Predictive Performance of Next Generation Physiologically Based Kinetic (PBK) Model Predictions in Rats Based on In Vitro and In Silico Input Data

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ABSTRACT

The goal of the present study was to assess the predictive performance of a minimal generic rat physiologically based kinetic (PBK) model based on in vitro and in silico input data to predict peak plasma concentrations (Cmax) upon single oral dosing. To this purpose, a dataset was generated of 3960 Cmax predictions for 44 compounds, applying different combinations of in vitro and in silico approaches for chemical parameterization, and comparison of the predictions to reported in vivo data. Best performance was obtained when (1) the hepatic clearance was parameterized based on in vitro measured intrinsic clearance values, (2) the method of Rodgers and Rowland for calculating partition coefficients, and (3) in silico calculated fraction unbound plasma and Papp values (the latter especially for very lipophilic compounds). Based on these input data, the median Cmax of 32 compounds could be predicted within 10-fold of the observed Cmax with 22 out of these 32 compounds being predicted within 5-fold, and 8 compounds within 2-fold. Overestimations of more than 10-fold were observed for 12 compounds, whereas no underestimations of more than 10-fold occurred. Median Cmax predictions were frequently found to be within 10-fold of the observed Cmax when the scaled unbound hepatic intrinsic clearance (Clint,u) was either higher than 20 l/h or lower than 1 l/h. Similar findings were obtained with a test set of 5 in-house BASF compounds. Overall, this study provides relevant insights in the predictive performance of a minimal PBK model based on in vitro and in silico input data.

Key words: Cmax; QIVIVE; rat; PBPK; PBK.

Substantial advances have been made over the last decades with the development of in vitro methods to capture biological effects of compounds that may serve as alternative test methods for animal toxicity testing (Jennings, 2015; Pamies et al., 2018; Pamies and Hartung, 2017). However, the quantitative chemical distribution in the body is frequently ignored when interpreting in vitro effect data. Without these considerations, the in vitro biological effect data as stand-alone may lead to incorrect conclusions about the in vivo potencies of compounds, because the ultimate in vivo effects will, besides the toxicodynamic effects (in the target tissue), also depend on the toxicokinetics (ie, the concentration of the chemical at the site of action) (Bessems et al., 2014; Blaauuboer, 2010; Yoon et al., 2015). Applying physiologically based kinetic (PBK) modeling concomitantly to in vitro toxicity testing provides an effective framework for the extrapolation of in vitro biological effect concentrations to equivalent (oral) doses (eg, Dejongh et al., 1999; Fabian et al., 2019; Forsby and Blaauuboer, 2007; Gubbels-van Hal et al., 2005; 18}
Louisse et al., 2010; Punt et al., 2019; Verwei et al., 2006; Wetmore et al., 2015. By simulating the plasma (or tissue) concentrations at different doses, one can infer the doses that are needed to reach the in vitro effect concentrations in the plasma (or tissue) and whether these effect doses are expected to be reached at defined exposure estimates.

Although PBK modeling is increasingly acknowledged to play a crucial role in the transition toward animal-free testing strategies for chemical safety evaluations to perform the required quantitative in vitro to in vivo extrapolations (QIVIVE), the development of PBK models solely on the basis of in vitro and/or in silico input data remains a challenge (Paini et al., 2019; Peters and Dolgos, 2019). Initial estimates of plasma and tissue concentrations of compounds can effectively be made with minimal generic PBK models that are defined based on (1) a first-order intestinal absorption rate ($k_a$) and a fraction absorbed ($f_a$), (2) intrinsic hepatic clearance ($Cl_{int}$), (3) tissue: plasma partition coefficients, (4) the fraction unbound plasma ($f_{up}$), and (5) passive renal excretion (defined as the glomerular filtration rate times the unbound concentration of the compound in plasma ($CFR \times Cl_{up}$)). For each of these different kinetic processes, different input approaches can be applied. For example, $k_a$ and $f_a$ can be estimated from Caco-2 absorption studies or calculated based on in silico tools (Hou et al., 2004). $Cl_{int}$ data can either be obtained with primary hepatocytes, S9, or microsomes, or can be predicted using in silico methods (Zhang et al., 2018). The $f_{up}$ can also be calculated in silico or experimentally derived using, for example, microdialysis experiments (Rottroff et al., 2010). Partition coefficients are generally calculated in silico, for which different approaches exist (Berezhzkovskiy, 2004; Poulin and Theil, 2003; Rodgers and Rowland, 2006; Schmitt, 2008). It must be noted that available in silico tools are often trained based on available in vitro data, indicating that the in vitro and in silico methods cannot be regarded as being totally independent.

