Estimation of fetal-to-maternal unbound steady-state plasma concentration ratio ($K_{p,uu,fetal}$) of P-gp and/or BCRP substrate drugs using a maternal-fetal PBPK model

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d) Abbreviations used:

AAFE: absolute average fold error; AAG: α 1-acid glycoprotein; ADME: absorption, distribution, metabolism, and excretion; AUC: area under the curve of the total plasma concentration-time profile; AUC_{fetal} : area under the curve of the umbilical vein total plasma concentration-time

profile; AUC_m : area under the curve of the maternal total plasma concentration-time profile;

$AUC_{fetal,u}$: area under the curve of the umbilical vein unbound plasma concentration-time profile;

$AUC_{m,u}$: area under the curve of the maternal unbound plasma concentration-time profile; BCRP:

breast cancer resistance protein; B/P: blood-to-plasma partition ratio; $CL_{PD,placenta}$: placental passive diffusion clearance; $CL_{int,PD,placenta}$: intrinsic placental passive diffusion clearance; $CL_{efflux,placenta}$: placental efflux clearance; $CL_{int,efflux,placenta}$: intrinsic placental efflux clearance; CL_{iv} : intravenous clearance; $CL_{int,CYPx}$: intrinsic clearance via CYPx isozyme; $CL_{int,bile}$: intrinsic biliary clearance; C_{max} : maximum plasma drug concentration; CL_R : renal clearance; C-T profile: drug concentration-time profile; CYP: cytochrome P450; $E_{max,CYP3A}$: maximal fold induction of CYP3A relative to control; $EC50_{CYP3A}$: nelfinavir concentration that produces half-maximal induction of CYP3A; REF: relative expression factor; f_{efflux} : fraction of a drug transported by placental P-gp or BCRP; f_{CLbile} : fraction of drug excreted in the bile; f_e : fraction of drug excreted in the urine; f_u : fraction unbound in plasma; $f_{u,m}$: fraction unbound in maternal plasma; $f_{u,f}$: fraction unbound in fetal plasma; f_m : fraction of drug metabolized; $f_{m,CYP}$: fraction metabolized by a CYP enzyme; GW: gestational week; HIV: human immunodeficiency virus; HSA: human serum albumin; IV: intravenous; IVIVE: *in vitro* to *in vivo* extrapolation; $K_{app,CYP3A}$: concentration of mechanism-based inhibitor associated with half-maximal inactivation rate of the CYP3A enzyme; K_p : tissue-to-plasma partition coefficient; $k_{inact,CYP3A}$: maximum inactivation rate of the CYP3A enzyme; $k_{i,CYPx}$: concentration of inhibitor that produces half-maximal inhibition of CYPx isozyme; $K_{p,uu,fetal}$: fetal-to-maternal steady-state unbound plasma concentration ratio; m-f-PBPK model: maternal-fetal physiologically based pharmacokinetic model; MP: maternal plasma; P_{app} : apparent permeability; P-gp: P-glycoprotein; pKa: acid dissociation constant; p.o.: oral administration; P_{ow} : octanol-water partition coefficient; PK: pharmacokinetics; UV: Umbilical vein; V_{ss} : steady-state volume of drug distribution; HLMs: human liver microsomes; UGT2B7: UDP Glucuronosyltransferase family 2 member B7

Abstract(249/250)

