained from human postmortem tissues. The results were less adequate when the liver partition coefficient was based on the experimental in vitro data (11.1). The in vivo doses necessary to reach the in vitro concentrations in the liver cells were 3610 mg using the best fitting model with a liver partition coefficient of 3.01 compared to 2840 mg with the in vitro liver partition coefficient of 11.1. We found that this difference is possibly attributable to the difference between protein binding in vivo (99.9 %) and in vitro (nearly zero) as the partition coefficient is highly dependent on protein binding. Hence, the fraction freely diffusible in the liver

tissue is several times higher in vitro than in vivo. In consequence, when extrapolating from in vitro to in vivo liver toxicity, it is important to consider non-intended in vitro/in vivo differences in the tissue concentration which may occur due to a low protein content of the medium.

**Keywords** In vitro–in vivo extrapolation · Reverse dosimetry · Role of protein binding in modeling

## Introduction

In vitro methods have been increasingly used to characterize pharmacological and toxicological properties of substances. This development is in part driven by the societal demand to respect animal protection in scientific research as much as possible (Directive 2010/63/EU on the protection of animals used for scientific purposes, European Commission, 2010, updating the Directive 86/609/EEC, Commission, 1986). The use of non-animal testing (e.g., read across strategies, available in vitro and in silico methods) has been repeatedly asked in many regulations in the European Union (EU), including the REACH (Registration, Evaluation, Authorization and Restriction of CHemicals) legislation; it is stipulated to avoid animal experiments whenever possible (Annex XI of Regulation (EC) No. 1907/2006, REACH).

In the past, identification of properties and hazard has been the focus of in vitro studies (Bessems et al. 2014). With the legislation banning animal testing for cosmetics and avoiding animal studies whenever possible, in vitro studies with the only aim of hazard assessment are not an option anymore. However, data are requested to enable risk assessment which requires deriving a dose–response relationship from in vitro toxicological studies. In this context,

✉ H. Mielke  
hans.mielke@bfr.bund.de

<sup>1</sup> Federal Institute for Risk Assessment, Berlin, Germany

<sup>2</sup> Istituto Superiore di Sanità, Environment and Primary Prevention Department, Mechanisms of Toxicity Unit, Rome, Italy

<sup>3</sup> Institute of Clinical Pharmacology and Toxicology, Charité – Universitätsmedizin Berlin, Berlin, Germany

<sup>4</sup> Institute for Occupational, Social and Environmental Medicine, Medical School, Göttingen, Germany

it is important to translate the *in vitro* concentration–effect information into *in vivo* dose–effect relationship. This is a crucial step for using the latter information for quantitative risk assessment.

In addition, several authors in the past years have given examples that in the *in vitro* system, the concentration to which cells are exposed is different from the nominal concentration (Gülden et al. 2001; Gülden and Seibert 2003). Hence, in a recent review it is recommended measuring the concentration–time course in the *in vitro* system and to determine corresponding *in vitro* kinetics for the identification of the actual toxic concentration in the *in vitro* system and its relevance in the *in vivo* context (Adler et al. 2011; Groothuis et al. 2015). Indeed, the use of *in vitro* toxicity data for the risk assessment of a chemical highly depends on the relevance of the *in vitro*-derived data and the possibility for the *in vitro*–*in vivo* extrapolation (Kramer et al. 2015). It has been shown that differences between *in vitro* effects and those observed *in vivo* may exist in drug development where the need has become evident to improve prediction to avoid high attrition rates in the late process (e.g., Smith et al. 2010). Recently, we have described discrepancies of carcinogen-induced gene expression alterations in rat liver and cultivated hepatocytes comparing the *in vivo* with the *in vitro* results. Those discrepancies were explained by the different concentration–time courses *in vitro* and *in vivo* (Schug et al. 2013), emphasizing the necessity to measure the *in vitro* biokinetics as indicated by Kramer et al. (2015).

The process by which the *in vitro* concentration–time course is then translated into the *in vivo* situation is called ‘reverse dosimetry’ or quantitative *in vitro*–*in vivo* extrapolation (QIVIVE; Bessems et al. 2014). In this regard, *in vitro* biokinetic studies complementing toxicity testing were recently reported from an EU FP7 Project (Predict IV) on a number of model compounds among which are amiodarone (Pomponio et al. 2015a, b), cyclosporin A (Bellwon et al. 2015) and ibuprofen (Truisi et al. 2015).

In this paper, we report on the quantitative *in vitro*–*in vivo* extrapolation starting with ibuprofen concentration–time data in the supernatant and in hepatic cells in an *in vitro* model with primary human liver cells as reported in Truisi et al. (2015).

## Materials and methods

We used a physiologically based human kinetic model (PBHKM) representing the features relevant for simulating the concentration–time profile of ibuprofen in blood and tissues of an adult male. The details of the basic model and the physiological parameters used to represent a male individual have been described elsewhere (Abraham et al.