Uncertainties exist with respect to the impact of these different approaches on the model predictions, the quality of the input data as well as the difficulty of determining whether additional kinetic processes need to be added to the model (eg, extrahepatic metabolism and/or transporter-mediated kinetics). As a result, PBK model predictions still need to be evaluated on a case-by-case basis against in vivo data (eg, plasma concentrations) (Peters and Dolgos, 2019; Tsamandouras et al., 2015). Moreover, when certain kinetic processes cannot be parameterized based on in vitro or in silico experiments, they are usually obtained by fitting model predictions to in vivo data (Peters and Dolgos, 2019; Tsamandouras et al., 2015). To facilitate the transition toward nonanimal testing strategies, it is important to move away from this case-by-case evaluation and optimization of PBK models against in vivo data, and to identify other strategies for the evaluation of the adequacy of in vitro- and in silico-based PBK models to estimate in vivo kinetics.

The goal of the present study was to assess the general predictive performance of a minimal generic rat PBK model based on in vitro and in silico input data to predict peak plasma concentrations ($C_{max}$) upon single dosing. To this end, $C_{max}$ predictions in rats upon single oral dosing were made for a set of 44 model compounds based on a range of input approaches for estimating the chemical specific parameters ($Cl_{int}$, $f_{up}$, partition coefficients, and $k_a$) and compared with observed $C_{max}$ values reported in the literature for these compounds in rats. We characterized the contribution of different input approaches to the wide variation in $C_{max}$ predictions for individual compounds. In addition, we assessed whether we could find a relation between chemical (kinetic) characteristics (such as extent of metabolic clearance, charge, lipophilicity, or uptake rate of the compounds) and the chance that the $C_{max}$ is predicted within 10-fold or not. The results obtained were applied on 5 in-house BASF compounds as case study.

**MATERIALS AND METHODS**

Chemical dataset. A dataset of 44 model compounds was formed based on the availability of in vivo data (maximum plasma concentrations [C$_{max}$]) in rats after single oral dosing, allowing to evaluate the performance of the $C_{max}$ predictions by the PBK model based on different in vitro and/or in silico input approaches. The majority of compounds in the dataset (38 compounds) were selected based on available plasma $C_{max}$ data for rat in the database of the R htkc package by Pearce et al. (2017). The dataset was extended with 4 food-relevant compounds (bisphenol A, genistein, daidzein, and ochratoxin A), for which in vivo oral kinetic studies in rats were available in the literature. In addition, rosuvastatin and fluvastatin were included for which transporter-mediated processes in liver and kidney play a main role in the kinetics (Chan et al., 2019). The final list of model compounds and related in vivo $C_{max}$ data (and related oral doses) is available as Supplementary Material. In addition to the 44 model compounds, 5 in-house BASF compounds were included as case study to test the minimal PBK modeling approach.

Generic PBK model code and input parameters. PBK model predictions in rat were performed based on a published generic (human) PBK model code by Jones and Rowland-Yeo (2013) that was implemented in R (R Core Team, 2021, version 4.1.1) and converted from human to rat by Punt et al. (2021) by inclusion of rat-specific parameters as obtained from Musther et al. (2017). The model consists of 13 compartments, corresponding to the major organs in the body and an arterial and venous blood compartment. The model requires chemical-specific parameters for intestinal uptake, distribution (ie, partition coefficients, blood:plasma ratio [assumed to be 1 in the present study for all compounds], $f_{up}$), hepatic clearance, and renal clearance (assumed to be the glomerular filtration rate times the free plasma concentration). Table 1 provides an overview on how these different input parameters were parameterized using a range of in vitro and/or in silico methods. Further details on these input approaches are given in the text below. The differential equations of the model are solved with the deSolve package (Soetaert et al., 2010). The R code of the PBK model is provided in the Github repository (https://github.com/wfsrqivive/rat_PBK.git; last accessed December 22, 2021).

Absorption from the gastrointestinal tract was described in the model by a first-order uptake process from the intestine to the liver compartment and requires an absorption rate constant ($k_a$) and fraction absorbed ($f_a$) as input (Jones and Rowland-Yeo, 2013). For the parameterization of these input constants an in silico approach based on a QSAR from Hou et al. (2004) was applied that predicts the Caco-2 apparent permeability ($P_{app}$) based on the topological polar surface area (TPSA) of the compounds (equation 1). Both ADMET Predictor software (v.9.0, Simulations Plus, Lancaster, California; www.simulations-plus.com; last accessed December 22, 2021) and ChemAxon (ChemAxon Ltd., Budapest, Hungary; www.chemaxon.com; last accessed December 22, 2021) were used to generate these TPSA values. Given that both software packages yielded the same TPSA results, no further distinctions were made.
between these 2 sets of results for assessing the PBK model’s predictive capacity. For 26 out of the 44 compounds, the QSAR-based approach was compared with in vitro measured apparent permeability ($P_{\text{app}}$) coefficients in Caco-2 transwell absorption experiments. Details on Caco-2 experiments are provided as Supplementary Material. Both the QSAR-derived $P_{\text{app}}$ values and the in vitro measured values were scaled to an uptake rate constant ($k_a$) and fraction absorbed ($f_a$) based on equations 2–5.

