Prediction of Pharmacokinetic Profile of Valsartan in Humans Based on *in vitro* Uptake-Transport Data

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The aim of this study was to evaluate a physiologically based pharmacokinetic (PBPK) model for predicting PK profiles in humans based on a model refined in rats and humans *in vitro* uptake-transport data using valsartan as a probe substrate. Valsartan is eliminated unchanged, mostly through biliary excretion, both in humans and rats. It was, therefore, chosen as model compound to predict *in vivo* elimination based on *in vitro* hepatic uptake-transport data using a fully mechanistic PBPK model. Plated rat and human hepatocytes, and cell lines overexpressing human OATP1B1 and OATP1B3 were used for *in vitro* uptake experiments. A mechanistic two-compartment model was used to derive the active and passive transport parameters, namely uptake *Michaelis–Menten* parameters (V_{max} and $K_{m,u}$) together with passive diffusion (P_{dif}). These transport parameters were then used as input in a whole body physiologically based pharmacokinetic (PBPK) model. The uptake rate of valsartan was higher for rat hepatocytes ($K_{m,u} = 28.4 \pm 3.7$ μM, $V_{max} = 1320 \pm 180$ pmol/mg/min, and $P_{dif} = 1.21 \pm 0.42$ μl/mg/min) compared to human hepatocytes ($K_{m,u} = 44.4 \pm 14.6$ μM, $V_{max} = 304 \pm 85$ pmol/mg/min, and $P_{dif} = 0.724 \pm 0.271$ μl/mg/min). OATP1B1 and -1B3 parameters were correlated to human hepatocyte data, using experimentally established relative activity factors (RAF). Resulting PBPK simulations were compared for plasma- (humans and rats) and bile- (rats) concentration–time profiles following iv bolus administration of valsartan. Plasma clearances (CL_P) for rats and humans were predicted within twofold relative to predictions based on respective *in vitro* data. The simulations were extended to simulate the impact of either OATP1B1 or -1B3 inhibition on plasma profile. The limited data set indicates that the mechanistic model allowed for accurate evaluation of *in vitro* transport data; and the resulting hepatic uptake transport kinetic parameters enabled the prediction of *in vivo* PK profiles and plasma clearances, using PBPK modelling. Moreover, the interspecies difference in elimination rate observed *in vivo* was correctly reflected in the transport parameters determined *in vitro*.

1. Introduction. – Prediction of pharmacokinetic parameters remains an important challenge during drug discovery and development. The ability to predict human pharmacokinetics based on a combination of *in vitro* ADME properties and physicochemical properties integrated into physiologically-based pharmacokinetic models has been demonstrated in a number of recent studies [1–3]. This approach proved to be very successful for lipophilic compounds mainly eliminated by liver metabolism and well permeable through cell membranes. For such compounds, a number of *in vitro* models such as liver microsomes or hepatocytes provide quantitative input parameters for metabolism, and are widely used for compound selection during the drug-discovery process and for quantitative predictions of *in vivo* clearance in animals and humans [4–7]. Such information proved also to be useful for quantitative prediction of full concentration–time profiles in animals and humans using physio-

logically based models [1–3]. Common approaches to optimize compounds during drug discovery are based on lipophilicity, permeability, and metabolic stability. The optimization of compound properties for improved metabolic stability, and, therefore, reduced lipophilicity and permeability has contributed to shift the elimination routes of compound towards non-metabolic processes [8]. As a consequence, transporter-mediated processes including uptake and efflux transport are increasingly found to be responsible for clearance of compounds [9–11]. Important limitations of the PBPK approach are realized for low-permeable BDDCS class-3 and -4 compounds for which hepatic uptake and biliary elimination are likely to be major components of the elimination process, and where the assumptions of flow-limited distribution and well-mixed compartments are not valid. Such processes can be addressed by the addition of permeability barriers for some tissues, and by the incorporation into a more complex liver model which includes active uptake into the liver, active efflux into the bile, biliary elimination, and enterohepatic recirculation. However, this improvement of current methodologies requires the availability of the appropriate quantitative input data as well as validation of the corresponding *in vitro* to *in vivo*-scaling approaches.

Biliary clearances have been measured *in vitro* in sandwich culture hepatocytes resulting in an acceptable scaling of biliary clearance to *in vivo* [12–15]. The uptake transport, measured *in vitro* as a clearance term, has already been integrated in the global hepatic intrinsic clearance together with metabolic, passive, and efflux transport clearances, in several studies [16][17]. *Paine et al.* successfully predicted *in vivo* hepatic clearance of three compounds [17] based on *in vitro* uptake data. In addition, a mechanistic two-compartmental model to derive active and passive uptake parameters from hepatocytes and overexpressing cells was recently described by *Poirier et al.* [18]. Using such transport parameters, rats *in vivo* profiles of metabolically stable drugs, fexofenadine and napsagatran, were successfully predicted using a whole body PBPK model [19].