Pregnant women are frequently prescribed drugs to treat chronic diseases (e.g., HIV infection), but little is known about the benefits and risks of these drugs to the fetus which are driven by fetal drug exposure. The latter can be estimated by fetal-to-maternal unbound plasma concentration at steady-state ($K_{p,uu,fetal}$). For drugs that are substrates of placental efflux transporters (i.e., P-gp or BCRP), $K_{p,uu,fetal}$ is expected to be <1 . Here, we estimated the *in vivo* $K_{p,uu,fetal}$ of selective P-gp and/or BCRP substrate drugs by maternal-fetal (m-f)-PBPK modeling of umbilical vein (UV) plasma and maternal plasma (MP) concentrations obtained simultaneously at term from multiple maternal-fetal dyads. To do so, three drugs were selected: nelfinavir (P-gp substrate), efavirenz (BCRP substrate), and imatinib (P-gp/BCRP substrate). A m-f-PBPK model for each drug was developed and validated for the non-pregnant population and pregnant women using the Simcyp simulator (v20). Then, after incorporating placental passive diffusion clearance, the *in vivo* $K_{p,uu,fetal}$ of the drug was estimated by adjusting the placental efflux clearance until the predicted UV/MP values best matched the observed data ($K_{p,uu,fetal}$ of nelfinavir=0.41, efavirenz=0.39, imatinib=0.35). Furthermore, $K_{p,uu,fetal}$ of nelfinavir and efavirenz at gestational week (GW) 25 and 15 were predicted to be 0.34, 0.23 and 0.33, 0.27 respectively. These $K_{p,uu,fetal}$ values can be used to adjust dosing regimens of these drugs to optimize maternal-fetal drug therapy throughout pregnancy, to assess fetal benefits and risks of these dosing regimens, and to determine if these estimated *in vivo* $K_{p,uu,fetal}$ values can be predicted from *in vitro* studies.

Keywords:

m-f-PBPK modeling, $K_{p,uu,fetal}$, placenta, P-gp, BCRP

Significance Statement (71 words, 3 sentences):

The *in vivo* $K_{p,uu,fetal}$ of nelfinavir (P-gp substrate), efavirenz (BCRP substrate), and imatinib (P-gp and BCRP substrate) was successfully estimated using m-f- PBPK modeling. These $K_{p,uu,fetal}$ values can be used to adjust dosing regimens of these drugs to optimize maternal-fetal drug therapy throughout pregnancy, to assess fetal benefits and risks of these dosing regimens, and to determine if these estimated *in vivo* $K_{p,uu,fetal}$ values can be predicted from *in vitro* studies.

Introduction

Pregnant women frequently take drugs (medication) throughout their pregnancy to treat the mother (e.g., hypertension, cancer) or the maternal-fetal pair (e.g., HIV infection) (McGowan and Shah, 2000; Mitchell *et al.*, 2011; Haas *et al.*, 2018). However, these drugs are often prescribed without knowledge of their fetal benefits and risks which are driven by fetal (and possibly by placental) drug exposure. Fetal drug exposure can be quantified only at delivery when simultaneous sampling of umbilical vein blood and maternal blood is possible. However, because these drug concentrations are time-dependent, they need to be collected in multiple maternal-fetal dyads to allow the estimation of fetal drug exposure (Zhang *et al.*, 2017). From these, fetal drug exposure, that is the fetal-to-maternal unbound steady-state plasma concentration ratio ($K_{p,uu,fetal}$), can be estimated (Anoshchenko, Storelli, *et al.*, 2021). For drugs that passively cross the placenta, provided there is no fetal or placental metabolism of the drug, $K_{p,uu,fetal}$ is easy to predict as it will be 1.0 (Zhang *et al.*, 2017). However, the placenta is richly endowed with efflux transporters, such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) at the maternal-placenta barrier (which efflux the drug from the placenta to the maternal blood). For drugs that are a substrate of these efflux transporters, $K_{p,uu,fetal}$ will be < 1 , and its deviation from unity will depend on the fraction of the drug effluxed by the transporter(s) (f_{efflux}). Estimation of a drug's $K_{p,uu,fetal}$ at term and at earlier gestational age, especially for those that are effluxed, is important for several reasons. First, it can be used to adjust dosing regimens of these drugs to optimize maternal-fetal drug therapy throughout pregnancy, provided the f_{efflux} of the drug at each gestational age can be estimated. Such estimation is now possible given our quantification of placental transporters in the first and second trimester as well as term by quantitative targeted proteomics (Anoshchenko *et al.*, 2020). Second, it can be used to assess fetal benefits and risks of these drug dosing regimens. Third, these $K_{p,uu,fetal}$ values can be used to determine if they can be predicted from *in vitro* studies, using the proteomics-