\begin{align}
\log P_{\text{app}} (\text{cm/s}) & = -4.36 - 0.01 \times \text{TPSA} \\
\log P_{\text{eff, human}} (10^{-4} \text{ cm/s}) & = 0.4926 + \log P_{\text{app}} (10^{-6} \text{ cm/s}) - 0.1454 \\
\frac{P_{\text{eff, rat}}}{P_{\text{eff, human}}} & = 11.04 \\
k_a (/h) & = \frac{P_{\text{eff}} \times 2 \text{ (cm/s)} / R \text{ (cm)} \times 3600 \text{ (s/h)}} \\
f_a & = 1 - \left(1 + (2 \times P_{\text{eff}} \times T_{\text{app}}) / (7 \times R)\right)^{-7},
\end{align}

in which equation 1 is derived from Hou et al. (2004), equation 2 scales the Caco-2 apparent permeability (QSAR predicted or in vitro measured) to the human effective permeability based on Sun et al. (2002), equation 3 scales the human effective permeability to a rat effective permeability based on equation that is derived from Wahajuddin et al. (2011), and equations 4 and 5 describe how the effective permeabilities are converted to $k_a$ and $f_a$ as derived from Yu and Amidon (1999). For the calculation of the $k_a$ and $f_a$ values with equations 4 and 5, an intestinal radius ($R$) of 0.18 cm for rat was used and a small intestinal transit time ($T_{\text{app}}$) of 1.47 h (Grandoni et al., 2019).

Physicochemical data ($\log P$, $\log D$, and $pK_a$ values, TPSA), which are used as input to calculate the $f_a$ and tissue: plasma partition coefficients and intestinal uptake were derived with ADMET Predictor software (v9.0, Simulation Plus, Lancaster, California; www.simulations-plus.com; last accessed December 22, 2021) and with ChemAxon (ChemAxon Ltd., Budapest, Hungary; www.chemaxon.com; last accessed December 22, 2021). Given that slight differences occur between the results of these 2 software packages with respect to the log $P$ and $pK_a$ and resulting $D$ estimates, the influence of these differences on the PBK model predictions was evaluated. The log $P$, $\log D$, and $pK_a(s)$ that were obtained for the 44 compounds with each of the 2 software packages are provided in the Github repository (https://github.com/wfsrqivive/rat_PBK.git; last accessed December 22, 2021).

An in silico approach for calculating the $f_a$ values was compared with measured values. For the in silico calculations of $f_a$, a method of Lobell and Sivarajah (2003) was used. Log $P$ and information on the $pK_a(s)$ are required input parameters for this calculation. The codes can be found in the Github repository (https://github.com/wfsrqivive/rat_PBK.git; last accessed December 22, 2021). The in vitro-derived rat-specific $f_a$ values for 39 compounds were taken from the httk package with the original data measured by Wetmore et al. (2013), Wood et al. (2017), and Honda et al. (2019).

For the calculation of partition coefficients, 3 approaches were compared including the in silico approaches of (1) Rodgers and Rowland (2006), (2) Berezhkovskiy (2004), which corresponds to the corrected method of Poulin and Theil (2002), and (3) the in silico approach of Schmitt (2008). Log $P$ and information on the $pK_a(s)$ are required input approaches for these calculations. The R codes for these different calculation methods were obtained from Utsey et al. (2020) and were adjusted to fit the pipeline of the PBK model calculations of the current study. The codes can be found in the Github repository as part of the input parameters (https://github.com/wfsrqivive/rat_PBK.git; last accessed December 22, 2021). As input for the prediction of the partition coefficients, the standardized tissue composition data from Utsey et al. (2020) were used. In case of the method of