In this study, valsartan was used as a reference compound to further validate our PBPK approach for transported compounds. Valsartan, an antihypertensive drug, was chosen as a low-permeable and well-soluble BDDCS class-3 compound [20]. Valsartan is mainly excreted unchanged through biliary excretion after iv bolus in rats [15] and in healthy male volunteers [21]. Valsartan is a very good test compound to assess the ability to predict uptake clearance based on *in vitro* data, since, in both rats and humans, hepatic clearance is much less than the hepatic blood flow [22][23]. In rats, valsartan is known to selectively accumulate in the liver and to be actively excreted into bile *via* the murine Mrp2 protein [22]. In man, the drug is taken up into hepatocytes mainly *via* OATP1B1 and OATP1B3, and excreted into bile possibly by the human MRP2 transporter [22]. In clinical trials, impairment of hepatic function doubled the area under the plasma-concentration–time curve compared to healthy volunteers, confirming the importance the hepatic elimination pathway in valsartan elimination [24]. In this context, drug–drug interactions (DDIs) at the transporter level were also investigated for their impact on the plasma profile.

The aim of this study was to evaluate the prediction strategy using physiologically based pharmacokinetic modeling by first verifying and possibly refining the PBPK simulations in rats, and then predicting the PK profile in humans together with the alteration of this PK profile through the interaction at the transporter level. For this

purpose, active transport parameters have been measured in rat and human hepatocytes, as well as in OATP-transfected cells, allowing the estimation of relative activity factors for the main liver OATPs. The performance of the physiologically based approach in predicting human pharmacokinetics was also compared to allometric scaling.

2. Strategy. – The strategy followed for the evaluation is similar to the one used in [18][19] and is presented in *Fig. 1*. *In vitro* uptake hepatocytes data were analyzed with a mechanistic two-compartment model. The calculated parameters were then put into a whole-body PBPK model to simulate plasma and bile profiles, and compared to the *in vivo* observations. The first step of the approach consisted in refinements of the rat PBPK model based on the rat physiology and rat hepatocytes *in vitro* data in order to comply with the *in vivo* observations. The refined rat PBPK could then be used as a model for the human PBPK, using human physiology and human hepatocytes *in vitro* data.

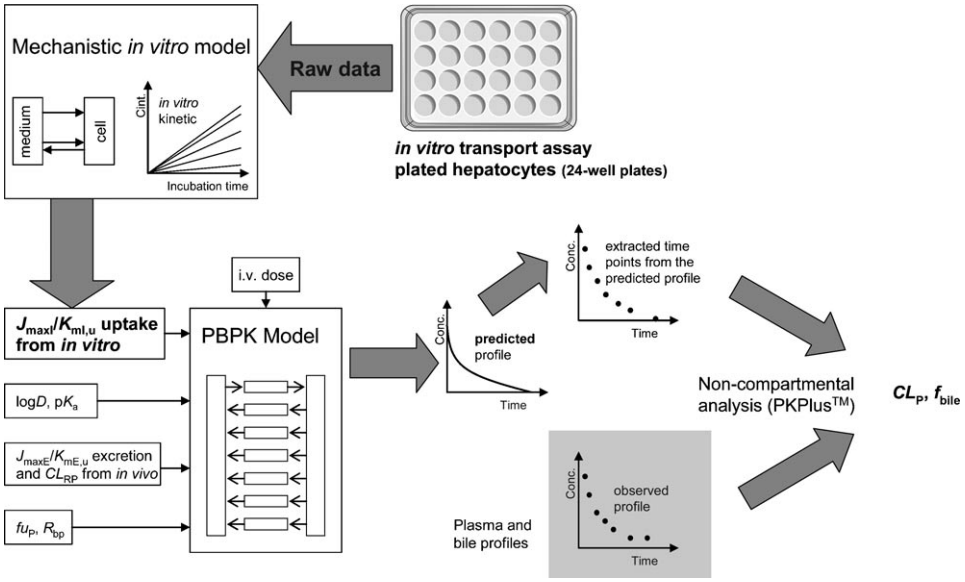

Fig. 1. *Steps taken in the evaluation of the use of* in vitro *transport data to predict* in vivo *plasma profiles* (adapted from [25])

3. Results. – Valsartan is a hydrophilic, poorly permeable acid highly bound to plasma protein in both rats and humans [22][23][26]. The drug affinity in plated hepatocytes (lumped value) was similar in both rats and humans ($K_{m,u} = 28.4 \pm 3.7$ μM in rats and $K_{m,u} = 44.4 \pm 14.6$ μM in humans), as well as the passive permeability through the basolateral membrane ($P_{dif} = 1.21 \pm 0.42$ μl/mg/min in rats and $P_{dif} = 0.724 \pm 0.271$ μl/mg/min in humans). However, the maximum velocity, V_{maxI}, in humans was more than fourfold lower than the value in rats ($V_{maxI} = 1320 \pm 180$ pmol/mg/min in rats and $V_{maxI} = 304 \pm 85$ pmol/mg/min in humans).