informed efflux ratio approach, as we have done before (Anoshchenko, Storelli, *et al.*, 2021). Therefore, to fulfill the above broad goals, we estimated the *in vivo* $K_{p,uu,fetal}$ of selective P-gp and/or BCRP substrate drugs by m-f-PBPK modeling of umbilical vein (UV) plasma and maternal plasma (MP) concentrations obtained simultaneously at term from multiple maternal-fetal dyads. Three drugs were studied: nelfinavir (P-gp substrate), efavirenz (BCRP substrate), and imatinib (P-gp/BCRP substrate). A m-f-PBPK model for each drug was developed and validated for the non-pregnant population and pregnant women using the Simcyp simulator (v20). Then, after incorporating placental passive diffusion clearance, the *in vivo* $K_{p,uu,fetal}$ of the drug was estimated by adjusting the placental efflux clearance until the predicted UV/MP values best matched the observed data.

Materials and Methods

Our search criteria for selecting the drug candidates were as follows: 1) candidate drug should be transported only by P-gp or BCRP or by P-gp/BCRP based on extensive *in vitro* studies; and 2) *in vivo* paired UV and MP drug concentrations data should be available, from a large number of maternal-fetal dyads, at multiple time points over the dosing interval (or for several half-lives) following the last maternal dose. A total of three candidate drugs that fulfilled these criteria were: nelfinavir, which is effluxed solely by P-gp and not by BCRP (Gupta *et al.*, 2004; Salama *et al.*, 2005), efavirenz, which is effluxed solely by BCRP but not by P-gp (Dirson *et al.*, 2006; Janneh *et al.*, 2009; Peroni *et al.*, 2011), and imatinib, which is effluxed by both BCRP and P-gp (Hamada *et al.*, 2003; Burger *et al.*, 2004; Oostendorp *et al.*, 2009; Zhou *et al.*, 2009).

PBPK model simulations and criteria for validation

PBPK simulation of the pharmacokinetics (PK) profiles of the above drugs was implemented as summarized in Fig. 1. Briefly (but detailed below), for each step of modeling, the predicted PK profiles and PK parameters (maximum plasma drug concentration [$C_{\max}$] and area under the curve of total plasma concentration-time profile [AUC]) of the drug were compared with the observed data. The observed plasma concentration-time profiles in graphical format were digitized using WebPlotDigitizer (<https://apps.automeris.io/wpd/>). These values were reported in the publications as geometric mean, arithmetic mean or median. Therefore, our PBPK predicted values are also reported in the same format. The PK profiles of the drugs were simulated using 100 virtual subjects (10 trials $\times$ 10 subjects). The PBPK model was considered validated if the observed PK profile fell within the 95th and 5th percentile of predicted data and the simulated PK parameters fell within the range 0.80-1.25-fold of the observed data (Ladumor *et al.*, 2019a; Ladumor *et al.*, 2019b). All the PBPK simulations were performed with trial designs (age range, proportion of female, gestational age, and dosing regimens) that matched the corresponding *in vivo* study (Table S1).

Development and validation of drug PBPK models for non-pregnant adults

A full PBPK model was constructed for nelfinavir using the Simcyp simulator (v20). Drug-related parameters for nelfinavir were collected from the literature (Table 1). A whole-body PBPK model was applied for the distribution of nelfinavir, and tissue-to-plasma partition coefficient (K_p) values were predicted using Simcyp Method 1 (Poulin and Theil, 2009). Nelfinavir binds extensively to α 1-acid glycoprotein (AAG) with a fraction unbound in human plasma (f_u) of 0.014 (Zhang *et al.*, 2001; Motoya *et al.*, 2006). Nelfinavir is metabolized by the cytochrome P450 (CYP) 3A, CYP2C19, CYP2D6, CYP2C9, CYP1A2, and CYP2E1 isoforms, and the fraction of drug metabolized (f_m) by each isoform were based on the inhibition of nelfinavir metabolism in pooled human liver microsomes (HLMs) in the presence of selective cytochrome P450 inhibitors