### Table 1. Input Approaches Applied in the PBK Model Predictions

| Applied Input                              | Method Reference | Method Name Used in the Figures | Number of Compounds for Which the Respective Data Are Available |
|--------------------------------------------|------------------|---------------------------------|---------------------------------------------------------------|
| Intestinal uptake ($k_a$ and $f_a$)        | Hou et al. (2004) | QSAR                             | 44                                                            |
| QSAR based on the topological surface area (TPSA) | This work        | In vitro                         | 26                                                            |
| Caco-2 $P_{\text{app}}$                    |                  |                                 |                                                               |
| Physicochemical characteristics            |                  |                                 |                                                               |
| $\log P$, $\log D$, $pK_a$, TPSA           | ADMET predictor  | ADMET                            | 44                                                            |
| $\log P$, $\log D$, $pK_a$, TPSA           | ChemAxon         | ChemAxon                         | 44                                                            |
| Tissue: plasma partition coefficients       |                  |                                 |                                                               |
| Berezhkovskiy                              | Berezskiovskiy   | Berezskiovskiy                   | 44                                                            |
| Rodgers and Rowland                        | Rodgers and Rowland | RodgersRowland                  | 44                                                            |
| Schmitt                                    | Schmitt          | Schmitt                          | 44                                                            |
| Intrinsic hepatic clearance (Cl_{in})      |                  |                                 |                                                               |
| (Cryopreserved) primary hepatocytes         |                  | Hep                              | 38                                                            |
| Liver S9                                   |                  | S9                               | 25                                                            |
| In silico predicted CYP clearance           |                  |                                 |                                                               |
| Fraction unbound plasma                    |                  |                                 |                                                               |
| In vitro with rapid equilibrium dialysis    |                  |                                 |                                                               |
| In silico predicted based on $\log P$, $\log D$, and $pK_a$ | Lobell and Sivarajah (2003) | In silico                         | 44                                                            |
Schmitt, the membrane affinity (log MA) was calculated from the log P based on a QSAR from Yun and Edginton (2013) as provided in the R code.

Three different approaches to obtain model parameter values for hepatic intrinsic clearance were compared. These include (1) an in silico approach, (2) an in vitro approach based on clearance studies with primary rat hepatocytes, and (3) an in vitro approach based on clearance studies with rat liver S9. In silico clearance calculations (rat microsomal P450 clearance) were carried out with the ADMET Predictor (v.9.0, Simulations Plus, Lancaster, California). The primary rat hepatocyte clearance data were derived from the database of the R httk package, containing the values that were originally measured by Wetmore et al. (2013), Wood et al. (2017), and Honda et al. (2019).

For 25 compounds out of the 44 compounds, the intrinsic hepatic clearance was measured in the current study in incubations with rat liver S9 in the presence of a mix of cofactors (NADPH, UDPGA, and PAPS) as described in the Supplementary Material. The in vitro compounds for which the S9 clearance rates were determined, were selected based on the expected clearance to include compounds with low (0–20 μl/min/mg S9 protein), medium (20–100 μl/min/mg S9 protein), and high (>100 μl/min/mg S9 protein) in vitro intrinsic hepatic clearance.

All in vitro-derived hepatic clearance values were corrected for unspecific binding to hepatocytes or S9 using a calculation method of Kilford et al. (2008) for primary hepatocytes and a method of Hallifax and Houston (2006) for the S9 clearance measurements. Although the latter calculation method was developed to predict the unbound intrinsic clearance in microsomal incubations, it was assumed to also be applicable to S9 incubations. The codes can be found in the GitHub repository as part of the input parameters (https://github.com/wfsrqivive/rat_PK.git; last accessed December 22, 2021). The in silico calculated clearance rates already represent the unbound clearance rates and no correction was required.

Rat PBK model predictions and data analysis. By combining different input approaches, a total of 3960 Cmax predictions were made for the different model compounds at the same exposure conditions as used in the in vivo studies from which the reported Cmax values were obtained. For each chemical, the predicted Cmax was divided by the observed Cmax as marker of the quality of the of PBK model prediction for that compound. As a result of the different input combinations a range in predicted:observed ratios is obtained for each chemical of which the median was calculated. When these median predicted Cmax outcomes were within 5-fold of the observed Cmax, the PBK model predictions were considered adequate for a first estimation of internal dose metrics by a minimal generic PBK model. Cmax predictions within 10-fold of the observed Cmax are less precise, but still considered relevant. A 2-fold cut-off value is also included in the different figures of the present study, as this cut-off value is frequently used in a regulatory context, though particularly for PBK models that are optimized to available in vivo data (Shebley et al., 2018). Median predicted Cmax values that are more than 10-fold higher than the observed Cmax values were considered as overestimated, and median Cmax values that are more than 10-fold lower than the observed Cmax values were considered as underestimated, though the latter did not occur in the present data set (see Results section). The effect of different input approaches on the Cmax predictions was determined, by comparing for each input approach and compound the median Cmax and predicted:observed ratios and determining the differences between the input approaches in predicted median Cmax values.

A sensitivity analysis was performed for the predictions by changing the input value of a parameter by 1% and determining the relative change in Cmax expressed as the normalized sensitivity coefficient (NSC). The sensitivity analysis was performed at an equal oral dose of 1 mg/kg bw for all compounds and input combinations. Each parameter was analyzed individually, keeping the other parameters to their initial values. The R codes for the above analyses can be found in the in the GitHub repository (https://github.com/wfsrqivive/rat_PK.git; last accessed December 22, 2021).