3.1. *Refinement of the Rat PBPK Model.* The possibility to simulate *in vivo* profiles based on *in vitro* uptake data was initially verified in the rat based on plasma-concentration–time profiles and biliary-excretion rates. The simulations using the input data measured *in vitro* in the absence of any further modification of the rat PBPK model led to a simulation in reasonable agreement with the observed data, and the plasma clearance was underpredicted by twofold. However, the simulated biliary-excretion profile underestimated the observed data, with a predicted fraction excreted in bile (f_{bile}) of 50 *vs.* 70–85% observed [15][22]. A sensitivity analysis was performed in order to identify the parameters to be optimized in order to match the *in vivo* profile in the rat. As shown previously, uptake capacity can be a very sensitive parameter for both the plasma and biliary-excretion profiles [19]. Thus, the uptake maximum velocity, J_{maxI}, had to be adjusted by a factor of 5 to match both plasma-concentration and biliary-excretion profiles. Furthermore, the excretion parameter, J_{maxE}, which was associated with a high uncertainty due to the lack of information on liver concentration, was fitted to a lower value: 0.0002 *vs.* 0.0126 mg/s initially (63-fold difference with uptake rate). These adjustments led to an improved simulation of the *in vivo* plasma and biliary-excretion profiles (*Fig. 2*,*a* and *b*). To capture all features of the concentration–time profile, *i.e.*, the terminal phase as well as the volume of distribution, which was underpredicted by a factor of 10, enterohepatic recirculation (EHR) had to be considered. In Fig. 2,*c*, EHR was added in the model and allowed for correct prediction of both clearance and volume of distribution, and of the full concentration–time profile. The intestinal permeability was fitted to 1.3×10^{-4} cm/s.

3.2. *Prediction of Human PK Based on the Adjusted Rat Model and* in vitro *Human Data.* The adjusted model developed in rats was transferred to the human situation: a scaling factor of 5 was applied to humans J_{maxI} (1.21 mg/s), and the unknown excretion parameter J_{maxE} was set to a 63-fold lower value than uptake (0.00382 mg/s) as in rats. In addition, the same intestinal permeability as in the rat was used. Based on its physicochemical properties (low log *D* and PAMPA values), intestinal permeability of valsartan is expected to be very low. However, OATP transporters are among the transporters responsible for drug uptake in the gastrointestinal tract, and the compound is substrate of several drug transporters including OATPs [22]. These processes are most likely responsible for the good bioavailability of 40% observed for valsartan in man [23]. The resulting prediction of human iv and po profiles based on the refined rat PBPK model and including EHR is shown in *Fig. 3* and yielded the best plasma clearance prediction (*Table*).

3.3. *Human OATP1B1 and OATP1B3 Data, and Relative Activity Factors, and Application to Human PBPK Prediction and DDI on OATPs.* Valsartan was characterized by similar $K_{mI,u}$ and V_{maxI} for both hepatic OATPs ($K_{mI,u1B3} = 23.5 \pm 2.2$ μM, $V_{maxI1B3} = 261 \pm 19$ pmol/mg/min, $K_{mI,u1B1} = 17.8 \pm 1.3$ μM, $V_{maxI1B1} = 261 \pm 15$ pmol/mg/min). CCK8 was chosen as selective substrate of OATP1B3, transfected cells data, and cryopreserved human hepatocytes data, *i.e.*, 3 kinetics each, 42 data points per kinetics, were fitted simultaneously using *Eqns. 1* and *2* to obtain $RAF_{OATP1B3}$. In the absence of any true selective substrate of OATP1B1, valsartan data were used to evaluate $RAF_{OATP1B1}$ fitting simultaneously the transfected cells (OATP1B1 and -1B3) and human hepatocytes data, *i.e.*, 3 kinetics each, 42 data points per kinetics, with a fixed $RAF_{OATP1B3}$ applying the two-compartment model. Resulting