**Table 1** Parameters of the PBHKM used

Physiological data <sup>a, b</sup>	Standard	Alternatives
Cardiac output (Qc) (l/h)	390	
Body weight (bw) (kg)	73	79 <sup>a</sup> (for model evaluation)
Blood flow through the organs (l/h)		
Adipose tissue	19.5	
Liver	99.5	
Brain	46.8	
Kidney	74.1	
Muscle	65.8	
Other organs	65.5	
Skeleton	7.8	
Skin	10	
Organ volumes (l)		
Adipose tissue	18.2	
Liver	1.8	
Brain	1.45	
Kidney	0.31	
Muscle	0.4 bw	
Vessel-rich tissue	3.8	
Skeleton	9.3	
Skin	0.037 bw	
Substance-specific data		
Molecular mass (g/mol)	206.29	
Partition coefficients		
Fat/blood	0.52	
Liver/blood	3.01 <sup>b</sup>	11.1 <sup>c</sup>
Brain/blood	2.9	
Kidney/blood	2.42	
Muscle/blood	0.31	
Other tissue/blood	3.01 <sup>b</sup>	11.1 <sup>c</sup>
Skeleton/blood	0.32	
Skin/blood	0.78	
Clearance (l/h)	0.06 kg bw <sup>a</sup>	3.6 <sup>c</sup>
Absorption half-life (min) <sup>d</sup>	20	
Extent of absorption (% of the dose) <sup>d</sup>	100	
Protein binding (%) <sup>d</sup>	99.9	

<sup>a</sup> Greenblatt et al. (1984)

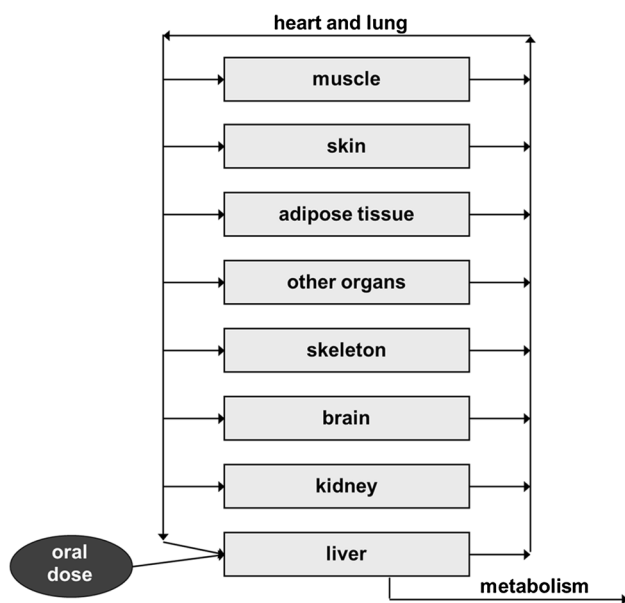
<sup>b</sup> Calculated according to Schmitt (2008)

<sup>c</sup> Calculated from the *in vitro* data in Truisi et al. (2015)

<sup>d</sup> Cristofolletti and Dressman (2014)

2004; see Table 1). The model includes eight organs/tissues as well as arterial and venous blood (Fig. 1). The organs are connected via blood flows, and the circulation system is closed via the lung and the heart.

Ibuprofen is a nonsteroidal anti-inflammatory drug with a low molecular weight (molecular weight 206.27 mol/g), and tissue membranes do not represent a significant barrier



**Fig. 1** Structure of the model

to distribution of ibuprofen as indicated by its in vivo volume of distribution of 0.17 l/kg bw (Greenblatt et al. 1984). Hence, its distribution is best described by perfusion rate-limited kinetics. Ibuprofen is highly protein bound (99 %; Cristofolletti and Dressman 2014) which is relevant for the partitioning between blood and tissues.

The rate of change of concentration is described by the equation  $V_T \frac{d}{dt} C_T = Q_T(C_A - C_{VT})$  in non-metabolizing tissues and by  $V_T \frac{d}{dt} C_T = Q_T(C_A - C_{VT}) - RAM$  in metabolizing tissues, where  $V_T$  denotes the volume of tissue T,  $C_T$  the concentration in tissue T,  $Q_T$  the blood flow through tissue T, and  $C_A$  the concentration in the arterial blood.  $C_{VT} = \frac{C_T}{P_T}$  describes the concentration in the venous blood leaving the tissue, with  $P_T$  as the tissue: blood partition coefficient. Excretion of ibuprofen (RAM) was modeled by metabolism in the liver via constant clearance. Other tissue compartments were regarded as non-metabolizing. Input was by the oral route (Fig. 1).