RESULTS

Performance of the Generic PBK Model Based on Different Input Approaches

By combining different input approaches to parameterize the PBK model (Table 1), a total of 3960 Cmax predictions were made for 78 in vivo Cmax results (ie, exposure situations described in the literature with reported Cmax values) for a total of 44 compounds. In Figure 1, the ratios between PBK model-predicted Cmax values and in vivo-observed Cmax values are shown. The results for bisphenol A, curcumin, daidzein, fluvastatin, genistein, resveratrol, rosuvastatin, and ochratoxin A represent the comparison of the predicted Cmax with observed Cmax values form multiple in vivo studies, whereas for the other compounds in vivo data were obtained from single studies. Figure 1 reveals a large variation (1–6 orders of magnitude) in the prediction:observed ratios. The largest range in predicted:observed ratios are observed for bisphenol A, curcumin, perethrin, and resmethrin. For these compounds, some of the individual Cmax predictions were 210-, 3343-, 553-, and 806-fold higher than the observed Cmax, respectively, and some predictions were 76-, 1.8-, 1183-, and 208-fold lower, respectively. Within the dataset as a whole, median Cmax predictions of 30 compounds were within 10-fold, whereas 14 compounds were predominantly overestimated, that is, having a median predicted Cmax that is >10-fold higher than the observed Cmax. Twenty-three compounds could be predicted within 5-fold and 12 compounds within 2-fold of the observed Cmax.

Sensitivity Analysis

A sensitivity analysis was performed by changing the input value of a parameter by 1% and determining the relative change in Cmax expressed as the NSC. Figure 2A shows the results for the most influential input parameters that affect the Cmax predictions (maximum observed NSCs >0.5 in absolute value). The NSCs of remaining input parameters are presented in Supplementary Figure 1. The results of the sensitivity analysis show that the chemical-specific input parameters related to the extent of metabolic clearance are most influential. This includes the intrinsic clearance parameter (Clint) itself, but also parameters that relate to the free available concentration for the metabolic conversion (fup, Kpli) and parameters that determine the blood flow and the volume of the liver (FQh, QC). Other important input parameters that affect the Cmax predictions relate the oral absorption (ka and f0). B:P ratio also showed to be a sensitive parameter. This parameter was set to a default value of 1 for all compounds in the present study, because measured data on B:P ratios are generally lacking and no in silico tools are available to estimate the B:P ratio. The observed differences between compounds in sensitivity toward the input parameters (see range in NSCs in Figure 2A) were found to primary relate to the intrinsic metabolic clearance of each compound (Figure 2B).
The sensitivity increases with increasing Cl_{int,u} until a maximum sensitivity is reached for compounds with a high Cl_{int,u} (Figure 2B). A similar relation is observed between the Cl_{int,u} of the compounds and the sensitivity toward the other input parameters that are depicted in Figure 2A, with low clearance compounds being less sensitive to changes in the input parameters than high clearance compounds (Supplementary Figure 2).

**Effect of the Input Approaches on the C_{max} Predictions**

Given the wide range in C_{max} outcomes and corresponding predicted:observed ratios in Figure 1 and the sensitivity of the C_{max} predictions to chemical-specific input parameters like Cl_{int}, f_\text{up}, f_\text{a}, and k_a, it is important to identify how the C_{max} predictions are affected by the different input approaches. To this end, we defined for each input approach the range in C_{max} predictions that are obtained and evaluated if systematic differences occurred between the different input approaches that were used to parameterize the generic PBK model. Figure 3 highlights those predictions for which differences (>3-fold) in predicted:observed ratios are observed for a specific chemical as a result of the applied input approach. These results reveal that differences in C_{max} predictions (and corresponding predicted:observed ratios) most frequently occur as a result of differences in calculation methods for the partition coefficients (Figure 3A) and the methods used to parameterize hepatic clearance (Figure 3B).

In case of the different approaches for the calculation of partition coefficients, the method of Rodgers and Rowland performed best. The median C_{max} of 32 compounds was predicted within 10-fold of the observed C_{max} values with the Rodgers and Rowland method. With the methods of Berezhkovskiy and Schmitt, 30 and 27 compounds were predicted within 10-fold of the observed C_{max} respectively. Particularly for acidic
compounds (pKa < 6), like ochratoxin A and pentadecafluorooctanoic acid, low Cmax predictions are obtained with the method of Berezhkovskiy compared with the other input approaches. For the highly lipophilic compounds (log P > 5), like etoxalane, novaluron, permethrin, and resmethrin, the method of Schmitt appears to result in overpredictions of the Cmax.

In case of the parameterization of the intrinsic hepatic clearance, the Cmax predictions based on in silico-derived hepatic intrinsic clearance values appear to be frequently different from the predictions based on in vitro S9 and/or hepatocyte intrinsic clearance data. Particularly in case of curcumin, metoprolol, and resveratrol, the in silico-calculated clearance values lead to an overprediction of the in vivo-observed Cmax. In case of curcumin, this overprediction is even up to 33436-fold. For the compounds for which both in vitro S9 and hepatocyte data are available, significant differences in intrinsic hepatic clearance were only found for metoprolol (Figure 3B).