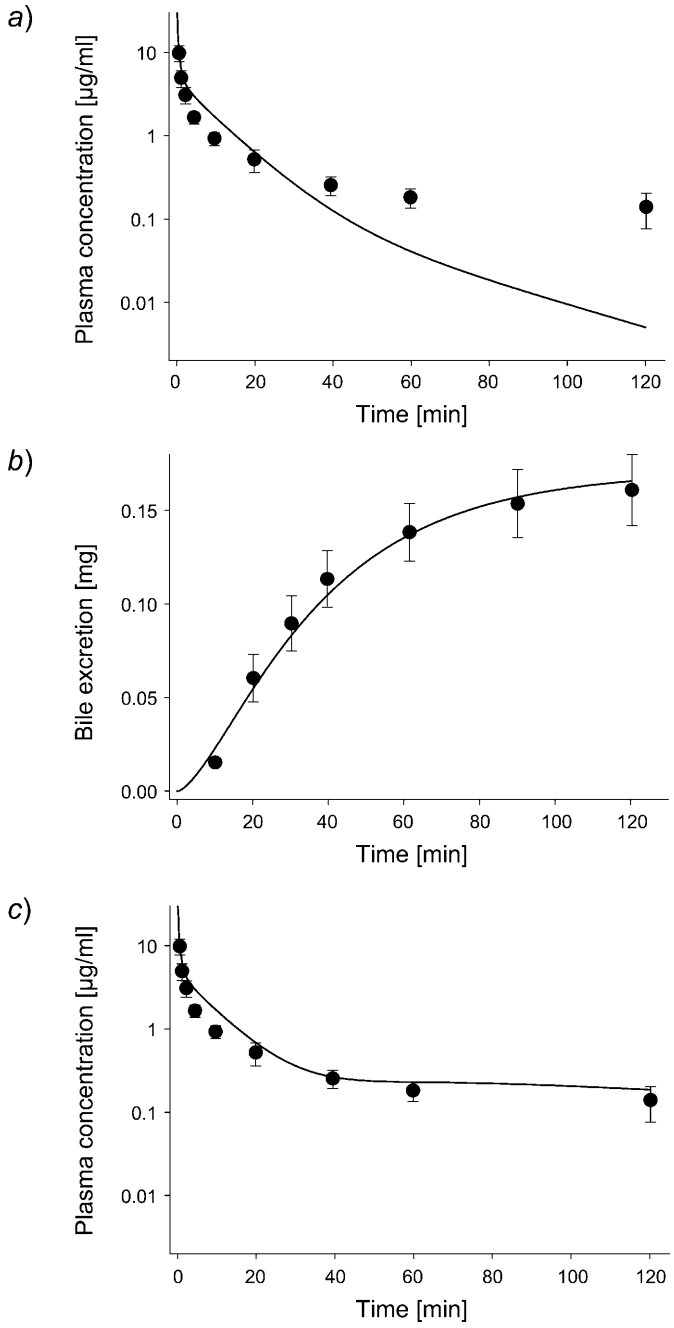

Fig. 2. *Valsartan rat plasma and biliary-excretion profiles* (simulated (line) and observed (symbols) data (extracted from [22])). Rats received an iv bolus of 1 mg/kg. PBPK Simulations in *a* and *b* are based on adjusted parameters: $J_{maxI} = 0.0630$ mg/s and $J_{maxE} = 0.0002$ mg/s. PBPK Simulation in *c* includes enterohepatic recirculation with enhanced intestinal permeability ($P_{eff} = 1.3 \times 10^{-4}$ cm/s).

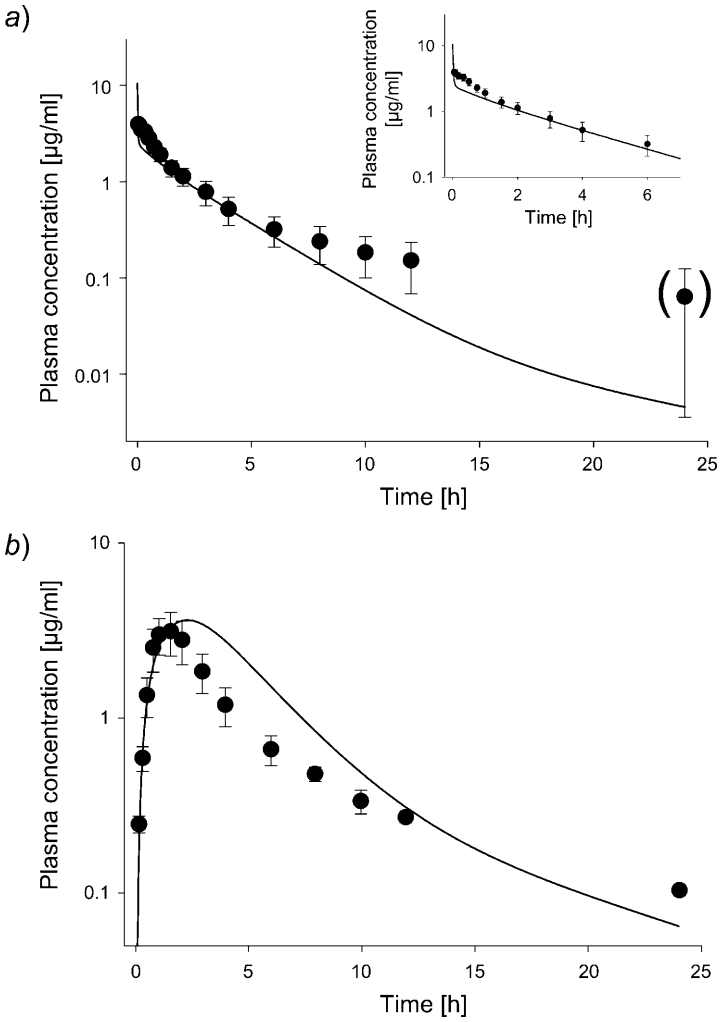

Fig. 3. *Valsartan human plasma and biliary-excretion profiles* (simulated (line) and observed (symbols) data (extracted from Fig. 3 in [23])). Healthy volunteers received an iv bolus of 20 mg (*a*) or were administered an oral solution of 80 mg (*b*). PBPK Simulations were obtained applying the adjusted model: $J_{maxI} = 1.21$ mg/s and $J_{maxE} = 0.00382$ mg/s. Enterohepatic recirculation with enhanced intestinal permeability ($P_{eff} = 1.3 \times 10^{-4}$ cm/s) was included in the PBPK model used for predictions. The brackets represent the uncertainty linked to the value of the data point, as it was extracted from a graph in linear scale.