For calculation of the partition coefficient ( $P_T$ ) the algorithm of Schmitt (2008) was used. This algorithm is based on physiological considerations concerning the factors influencing the partitioning between the molecules in plasma and in the cell considering that it is the concentration of the unbound fraction in plasma water and in the intracellular water which is in equilibrium. Binding to plasma proteins can be considered as substance concentration independent as the number binding sites on the main plasma protein albumin is infinitively large, compared to the molar concentration of the drug or chemical in question as long as the plasma albumin concentration remains in the

normal range. There are exceptions when binding occurs to specific plasma proteins, which is not known for ibuprofen which is bound to albumin only. Also for the binding of a substance in a given tissue, it can generally be assumed to be determined by unspecific binding and the partition coefficients can be considered concentration independent in the concentration range which is of biological relevance. The main constituents in the cell which contribute to the binding are proteins and lipids, whereby it is to be distinguished between neutral lipids and phospholipids, the latter being further subdivided into those which possess an overall neutral head group with two oppositely charged moieties, and those carrying only the negative charge of the acidic phosphate group. It is also important to consider that only the uncharged fraction of the molecules can freely diffuse and this can be taken into consideration the different pH in the plasma and intracellular and applying the Hendersen–Hasselbalch equation.

The algorithm developed by Schmitt (2008) uses for every tissue the tissue-specific composition for water, proteins, neutral lipids and phospholipids and considers pH dependency. The algorithm captures only physical processes of the distribution which depend on the physicochemical properties of the drug or chemical in question. The algorithm does not capture a transport process by transporter proteins against a concentration gradient. However, the predictions using the algorithm are similar to the experimental results as shown in the publication (Schmitt 2008). The algorithm is also recommended as one of the helpful and usable tools for prediction of partition coefficient between blood and tissues in report on a joint EPAA–EURL ECVAM ADME workshop (Bessemers et al. 2014). Details on concept and the formulas used are found in Schmitt (2008). The tissue/blood partition coefficients were calculated using  $\log P = 2.23$  (Hansch et al. 1995) and a pKa of 4.5, and a nonprotein-bound fraction of 0.01 (see Table 1).

In an alternative approach, the concentration in the cell lysate and the supernatant as reported by Truisi et al. (2015) was used to calculate the liver tissue/blood partition coefficient, whereby cell lysate and the supernatant were taken to represent the liver tissue and the blood, respectively. The concentrations per well, as given in the reference, were transformed to concentrations in  $\mu\text{M}$  taking into consideration the well volume of 2 ml for the supernatant and a cell volume of 8  $\mu\text{l}$  for the cell lysate and a number of  $2 \times 10^6$  cell per well (see supplementary material in Truisi et al. 2015). The ratio was calculated for the concentrations at the different time points, where measurements were done, and the mean of the ratios was taken. In a similar way, the ratio of the concentrations in the hepatic cells and the supernatant was calculated for primary rat hepatocyte cultures and for Hepa RG cell cultures.

In order to evaluate what influence the protein binding would have on the calculated distribution between liver tissue and blood, we calculated the liver tissue/blood partition coefficient by setting the nonprotein-bound fraction of ibuprofen on values between 0.01 and 1.0.

The clearance was (1) taken from Greenblatt et al. (1984) who performed an *in vivo* study in human volunteers and (2) calculated from the publication of Truisi et al. (2015) (see Table 1). The oral absorption half-life was estimated to be 20 min, using data published by Cristofolletti and Dressman (2014). The extent of absorption by the oral route was set to 100 % (Cristofolletti and Dressman 2014).

Simulation was performed using MATLAB (version R2015b). As results of the simulation, we obtained the concentration–time profiles.

Evaluation of PBHKM was performed by comparing the simulation results with plasma concentration–time data obtained in an elderly volunteer after oral intake of a single dose of 600 mg ibuprofen as reported by Greenblatt et al. (1984). In this evaluation process, we used the hepatic partition coefficient calculated by the algorithm of Schmitt (2008) and alternatively determined the coefficient by using the experimental *in vitro* data. Accordingly, we used alternatively *in vivo* data and *in vitro* data for the calculation of clearance data (Table 1).

To determine the *in vivo* dose generating the same concentration–time profile as in the *in vitro* study for both the concentrations in the supernatant and in the liver cells, we simulated the concentration–time profile in an iterative process using different doses. For scaling the dose, we took the concentrations measured at different time intervals in the supernatant and the human hepatic cells from the publication of Truisi et al. (2015). The decision for the optimized dose was made by comparing the sum of squared (SSQ) differences in each observation from the predicted concentration–time profile of different doses and taking the dose with the lowest SSQ, respectively. In a second series, we determined the dose which best fitted the concentrations in the human hepatic cells because this is the relevant metric related to hepatic toxicity measured at different time intervals (Truisi et al. 2015).

## Results

### Model evaluation

In the evaluation step, we first used the tissue/blood partitioning coefficient calculated by the algorithm of Schmitt (2008) of 3.01, the *in vivo* protein binding of 99 % with a fraction of non-protein-bound ( $f_u$ ) ibuprofen of 0.01 and the *in vivo* clearance published by Greenblatt et al. (1984) of 0.06 kg bw (L/h) (corresponding to 79 ml/min). We

predicted the concentration–time profile in the blood and in the liver for the 600 mg dose and body weight of 79 kg as reported for the volunteer (presented in Fig. 1 in the publication of Greenblatt et al. 1984). To evaluate the goodness of the prediction, we compared the simulated concentration–time profile in blood with the concentration–time data. Figure 2a illustrates the outcome which confirms the validity of the PBHKM and the parameters we have applied.