Occasional outliers were found to occur in the Cmax predictions as a result of the uptake parameters (ka and fa) (Figure 3B), and the fup (Figure 3C). The most important outlier was found for bisphenol A, for which the Cmax was significantly (up to 806-fold) underestimated when the in vitro-measured fup is used in the simulations compared with the in silico-calculated fup. Given that the relatively high in vitro-measured fup of 0.71 does not match with another reported in vitro fup by Csanády et al. (2002) of 0.05 and the in silico-predicted fup of 0.04, the simulations based on the fup of 0.71 were considered to be incorrect. In case of permethrin and resmethrin, the in silico-predicted uptake rates (Figure 3B) were found to provide Cmax results that are closest to the in vivo-observed Cmax values. A key challenge with these compounds, that might have caused the high variation in Cmax predictions, is the relatively high lipophilicity of these compounds (log P values larger than 5), which may hamper reliable performance of the in vitro studies with Caco-2 cells, providing unreliable Papp values to derive the uptake rate.

**Performance of the Optimized PBK Model**

Figure 4 depicts the results of the dataset in which the most significant outliers as described above are removed. This includes a removal of the simulations based on (1) the methods of Berezhkovskiy and Schmitt for the partition coefficients, (2) the in silico-derived intrinsic hepatic clearance data, and (3) the in vivo-derived fup values. In addition, the in vitro-derived uptake rates for permethrin and resmethrin were removed as identified significant outliers. The results of the reduced dataset that was obtained shows a significant reduction in the variation in Cmax predictions and the related predicted:observed Cmax ratios. The remaining relatively high variation in predicted:observed Cmax ratios as observed for bisphenol A, daidzein, genistein, and resveratrol in Figure 4 can be attributed to the variation in the related in vivo studies.

The median Cmax prediction for curcumin, boscalid, and chlorpyrifos improved and could be predicted within 10-fold in the optimized dataset. In contrast, the median Cmax of fluvastatin becomes more than 10-fold overestimated in the reduced dataset. Overall, the removal of the most significant outliers mainly resulted in an increased number of predictions within 10-fold. Median Cmax predictions of 32 compounds were within 10-fold within the reduced dataset, whereas 12 compounds were predominantly overestimated, that is, having a median predicted Cmax that is >10-fold higher than the observed Cmax. Twenty-two compounds could be predicted within 5-fold and 8 compounds within 2-fold of the observed Cmax.

We assessed whether we could find a relation between chemical (kinetic) characteristics (such as extent of metabolic clearance, charge, lipophilicity, or uptake rate of the compounds) and the chance of being predicted within 10-fold as
are available for the 5 compounds as well as the C in vitro provides an overview of the fold (Supplementary Material).

lipophilicity, etc.) and the chance of being predicted within 10-fold (ie, low or high clearance compounds). Table 2 obtained with respect to the range in predicted:observed ratios and the type of compounds that are likely to be predicted within 10-fold (Supplementary Material). For the compounds that had a clearance between 1 and 20 l/h, no relations were identified between chemical characteristics (clearance, charge lipophilicity, etc) and the chance of being predicted within 10-fold (Supplementary Material).

Application of the Minimal PBK Model Approach on In-House BASF Compounds as Case Study

The minimal PBK model approach was applied on 5 in-house BASF compounds as case study to test whether similar results are obtained with respect to the range in predicted:observed ratios and the type of compounds that are likely to be predicted within 10-fold (ie, low or high clearance compounds). Table 2 provides an overview of the in vitro and in silico input data that are available for the 5 compounds as well as the C max predictions that are obtained with the generic PBK model. The physicochemical characteristics were estimated with ChemAxon, the intrinsic hepatic metabolic clearance data were derived from experiments with rat liver S9, and Caco-2 apparent permeability coefficients were derived from in vitro experiments (BASF1-3 and 5) or estimated in silico (BASF4). The partition coefficients were calculated with the calculation method of Rodgers and Rowland. Based on the analysis with the 44 reference compounds, these applied input approaches can be considered to provide the best predictions when applied in this minimal PBK model.

Based on the scaled unbound hepatic clearance values (Table 2), it was expected that the C max predicted for BASF1, 3, and 4 had a high chance to be predicted within 10-fold compared with the in vivo data as these are all low clearance compounds (C int,u <1 l/h) (see Performance of the Optimized PBK Model section). Running the PBK models indicated that the C max of these 3 compounds are indeed within 10-fold of the observed C max. The scaled C int,u values of BASF2 and BASF5 of 2.7 and 14.1 l/h were in the range of scaled C int,u values of compounds for which it was more difficult to discriminate whether overprediction is likely to occur or not (see Performance of the Optimized PBK Model). BASF2 was overpredicted (18- to 65-fold, depending on the dose), whereas the C max of BASF5 was predicted within 5-fold of the observed C max. Additional information (eg, on the solubility, transporter involvement, or extrahepatic metabolism) is expected to be essential to further discriminate whether certain compounds are likely to be predicted by the generic PBK model within 10-fold of the observed C max or not.