RAFs will be specific of the cells used in the *in vitro* experiments, namely recombinant cells and the batch of cryopreserved human hepatocytes. To extend the use of those factors from one donor to a wider population, five batches of fresh human hepatocytes were incubated with seven tests substrates: estrone-3-sulfate, taurocholate, CCK8, valsartan, fexofenadine, napsagatran, and pitavastatin. No significant difference could be observed between the cryopreserved batch and the five batches of fresh cells.

Table. *Comparison of Observed and Predicted Systemic and Hepatic Plasma Clearances in Both Rats and Humans* (iv bolus)

	Rats [ml/min/kg]		Humans [ml/min/kg]	
	CL_P	CL_{HP}	CL_P	CL_{HP}
Observed				
As described in original publication	17.8 ± 2.5	12.5 ± 1.0	0.493 ± 0.088	0.353
As evaluated in present study extracting data	11.4	8.0	0.439	0.309
Predicted[a])				
Allometry	na[b])	na	4.30	3.00
Well-stirred model using respective *in vitro* CL_{int}		9.3		0.534
PBPK Basic prediction	6.6	4.6	0.286	0.200
PBPK Refined model	16.6	11.6	0.827	0.579
PBPK Refined model + EHR	na	na	0.682	0.477

[a]) For allometric and PBPK predictions, CL_{HP} was estimated as 70% of the CL_P in both species, mainly for comparison with the observed data and the well-stirred model evaluation. [b]) na: Non-applicable.

To evaluate the impact of inhibition of transporters on the plasma-concentration profiles, PBPK simulations were performed using *Eqn. 2* with two distinct uptake transporters, OATP1B1 and OATP1B3, keeping the adjusted human model. The outcome simulation was very similar to that obtained with full hepatocyte data (*Fig. 4*). Individual $K_{mI,u}$ values were subsequently increased to simulate inhibition of each uptake transporter, outcome prediction are shown in *Fig. 4*.

4. Discussion. – The use of PBPK modeling based on *in vitro* ADME properties and physicochemical properties has been shown to be successful for high-permeability compounds, but further validation needs to be performed to extend its applicability to compounds with permeability-limited disposition. As transporter-mediated processes are increasingly found to be responsible for clearance of permeability-limited compounds, such processes need to be taken into account for accurate predictions into man. Thus, improvements of current methodologies require the appropriate quantitative input data as well as validation of the corresponding *in vitro* to *in vivo*-scaling approaches. Successful attempts to predict pharmacokinetics *in vivo* based on *in vitro* transport data have been reported recently by our group [19] and by others [17]. These studies are, however, limited to the rat. This work represents an attempt to validate the strategy to predict human pharmacokinetics using physiologically based model following the previously published strategy which involves initial verification and optimization in the rat [3]. Such a methodology applied to metabolized and renally cleared compounds has been shown to significantly improve the predictions over empirical techniques such as allometric scaling for example [1–3]. Attempts to apply allometric scaling techniques to biliary excretion have resulted in poor predictions of human clearance. Thus, the biliary clearances of susalimod [27] and napsagatran [28] were overestimated by 20- and sevenfold, respectively, and other similar failures to predict the human pharmacokinetics from pre-clinical data have also been reported [29–31]. These failures are probably due to species differences in the transport rate, protein isoform, and abundance [32][33]. This was confirmed in this study with

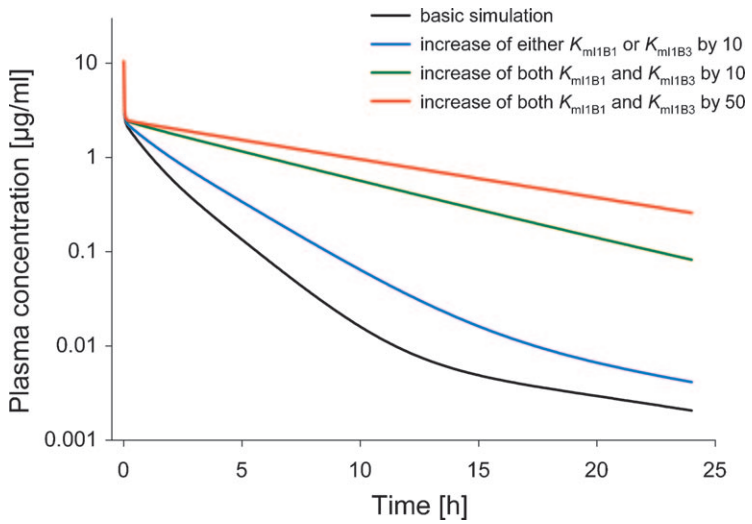

Fig. 4. *PBPK Predictions of valsartan concentration–time profile using two distinct uptake transporters: OATP1B1 and -1B3.* To simulate competitive inhibition of the transporters, their respective $K_{mI,u}$ values were increased by a factor 10 (90% inhibition) or 50 (98% inhibition).

valsartan: human predicted plasma clearance based on rat value was overpredicted by ninefold compared to the published data. Thus, mechanistic approaches based on *in vitro* human transport data and physiologically based scaling methods are recommended rather than empirical allometric scaling for prediction of human biliary clearance. This was exemplified with valsartan in this study and by other examples [16][17], for which encouraging results were achieved: prediction of the hepatic clearance based on *in vitro* intrinsic clearance using the well-stirred model led to a more accurate prediction in human than allometric scaling.