### Using *in vitro* data instead of *in vivo* data

In a second step, we used the ratio of the *in vitro* concentration in the hepatic cell lysate and in the supernatant as the partition coefficient of liver tissue/blood for the modeling which is 11.1. The simulated concentration–time profile did not fit well with the experimental data (Fig. 2b). Comparing the SSQs for the distance between experimental and modeled data demonstrated clearly that the simulation with the calculated liver tissue/blood partitioning coefficient fitted the experimental data better.

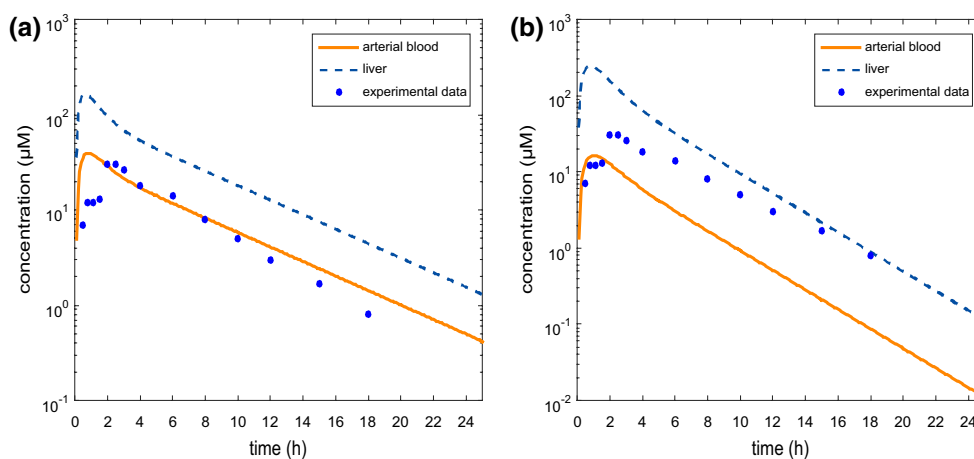
It is to be noted that the ratio of the *in vitro* concentration in the hepatic cell lysate and in the supernatant is 24.1 for primary rat hepatocyte cultures and 6.6 for Hepa RG cell cultures.

When we evaluated the influence of the nonprotein-bound fraction ( $f_u$ ) on the *in silico* predicted liver/blood partition coefficient, it became obvious that with increasing  $f_u$ , the liver/blood partition coefficient increased. For example, for a  $f_u$  of 0.01, the ratio was 3, for  $f_u$  of 0.035, the ratio was 10, and in the case that no protein binding was present ( $f_u = 1$ ), the ratio was 296.

Concerning the clearance, the results did not differ noticeable when we used the *in vitro* clearance instead of the *in vivo* value (not shown) which was anticipated because the clearance values were similar.

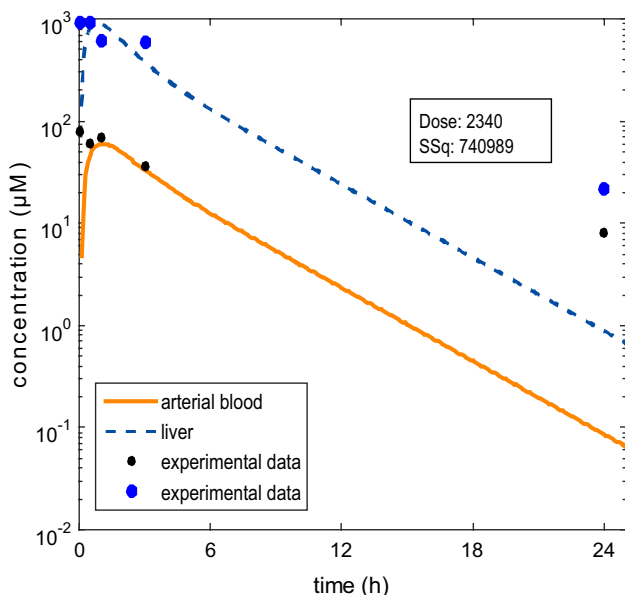
### Dose finding

To determine the dose which would lead to the same *in vivo* concentrations in humans in plasma and liver as in the *in vitro* experiment in the supernatant and the hepatic cells, we used the *in vitro* clearance and the *in vitro* hepatic tissue: supernatant partition coefficient. It is obvious that the concentration–time profile up to 3 h was in line with the experimental data, whereas the last data point (at 24 h) was higher than estimated by the prediction (Fig. 3). It should be mentioned that the data point at 24 h is characterized by high uncertainty as it represents the mean value of three measurements of which two measurements were below the level of detection (Truisi et al. 2015). We changed the dose in steps of 10 mg given into the human system and compared the SSQs for the different doses. The dose which gave the lowest SSQ was 2340 mg (Table 2).