**DISCUSSION**

Predictions of internal dosimetry, such as C max, are crucial in the transition toward next generation (animal-free) testing strategies for chemical safety evaluations to convert in vitro toxicity data into in vivo dose-response or at least potency information (eg, DeJongh et al., 1999; Fabian et al., 2019; Forsby and Blaauboer, 2007; Gubbels-van Hal et al., 2005; Louisse et al., 2010; Punt et al., 2019; Verwei et al., 2006; Wetmore et al., 2015). At present, PBK model predictions still need to be evaluated on a case-by-case basis against in vivo kinetic data (Peters and Dolgos, 2019; Tsamandouras et al., 2015). For a transition to next generation (animal-free) regulatory risk evaluations to happen, other means to gain confidence in PBK model predictions are needed. The goal of the present study was to assess the predictive performance of a minimal generic rat PBK model based on in vitro and in silico input data to predict peak plasma concentrations (C max) upon single oral dosing.

Different cut-off values (2-, 5-, and 10-fold) were used as performance indicators in the current study. Discussions are presently still ongoing on what level of deviation between predicted and observed kinetics is acceptable within a regulatory context (Shebley et al., 2018). The required precision of PBK model predictions may depend on the use application. The 10-fold cut-off
value used in the present study provides a relevant indication of whether compounds can be captured with the minimal generic PBK model. For the compounds falling outside this value, deviations between the predicted and observed $C_{\text{max}}$ values ranged between 16-fold (daidzein) and 783-fold (alachlor), which cannot be resolved by optimization of the different input parameters. A 2-fold cut-off value is frequently requested within a regulatory context to demonstrate that the proposed model is fit for purpose (Peters and Dolgos, 2019). A key challenge with this 2-fold is that the differences between in vivo studies, to which the model predictions are compared, tend to be higher than 2-fold themselves, possibly related to differences in biology or technical aspects (Sheley et al., 2018). For example, in the present study, the ranges in predicted:observed ratios for bisphenol A (between 0.15- and 12-fold) were primarily caused by the 60-fold difference in the respective in vivo results, to which the predictions were compared with (Domoradzki, 2004; Pottenger, 2000; Sun Dong Yoo et al., 2001; Tominaga et al., 2006; Upmieier et al., 2000). Taking the different cut-off values into account, the results reveal that most of the compounds fall within the 10-fold range, whereas predictions within the 5- or 2-fold are more difficult to obtain. Although some level of uncertainty in PBK model predictions can be considered acceptable for situations where the margin of exposure between exposure and biological effects is large (Wetmore et al., 2015), further work will be needed to determine how the generic PBK model can be further improved to increase the number of predictions that can be made within 5- or 2-fold to increase the overall precision of the model. Furthermore, based on the chosen cut-off value(s) of required model precision, related uncertainty factors may need to be applied when using in vitro- and in silico-based PBK model results in a regulatory context.

A key challenge with respect to PBK model development is to determine the design of the model structure for a specific compound. The results of the present study revealed that the minimal generic PBK model, based on partition coefficients, $C_{\text{int}}$, $F_{\text{int}}$, and $P_{\text{app}}$ as main chemical-specific input, worked best for compounds that are either extensively metabolized ($C_{\text{int}}>20\,\mu l/h$) or compounds that are metabolized to a limited extent ($C_{\text{int}}<1\,\mu l/h$), as these compounds were frequently found to be predicted within a 10-fold range. Three out of the 5 in-house BASF compounds fell into this category of low-clearance compounds and were also predicted within 10-fold of the observed $C_{\text{max}}$ (BASF1, BASF3, and BASF4). In case of the chemicals that are 10-fold overpredicted it is expected that, for example, a lack of inclusion of extrahepatic metabolism and/or transporter-mediated processes in the PBK model are underlying causes of the deviations between predicted and observed $C_{\text{max}}$ values. In addition, transporter-mediated processes might also be relevant to be included for some of the low-clearance compounds, which have been reported to be substrates of transporter proteins (like pentadecfluoroocotoanic acid, ochratoxin A, and tolbutamide) (Anzai et al., 2010; Bi et al., 2018; Worley and Fisher, 2015). Although the $C_{\text{max}}$ of many of these compounds could be predicted within 10-fold, refinement of the kinetic predictions is expected to be achieved by also including these transporter-mediated processes, particularly to better simulate repeated exposures. Further work will be needed to explore whether recommendations can be made based on these characteristics on the design of a PBK model for a specific compound, particularly to determine when a minimal PBK model will be sufficient and when additional kinetic processes need to be considered.