A further step in PK prediction is the use of PBPK models to simulate plasma-concentration–time profiles while allowing a mechanistic description of the pharmacokinetic processes. Following our proposed two-step strategy, the PBPK model was verified first and refined in the rat to confirm the validity of the assumptions used based on simulations of plasma and bile-excretion profiles. Based on rats, although clearance predictions were within twofold, it was necessary to adjust J_{maxI}, which turned out to be one of the most sensitive parameter, by a fivefold to match the biliary excretion and the shape of plasma-concentration–time profile more accurately. In agreement with the strategy proposed, such adjustment was then applied to the human parameter.

Several hypotheses can be proposed to rationalize the improvement of plasma-profile prediction with increasing J_{maxI}: *i*) reduced uptake activity in isolated hepatocytes *in vitro* compared to *in vivo*, as it has already been suggested [34][35], *ii*) missing process(es) (intracellular binding, efflux back into medium) in the *in vitro* two-compartment model used for data analysis, *iii*) misevaluation of the scaling factors (MTPMH, HPGL, cell volume) which were established for metabolism IVIVC, *iv*) bias in the incubation conditions and *in vitro* setting. More data need to be generated in this context using multiple drugs to evaluate the consistency of such J_{maxI} adjustments.

Similar adjustments are used, for example, for metabolic stability where refinement and validation of the PBPK model in pre-clinical species as a first step is a common strategy to apply for predicting human situation [3][36][37].

An additional complexity in this study was the need to account for enterohepatic recirculation. The refined model including enterohepatic recirculation (EHR) yielded the best estimate of plasma profile for valsartan. This is likely to be the case for substrates of hepatic drug transporter(s) in general, since such compounds can exhibit reasonable intestinal permeability and are likely to be substrates of intestinal transporters. The enterohepatic recycling can, therefore, be an important step in the disposition of actively transported drugs and an important process to include in a PBPK model [31]. As PBPK modeling allows mechanistic integration of multiple processes such as metabolism and transport which can be assessed independently *in vitro*, PBPK modeling is very valuable for compounds undergoing mixed elimination.

Apical export into bile was not determined *in vitro* in this study. However, several *in vitro* tools have been described to estimate this process. Double transfected cells or membrane vesicles expressing bile-export transporters can be useful to identify the involved transport protein; however, the issue which remains is the scaling to *in vivo* [8][38]. Recently, the expression levels of 34 transport proteins in liver and other organs of mouse was determined using LC/MS/MS, providing very useful information for *in vitro*–*in vivo* extrapolation of active transport [39]; however, a similar picture for rat and mainly human tissues is still missing. Rat hepatocytes in sandwich culture have been used to calculate *in vitro* biliary clearances characterizing in a single-tool basolateral uptake and canalicular excretion [12–15]. Whereas the rank order of global biliary clearances was consistent from *in vitro* to *in vivo* [14], this method does not allow isolation of the apical transport process nor the calculation of corresponding *Michaelis–Menten* parameters necessary for the parameterization of the bile excretion in a PBPK model. Further development seems to be necessary to use *in vitro* tools for quantitative measurement of the hepatic export rate. However, accurate predictions of plasma-concentration–time profile depend solely on an accurate estimate of uptake into hepatocytes. A good estimate of export rate is, however, essential for predicting liver concentrations.

Relative activity factors (RAF) were first described in the field of CYPs as scaling factors that account not only for the relative abundance of isoform in human liver microsomes but also for the turnover of the protein [26][40]. Thus, RAFs are based on the activity differences between systems rather than their relative abundance. In this study, RAF was defined as ratio of V_{maxI} as the original method for enzymes, but applied to transporters. To evaluate the $RAF_{OATP1B3}$ and $RAF_{OATP1B1}$, CCK8, an OATP1B3-specific substrate, was used together with valsartan, transported by both OATPs. Thus, both RAFs reflected the transporter's activity in our OATP-transfected cells relative to their activity in the batch of cryopreserved hepatocytes, independently of the substrate used. As observed by *Yamashiro et al.*, valsartan was consequently evaluated to be taken up equally by OATP1B1 and OATP1B3 for the unique batch of human hepatocytes used [22]. Data obtained with five different substrates indicated that no significant differences are expected between the cryopreserved batch and fresh cells, meaning that valsartan parameters and the RAF are probably relevant for a wider population. Established RAFs for transport proteins will allow *i*) to relate transporter