**Fig. 2** **a** Model-based simulated concentration–time profile in blood and liver compared to experimental in vivo data taken from the literature to assess the performance of the model. Condition: hepatic tissue/blood partition coefficient calculated by the algorithm of Schmitt (2008) of 3.01; clearance of 0.06 kg bw L/h calculated from in vivo data (Greenblatt et al. 1984) compared with the blood concentration–time data of one volunteer taken from Fig. 1 in Greenblatt et al. (1984). **b** Model-based simulated concentration–time profile in blood and liver compared to experimental in vivo data taken from the literature to assess the performance of the model. Condition: hepatic tissue/blood partition coefficient of 11.1 derived from the in vitro data (con-

centration in human hepatic cells/concentration in the supernatant); clearance of 3.6 l/h calculated from the in vitro concentration–time data (Truisi et al. 2015) compared with the blood concentration–time data of one volunteer taken from Fig. 1 in Greenblatt et al. (1984). The concentration–time profile in blood is in better agreement with the experimental data if the hepatic tissue/blood partition coefficient is 3.01 (as calculated by the algorithm of Schmitt 2008). The dose was 600 mg and the weight of the subject 79 kg as in Greenblatt et al. (1984). *Solid orange line* predicted concentration in the arterial blood; *dashed blue line* predicted concentration in the liver; *blue dots* concentrations over time measured in a human volunteer (color figure online)



**Fig. 3** Optimized dose in reverse dosimetry with optimization for concentrations both in the supernatant (*small black symbols*) and in the human hepatic cells (*large blue symbols*) using the in vitro parameters. Condition: hepatic tissue/blood partition coefficient of 11.1 derived from the in vitro data (concentration in human hepatic cells/concentration in the supernatant); clearance of 3.6 L/h calculated from the in vitro concentration–time data (Truisi et al. 2015). *Solid orange line* predicted concentration in the arterial blood; *dashed blue line* predicted concentration in the liver; *small black dots* concentrations over time measured in the supernatant; *large blue dots* concentrations over time measured in human hepatic cells (color figure online)

It should be noted that the dose of 2340 mg was found with the in vitro partition coefficient between hepatic cells and supernatant of 11.1 which is 3.67-fold higher than the validated calculated value of 3.01. Therefore, we have undertaken dose optimization by targeting the concentration in the liver, the target for toxicity by using (1) the same parameters as in the validation step (tissue/blood partitioning coefficient calculated by the algorithm of Schmitt 2008), the in vivo protein binding of 99 % with an unbound fraction ( $f_u$ ) of 0.01 and the in vivo clearance published by Greenblatt et al. (1984) and (2) the in vitro clearance and the in vitro hepatic tissue: supernatant partition coefficient. The dose with the lowest SSQ was 3610 mg for the situation (1) and 2340 mg for situation (2), the same dose as found when the concentrations in both the supernatant and in the liver cells were used for scaling (Fig. 4a, b).

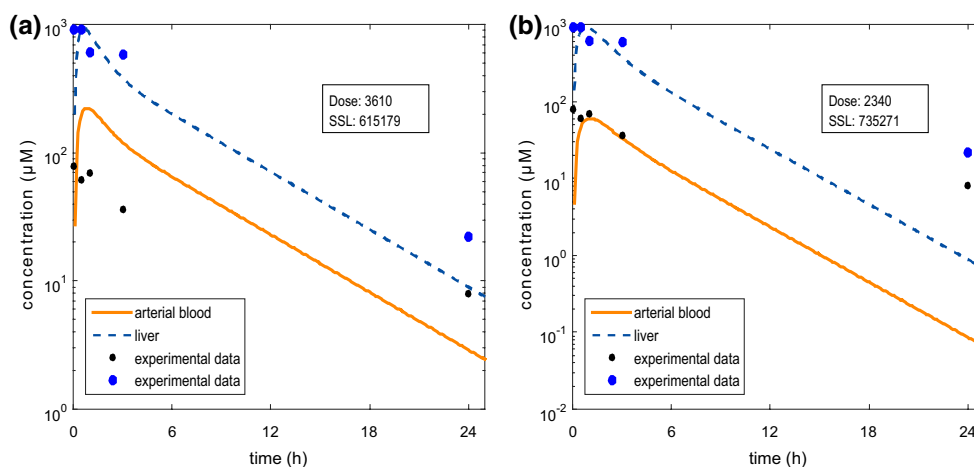
## Discussion

The current paper describes the reverse dosimetry for ibuprofen for which measured concentration–time data were available from an in vitro study in a human hepatic cell model.