Comparison of the different input approaches revealed a high influence of the selected input parameters on the PBK model predictions. For example, the results of the present study show that the method of Berezhkovskiy should not be used for acids ($pK_a<6$) as this might lead to underprediction of the $C_{\text{max}}$. This is probably caused by the fact that the impact of drug ionization on partitioning is not explicitly taken into account in the method of Berezhkovskiy (Utsey et al., 2020). The calculation method of Schmitt was found to frequently lead to relative overpredictions of the $C_{\text{max}}$ of highly lipophilic compounds ($log P>5$) and may therefore not be appropriate for this group of compounds. The results of the calculation method of Schmitt largely depend on the membrane affinity as input. As this value was not available for all compounds, it was calculated in the present study based on $log P$ based on a QSAR from Yun and Edginton (2013). Highly lipophilic compounds might not fall into the applicability domain of this QSAR. In case of the hepatic clearance, the in vitro-observed data are preferred above in silico-generated clearance data. The $C_{\text{max}}$ values of various compounds were overpredicted as a result of the use of in silico clearance values, and more importantly a direct comparison of the in silico-estimated clearance values with the in vitro values revealed a relatively poor correlation ($R^2=0.3$, Supplementary Figure 3), indicating that challenges still exist with the

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**Table 2. Chemical-Specific Data of 5 In-House BASF Compounds and Information on PBK Model-Predicted $C_{\text{max}}$ Values**

| Parameter                                                                 | Test Compounds |
|---------------------------------------------------------------------------|----------------|
| Parameter                                                                 | BASF1 | BASF2 | BASF3 | BASF4 | BASF5 |
| Log P ChemAxon                                                            | 1.19  | 2.96  | 3.45  | −2.82 | 3.99  |
| $pK_a(s)$ ChemAxon                                                        | 3.38 (A); 10.7 (A); −0.11 (B) | Neutral | Contains N+ | 0.44 (B) |
| $k_{\text{h}}$ (1/h) and $f_u$ scaled based on the Gac-2 $P_{\text{app}}$ | 1.72 and 0.91; 1.55 and 0.88; 1.27 and 0.84; 1.65 and 0.90 | 46.8  | 37.9  | 25   | 43 a  | 13.8  |
| $f_u$ (in silico predicted)                                               | 0.124 | 0.124 | 0.079 | 0.982 | 0.047 |
| Liver S9 clearance ($\mu l/min/S9\,mg$ protein)                          | 1.9   | 17.3  | 0     | 0     | 53.9  |
| Scaled unbound hepatic clearance ($C_{\text{int,app}}$ $l/h$)              | 0.2   | 2.3   | 0     | 0     | 14.1  |
| Doses (mg/kg bw)                                                          | 4; 40 | 200   | 50; 500 | 3; 30 | 300   | 1.2; 12 | 5; 50 |
| $C_{\text{max}}$ Predicted (mg/l)                                         | 12; 119; 594 | 7; 72 | 0.4; 3.7; 37 | 0.9; 9.4 | 0.2; 1.6 |
| $C_{\text{max}}$ Observed (mg/l)                                          | 7; 14 | 260   | 0.4; 1.1 | 0.2; 3.6; 26.0 | 0.23; 2.3 | 0.09; 0.4 |
| Predicted:observed ratio                                                  | 1.7; 8.5; 2.3 | 18; 65 | 2; 1.4 | 4; 4 | 2; 4 |

A, acid; B, base.

*In silico estimated based on a topological surface area of 0 (zero) $A^2$, using the calculation method of Hou et al. (2004).
predictive value of in silico tools for $C_{\text{int}}$. In case of the $F_u$ and $P_{\text{app}}$ values, a significant influence of in vitro experimental variation on the model predictions was observed. Particularly, for highly lipophilic compounds, like permethrin and resmethrin, the in vitro input data led to underpredictions by the PBK model. These results indicate the importance of standardizing and harmonizing the in vitro approaches to obtain robust results, including in vitro protocol adjustments to work with very lipophilic compounds (Ferguson et al., 2019; Wambaugh et al., 2019).

Overall, the current study provided relevant insights into the predictive performance of a minimal PBK model and the influence of different input approaches on the model predictions. Best performance was obtained when the hepatic clearance was parameterized based on (1) in vitro (hepatocytes or liver S9)-measured intrinsic clearance values, (2) the method of Rodgers and Rowland for calculating partition coefficients, and (3) in silico-calculated fraction unbound plasma and $P_{\text{app}}$ values (the latter especially for very lipophilic compounds). Further work will particularly be needed to find ways to determine, in the absence of prior knowledge on the chemical’s in vivo toxicokinetics, when and which additional kinetic processes (like extrahepatic metabolism or transporter-mediated processes) need to be added to obtain adequate predictions of the in vivo kinetics.

**SUPPLEMENTARY DATA**

Supplementary data are available at Toxicological Sciences online.

**DECLARATION OF CONFLICTING INTERESTS**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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