overexpressing cells activity to human hepatocyte – and so *in vivo* – activity for a new compound in development, *ii*) to build human PBPK models based on individual uptake transporter values, *iii*) to evaluate the impact on plasma profile of the modulation of a specific transporter using such PBPK model. Thus, using the transporter-transfected cell values and the RAFs, PBPK predictions were made for valsartan human plasma profile including predictions of transport inhibition. As OATP1B1 and -1B3 contributed equally to the valsartan uptake, 90% inhibition of either protein produced the same plasma profile and a 37% decrease of CL_P compared to the basic simulation. If both transporters were to be inhibited at the same time (90% inhibition), the clearance was decreased by 77%. Together with the immortalized cell results, those simulations could be used in drug development as a starting point for potential clinical DDI studies. In clinic, impairment of the hepatic function is already known to decrease significantly valsartan CL_P, and its plasma AUC has been shown to be sensitive to OATP1B1 polymorphisms [24][41]. Several drugs on the market are known to interact with OATPs such as statins, other sartans, antibiotics, or antidiabetic drugs [8][42]; however, no specific inhibitor is known.

5. Conclusions. – In summary, the limited data set indicated that the mechanistic two-compartment model allowed for accurate evaluation of *in vitro* transport data, and the resulting hepatic uptake transport kinetic parameters enabled the prediction of *in vivo* PK profiles and plasma clearances, using PBPK modeling. Moreover, the interspecies difference in elimination rate observed *in vivo* was correctly reflected in the transport parameters determined *in vitro*. The PBPK model refined in preclinical species such as the rat allowed for accurate prediction of the human plasma and bile profiles based on the respective *in vitro* transport data. With regard to plasma clearance, the active OATP-mediated hepatic uptake was the rate-limiting step in valsartan hepatic elimination; and the established model was successfully extended to investigate the impact of transporter inhibition on plasma profile.

Experimental Part

1. *Material and Methods.* 1.1. *Preparation and Plating of Hepatocytes.* Isolation and conventional primary culture of rat hepatocytes were performed as described in [43][44]. Human hepatocytes were from *KaLy-Cell*, F-Besancon, or *Hepacult GmbH*, D-Munich. Plateable cryopreserved human hepatocytes were purchased from *BD Gentest*™ (Woburn, MA; lot 77), the donor was a 27 year-old female Caucasian with brain tumor. Conventional primary culture of plateable cryopreserved human hepatocytes was performed as recommended by *BD Gentest*™.

1.2. *Kinetic* in vitro *Experiment.* Recombinant cells or hepatocytes were prepared as outlined above, and the uptake assays were performed as described in [18]. Kinetic experiments were carried out as described above on 24-well plates. One experiment consisted of the drug of interest incubated at seven or eight different concentrations which were adjusted, after a first experiment, to allow proper K_m and P_{dif} evaluation. Three different time points were used per drug concentration (30, 60, and 90s), and each time point was performed in duplicate. Kinetic parameters were calculated using the whole data set in a single step as described later, and three independent experiments were performed using either different batches of recombinant cells, rat hepatocytes prepared from three different rats, or cryopreserved human hepatocytes (lot 77) thawed on three different days.

1.3. In vivo *Studies.* Rat plasma- and bile-concentration–time profiles were extracted using i-extractor 1.0 (*Linden Software Ldt*, UK) from Fig. 8 in [22] (iv bolus 1 mg/kg). Human plasma-

concentration–time profiles iv and po were extracted from the main study by *Flesch et al.* [23] using i-extractor 1.0 (*Linden Software Ldt*, UK).

2. *Data Analysis.* 2.1. In vitro: *Data Analysis of Uptake Experiments Using Compartmental Model.* The cellular uptake process consists in both an active, saturable process and a passive component. The active transport can be characterized by *Michaelis–Menten* parameters (V_{maxI}, $K_{mI,u}$), while the passive process is represented by the passive diffusion, P_{dif}. A mechanistic two-compartmental model was used to determine those three parameters from the *in vitro* uptake experiment as described previously by us and was used to simultaneously estimate the fraction bound to culture material (f_b), V_{maxI}, P_{dif}, and $K_{mI,u}$.

2.2. *Relative Activity Factors* (*RAF*s). RAF was defined as ratio of V_{maxI} determined in human hepatocytes and transfected cells. To evaluate the RAF for OATP1B3, *in vitro* kinetics were performed in cryopreserved human hepatocytes and OATP1B3, overexpressing cells for the OATP1B3-specific substrate, CCK8. In the absence of an OATP1B1 specific substrate, the evaluation of $RAF_{OATP1B1}$ was performed using valsartan as a substrate of both OATP1B1 and OATP1B3. $RAF_{OATP1B3}$ evaluated using CCK8 was fixed, and all valsartan *in vitro* data (3 kinetics in each cell types, OATP1B1/1B3 overexpressing CHO cells, and cryopreserved human hepatocytes) were fitted simultaneously.