In a first step, the PBHKM, we had constructed, was assessed by comparing the modeled plasma concentration–time profile with data from an in vivo pharmacokinetic

**Table 2** In vitro cytotoxicity data from literature

Cell line	50 % inhibition of cell growth in culture (mM)	Concentration with 10 % cytotoxicity (mM)	References
L, of mouse subcutaneous tissue origin	0.89		Mukaide and Kameyama (1975a)
Embryonic skin and muscle from humans	0.7		Mukaide and Kameyama (1975b)
EK 12	0.63		Mukaide and Kameyama (1975c)
EL	0.61		Mukaide and Kameyama (1975c)
Primary rat hepatocytes		0.1	Truisi et al. (2015)
Primary human hepatocytes		1	Truisi et al. (2015)



**Fig. 4** Comparison of the outcome for reverse dosimetry for the target organ liver using the in silico parameters versus using the in vitro parameters for the modeling. **a** Optimized dose in reverse dosimetry with optimization for concentrations in the target organ of toxicity (liver). Condition: hepatic tissue/blood partition coefficient of 3.01 calculated by the algorithm of Schmitt (2008); clearance of 0.06 kg bw calculated from in vivo data (Greenblatt et al. 1984). **b** Optimized dose in reverse dosimetry with optimization for concentrations in the target organ of toxicity (liver). Condition: hepatic tis-

sue/blood partition coefficient of 11.1 derived from the in vitro data (concentration in human hepatic cells/concentration in the supernatant); clearance of 3.6 l/h calculated from the in vitro concentration–time data (Truisi et al. 2015). *Solid orange line* predicted concentration in the arterial blood; *dashed blue line* predicted concentration in the liver; *small black dots* concentrations over time measured in the supernatant; *large blue dots* concentrations over time measured in human hepatic cells (color figure online)

experiment in humans. The modeling outcome showed an excellent conformity with the experimental data. This established confidence in the model when using a partition coefficient between hepatic tissue and blood calculated by an algorithm based on physicochemical properties of chemicals and the tissue composition (Schmitt 2008) and by using in vivo clearance data (Greenblatt et al. 1984). It was interesting to note that the partitioning between hepatic tissue and blood was different when calculated by the algorithm of Schmitt (2008) compared to calculating the partitioning using the in vitro data of Truisi et al. (2015). Using in vitro partition coefficient calculated as the cell/supernatant ratio to predict the concentration–time profile in vivo turned out not to be in conformity with the experimental in vivo data. In addition, the relevance of the value

calculated by an algorithm for the partitioning between liver tissue and blood was supported by postmortem data obtained in a human subject who committed suicide with an overdose of ibuprofen (Kunsmann and Rohrig 1993). In this subject, the ratio between the ibuprofen concentrations measured in the liver and in the blood of the arteria femoralis was 2.7.

We evaluated possible causes for this finding and found that a plausible explanation might be a difference in the protein binding and hence the fraction of unbound ibuprofen. Only the nonprotein-bound, free concentration is in equilibrium with the free concentration in the tissue (Pelkonen et al. 2008; Smith et al. 2010). Between the in vivo situation and the experimental conditions in vitro, there is a difference in the protein content in the blood, in particular

albumin which is the binding protein for ibuprofen, and the content of protein in the media used for the *in vitro* cultivation (Williams' E medium and the Geltrex<sup>TM</sup>, which was applied in serum-free culture media). The influence of a lower protein binding in plasma/cultivation medium on the partition coefficient was shown with the theoretical calculations using the Schmitt algorithm and showed an increasing tissue/cellular concentration with decreasing protein binding and an increase in the free fraction in plasma. Thus, the data support the notion that the lower protein concentration and the higher nonprotein-bound, free concentration in the medium *in vitro* is the underlying cause for the observed *in vitro*–*in vivo* difference.

In the *in vitro* experiment, the concentration in the human liver cells was 11-fold higher than the concentration in the supernatant. In human hepatocytes, Trusi et al. (2015) reported cytotoxic response (TC10, the concentration at which 10 % cell death was measured) to occur at about 1 mM. In the same study, the TC10 for rat hepatocytes was tenfold lower (100  $\mu$ M) which is similar to other cells of rat origin which had the same sensitivity (Mukaide and Kameyama 1975a, b, c; Table 2). When we calculated the ratio of the concentration in rat hepatocytes, the supernatant a value of 24.1 resulted. Hence, the intracellular concentration in rat hepatic cells is twice the concentration in human hepatocytes and explains the lower TC10 for rat hepatocytes compared to the human hepatocytes. Both results show the importance to consider the influence of the free fraction in the medium for the concentration in the cells when performing *in vitro* toxicity testing.

According to the validated human model, the *in vivo* concentration in the liver is threefold higher than in the blood and fourfold lower compared to the concentration in the human hepatic cells *in vitro*. In the modeling using the *in vitro* conditions (hepatic cell/supernatant 11) and optimized for the *in vitro* liver concentration–time profile, the dose of 2.340 mg ibuprofen fits to the hepatic cell concentrations. When we parametrized the model with the *in silico* predicted liver/blood partition coefficient, the dose of 3.610 mg ibuprofen was necessary to reach the *in vitro* hepatic cell concentrations. This is a about a 1.5-fold higher dose.

When comparing the two doses of 2340 and 3610 mg with clinical data, the daily therapeutic doses of 1200 mg up to 2400 mg given in three divided doses indicate that a dose-dependent toxicity is not expected at 2340 mg but could occur at 3610 mg. There are several reports in the literature on liver toxicity caused by ibuprofen for which the underlying mechanism remains unclear (Douros et al. 2015). Judged from the clinical findings, a direct toxic effect seemed less likely, since overdose did not lead to hepatic injury and was responsible for only minimal ALT elevation (Friis and Andreasen 1992). Two cases of liver injury with ibuprofen were clearly characterized by

a delayed response and clinical signs of cholestasis and immunological responses (Alam et al. 1996; Elkrief et al. 2007). There are three case reports on suicidal attempts. In one case, severe liver toxicity has been observed requiring liver transplantation. In this case, the dose was 9600 mg (Laurent et al. 2000), and in the second case, 20,000 mg ibuprofen was ingested with occurrence of acute liver toxicity (Lee and Finkler 1986). In the third case, the total dose is less clear, but could be up to an intake of 90,000 mg over a period of 2 weeks without obvious liver toxicity (Kunsmann and Rohrig 1993). Hence, the dose of 2340 mg is within the therapeutic range and is less likely to be predictive of liver toxicity *in vivo* in humans. However, 3610 mg which represents one-third of the toxic response in the case reported by Laurent et al. (2000) might be a conservative estimate for a low toxic dose.

In summary, our work shows that QIVIVE modeling can be performed with reliable results. When performing reverse dosimetry, several points have to be taken into consideration. The *in vitro* clearance might be different from the *in vivo* clearance. We have shown this feature in a former study causing prolonged exposure and effects *in vitro* which were not observed *in vivo* (Schug et al. 2013). In the current study, we have shown that there may be differences in the tissue/blood partitioning *in vitro* compared to the *in vivo* situation for which we identified the different protein content of human blood and the culture media resulting in a difference in the unbound concentration as the most probable underlying cause for a higher intracellular concentration with related toxicological response. As it is the nonprotein-bound concentration in the blood or in the culture medium which is in equilibrium with the nonprotein-bound intracellular or tissue concentration, the protein content in the culture medium influences the intracellular concentration. The results of this study on the importance of the free fraction in the cultivation medium can be generalized for substances with a high protein binding and a low nonprotein-bound fraction in plasma. Ibuprofen with a protein binding of 99 % and a free fraction of 0.01 is a paradigmatic substance in this respect. In these cases, it should be understood that a serum-free medium is not a perfect surrogate for blood.

The measurements *in vitro* are crucial to more precisely predict the dose necessary *in vivo* to reach the concentration and the effect in the target tissue. In particular, as this report shows, measurements of intracellular concentrations in the target tissue are a must to be able to correctly extrapolate the *in vitro* toxicological findings. Thus, when applying reverse dosimetry (QIVIVE) to reach the same effect *in vivo* as seen *in vitro*, it is necessary to know the time course of the concentration in the cells relevant for toxicity and not only the concentration–time course in the supernatant.