2.3. *Whole-Body PBPK Model.* A whole-body PBPK model in rats and humans was used in this study; the rat model has been described in [19]. The mathematical model was available in the simulation software *GastroPlus*™ 6.0.0 (*Simulations Plus Inc.*, CA, USA). The human PBPK model was built based on the physiology of a 27 year-old female Caucasian as the hepatocyte donor used in the *in vitro* experiments.

The input data for each compound were molecular weight, log *D*, pK_a, fu_P, R_{bp}, renal clearance, weight, and dose. As described in *Fig. 1*, the simulated plasma and bile profiles were analyzed by non-compartmental analysis to derive CL_P and f_{bile} values for comparison with the *in vivo* observations (no bile observation for humans).

In liver tissues, the extracellular partition coefficient, Kp_e, was used to estimate the concentration of the drug in the extracellular compartment. The drug entry into the liver consisted of a passive permeability process parameterized as a permeability–surface-area product (PS_{TC}) and of active uptake. Drug exit from the liver was driven by active efflux into bile. No active sinusoidal efflux back into the blood was considered. As valsartan is a metabolically stable compound, no metabolic clearance was included in the model.

The mass-balance equations for plasma (*Eqns. 1* and *2*) and liver (*Eqn. 3*) used in the model were as follows:

When using hepatocytes data:

$$\left(V_e + V_P \frac{R_{bp}}{Kp_e}\right)\frac{dC_e}{dt} = Q_L\left(Cb_i - \frac{C_e R_{bp}}{Kp_e}\right) - \left(\frac{J_{maxI} \times C_e, u}{K_{mI,u} + C_{e,u}} + PS_{TC}(C_{e,u} - C_{t,u})\right) \quad (1)$$

When using recombinant cells data:

$$\left(V_e + V_P \frac{R_{bp}}{Kp_e}\right)\frac{dC_e}{dt} = Q_L\left(Cb_i - \frac{C_e R_{bp}}{Kp_e}\right) - \left(\frac{J_{maxI1B1} \times RAF_{OATP1B1} \times C_{e,u}}{K_{mI,u1B1} + C_{e,u}} + \frac{J_{maxI1B3} \times RAF_{OATP1B3} \times C_{e,u}}{K_{mI,u1B3} + C_{e,u}} + PS_{TC}(C_{e,u} - C_{t,u})\right) \quad (2)$$

The liver blood flow (Q_L) was taken as 60 ml/min/kg in rat, and as 20 ml/min/kg in humans:

$$V_t \frac{dC_t}{dt} = \frac{J_{maxI} \times C_{e,u}}{K_{mI,u} + C_{e,u}} + PS_{TC}(C_{e,u} - C_{t,u}) - \left(\frac{J_{maxE} \times C_t \times fu_L}{C_t \times fu_L + K_{mE,u}} + PS_{TCAp} \times C_t \times fu_L\right) \quad (3)$$

The basolateral uptake parameters derived from *in vitro* experiments (V_{maxI}, $K_{mI,u}$, and P_{dif}) were scaled to *in vivo* as described below. The affinity $K_{mI,u}$ value estimated *in vitro* was assumed to be identical *in vivo*. PS_{TC} was reduced to a clearance term and scaled to *in vivo* according to *Eqn. 4:*

$$PS_{TC} = P_{dif} \times \text{MTPMH} \times \text{HPGL} \times \text{liver weight} \times fu_{inc} \quad (4)$$

where MTPMH was the milligrams of total protein per million hepatocytes, and HPGL was the hepatocellularity (120.10^6 cells per g of liver for both rats and humans [45]). The unbound fraction in the incubation, fu_{inc}, was considered to be 1, as no protein was added in the incubation medium, and non-specific binding was taken into account in the *in vitro* model [18].

The same scaling factors were applied to V_{maxI} where the molecular weight (MW) was used to convert J_{maxI} in mg/s (*Eqn. 5*):

$$J_{maxI} = V_{maxI} \times \text{MW} \times \text{MTPMH} \times \text{HPGL} \times \text{liver weight} \times fu_{inc} \quad (5)$$

The following equation was used to model the excretion of the non-metabolized compound into the bile (*Eqn. 6*):

$$\frac{\Delta M_{bile}}{\Delta t} = \frac{J_{maxE} \times C_L \times fu_L}{C_L \times fu_L + K_{mE,u}} + PS_{TCAp} \times C_L \times fu_L \quad (6)$$

The amount of drug cleared through bile in rats continuously recirculates into the duodenum, where it is available for reabsorption. However, in the rat, *Yamashiro et al.* studied the plasma kinetics in bile-duct cannulated rats [22], so reabsorption was not allowed in the rat PBPK model.

For humans, the amount of drug cleared through bile was split into two parts. 75% of the bile was assumed to go to the gallbladder where it was stored during fasted state [46]. The remaining 25% continuously recirculated to the duodenum, where it was available for reabsorption. Upon switching to fed state, the fraction of drug stored in gallbladder gets emptied into the duodenum over the specified time period (gallbladder emptying time, 30 min).

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Received March 13, 2009