## References

- Abraham K, Mielke H, Huisinga W, Gundert-Remy U (2004) Elevated internal exposure of children in simulated acute inhalation of volatile organic compounds: effects of concentration and duration. *Arch Toxicol* 79:63–73
- Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Andersen KE, Angers-Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S, Coecke S, Corvi R, Cronin M, Daston G, Dekant W, Felner S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Leite SB, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhrer S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, Tähti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM (2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. *Arch Toxicol* 85:367–485
- Alam I, Ferrell LD, Bass NM (1996) Vanishing bile duct syndrome temporally associated with ibuprofen use. *Am J Gastroenterol* 91(8):1626–1630
- Bellwon P, Truissi GL, Bois FY, Wilmes A, Schmidt T, Savary CC, Parmentier C, Hewitt PG, Schmal O, Josse R, Richert L, Guillouzo A, Mueller SO, Jennings P, Testai E, Dekant W (2015) Kinetics and dynamics of cyclosporine a in three hepatic cell culture systems. *Toxicol In Vitro* 30(1 Pt A):62–78
- Bessems JG, Loizou G, Krishnan K, Clewell HJ 3rd, Bernasconi C, Bois F, Coecke S, Collnot EM, Diembeck W, Farcal LR, Geraets L, Gundert-Remy U, Kramer N, Küsters G, Leite SB, Pelkonen OR, Schröder K, Testai E, Wilk-Zasadna I, Zaldivar-Comenges JM (2014) PBTK modelling platforms and parameter estimation tools to enable animal-free risk assessment: recommendations from a joint EPAA–EURL ECVAM ADME workshop. *Regul Toxicol Pharmacol* 68(1):119–139
- Cristofolletti R, Dressman JB (2014) Use of physiologically based pharmacokinetic models coupled with pharmacodynamic models to assess the clinical relevance of current bioequivalence criteria for generic drug products containing Ibuprofen. *J Pharm Sci* 103(10):3263–3275
- Douros A, Bronder E, Andersohn F, Klimpel A, Thomae M, Sarganas G, Kreutz R, Garbe E (2015) Drug-induced liver injury: results from the hospital-based Berlin case-control surveillance study. *Br J Clin Pharmacol* 79(6):988–999
- Elkrief L, Chrysostalis A, Moachon L, Franck N, Terris B, Chausade S, Sogni P (2007) Hépatite cholestatique sévère associée à un syndrome de Stevens-Johnson après la prise d'ibuprofène (Severe cholestatic hepatitis associated with a Stephens-Johnson syndrome after taking ibuprofen). *Gastroenterol Clin Biol* 31:1043–1045
- Friis H, Andreassen PB (1992) Drug-induced hepatic injury: an analysis of 1100 cases reported to the Danish committee on adverse reactions between 1978 and 1987. *J Intern Med* 232:133–138
- Greenblatt DJ, Abernethy DR, Matlis R, Harmatz JS, Shader RI (1984) Absorption and disposition of ibuprofen in the elderly. *Arthritis Rheum* 27(9):1066–1069
- Groothuis FA, Heringa MB, Nicol B, Hermens JL, Blaauboer BJ, Kramer NI (2015) Dose metric considerations in in vitro assays to improve quantitative in vitro–in vivo dose extrapolations. *Toxicology* 332:30–40
- Gülden M, Seibert H (2003) In vitro–in vivo extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations in vitro. *Toxicology* 189(3):211–222
- Gülden M, Mörchel S, Seibert H (2001) Factors influencing nominal effective concentrations of chemical compounds in vitro: cell concentration. *Toxicol In Vitro* 15(3):233–243
- Hansch C, Hoekman D, Leo A, Zhang L, Li P (1995) The expanding role of quantitative structure-activity relationships (QSAR) in toxicology. *Toxicol Lett* 79:45–53
- Kramer NI, Di Consiglio E, Blaauboer BJ, Testai E (2015) Biokinetics in repeated-dosing in vitro drug toxicity studies. *Toxicol In Vitro* 30(1 Pt A):217–224
- Kunsmann GW, Rohrig TP (1993) Tissue distribution of ibuprofen in a fatal overdose. *Am J Forensic Med Pathol* 14:48–50
- Laurent S, Rahier J, Geubel AP, Lerut J, Horsmans Y (2000) Subfulminant hepatitis requiring liver transplantation following ibuprofen overdose. *Liver* 20:93–94
- Lee CY, Finkler A (1986) Acute intoxication due to ibuprofen overdose. *Arch Pathol Lab Med* 110:747–749
- Mukaide A, Kameyama T (1975a) Effects of drugs on cell culture (I). *Nihon Yakurigaku Zasshi* 71(3):295–300
- Mukaide A, Kameyama T (1975b) Effects of drugs on cell culture (II). *Nihon Yakurigaku Zasshi* 71(3):301–305
- Mukaide A, Kameyama T (1975c) Effects of drugs on cultured cells (III). *Nihon Yakurigaku Zasshi* 71(4):379–385
- Pelkonen O, Kapitulnik J, Gundert-Remy U, Boobis AR, Stockis A (2008) Local kinetics and dynamics of xenobiotics. *Crit Rev Toxicol* 38(8):697–720
- Pomponio G, Savary CC, Parmentier C, Bois F, Guillouzo A, Romanelli L, Richert L, Di Consiglio E, Testai E (2015a) In vitro kinetics of amiodarone and its major metabolite in two human liver cell models after acute and repeated treatments. *Toxicol In Vitro* 30(1 Pt A):36–51
- Pomponio G, Zurich MG, Schultz L, Weiss DG, Romanelli L, Gramowski-Voss A, Di Consiglio E, Testai E (2015b) Amiodarone biokinetics, the formation of its major oxidative metabolite and neurotoxicity after acute and repeated exposure of brain cell cultures. *Toxicol In Vitro* 30(1 Pt A):192–202
- Schmitt W (2008) General approach for the calculation of tissue to plasma partition coefficient. *Toxicol In Vitro* 22:457–467
- Schug M, Stöber R, Heise T, Mielke H, Gundert-Remy U, Godoy P, Reif R, Blaszkewicz M, Ellinger-Ziegelbauer H, Ahr HJ, Selinski S, Günther G, Marchan R, Blaszkewicz M, Sachinidis A, Nüssler A, Oberemm A, Hengstler JG (2013) Pharmacokinetics explain in vivo/in vitro discrepancies of carcinogen-induced gene expression alterations in rat liver and cultivated hepatocytes. *Arch Toxicol* 87(2):337–345
- Smith DA, Di L, Kerns EH (2010) The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. *Nat Rev Drug Discov* 9:929–939
- Truissi GL, Consiglio ED, Parmentier C, Savary CC, Pomponio G, Bois F, Lauer B, Jossé R, Hewitt PG, Mueller SO, Richert L, Guillouzo A, Testai E (2015) Understanding the biokinetics of ibuprofen after single and repeated treatments in rat and human in vitro liver cell systems. *Toxicol Lett* 233(2):172–186