(US FDA, 2003). The intrinsic hepatic clearance (CL_{int}) of nelfinavir by each isoform was back-calculated from the intravenous (IV) total systemic clearance ($CL_{iv} = 37.7$ L/h) using the Simcyp simulator (Sarapa et al., 2005) after correcting for renal clearance ($f_e = 2\%$) and biliary clearance ($f_{CL,bile} = 10\%$) (US FDA, 2003). Our previous reported mechanism-based inhibition and induction of CYP3A by nelfinavir in HLMs and hepatocytes, respectively (Dixit *et al.*, 2007; Kirby *et al.*, 2011) and competitive inhibition of CYP3A, CYP2C9 and CYP1A2 by nelfinavir (Lillibridge et al., 1998) were incorporated into the PBPK model. Then, PK data after IV administration were simulated and validated using the observed data. Thereafter, the Advanced Dissolution, Absorption and Metabolism (ADAM) model of Simcyp, with integrated *in vitro* dissolution profiles in the fed and fasted state, was used to describe nelfinavir absorption (Shono *et al.*, 2011; Chapa *et al.*, 2020). Then, nelfinavir PK after single oral administration in the fed/ fasted state, multiple doses, and coadministration with ritonavir (inhibitor of CYP3A and CYP2D6, inducer of CYP3A and CYP2C9, Simcyp default compound file) were predicted and validated. Efavirenz and imatinib PBPK models for the non-pregnant adults were reproduced, without modification, from previous publications (Atoyebi *et al.*, 2019; Adiwidjaja *et al.*, 2020) and validated with the additional published *in vivo* data.

Development and validation of drug PBPK models for pregnant women

After validating the PK of the drug in the non-pregnant population, drug-specific parameters were fixed, and, except for the changes in CYP activity, the pregnancy-induced changes in physiological parameters specified in the Simcyp pregnancy module were implemented. The pregnancy-induced changes in hepatic CYP activity were based on our previously published data: CYP3A was induced 2-fold during the 2nd trimester, and 3rd trimesters (Ke *et al.*, 2012; Zhang *et al.*, 2015), CYP2D6 was induced 1.9 and 2-fold during the 2nd and 3rd trimester, CYP1A2 was suppressed by 48, and 65% during the 2nd and 3rd trimesters (Ke et al., 2013),

CYP2B6 activity was induced by 1.1 and 1.3-fold during the 2nd and 3rd trimesters, CYP2C9 activity was induced by 1.5 and 1.6-fold during the 2nd and 3rd trimesters (Ke *et al.*, 2014). CYP2C19 activity was suppressed by 62% and 68% during the 2nd and 3rd trimesters (Dickmann and Isoherranen, 2013; Ke *et al.*, 2014). Then, nelfinavir and efavirenz PK in postpartum, 2nd and 3rd trimester women were predicted and validated using the observed data. Corresponding *in vivo* data for imatinib are not available. We assumed physiological parameters in postpartum women (6-12 weeks) had returned to levels in the non-pregnant women prior to pregnancy (gestation age = 0). In addition, the gestational stage in our study was defined per U.S. Department of Health and Human Services (HHS) recommendations: 1-12 weeks for the 1st trimester, 13-28 weeks for the 2nd trimester, and 29-40 weeks for the 3rd trimester.

Estimating human steady-state unbound fetal-to-maternal plasma concentration ratio ($K_{p,uu,fetal}$) at term

Maternal pharmacokinetics of nelfinavir, efavirenz, and imatinib (per os, p.o.) were predicted using pregnant-PBPK models and compared to the observed PK profiles. Then, the bidirectional placental passive diffusion clearance ($CL_{PD,placenta}$) of the drug at maternal-placental and placental-fetal barriers was estimated as we have previously described (Zhang and Unadkat, 2017). Briefly, we chose midazolam as an *in vivo* calibrator to estimate $CL_{PD,placenta}$ of nelfinavir, efavirenz or imatinib. The $CL_{PD,placenta}$ of the drug (nelfinavir, efavirenz or imatinib) was estimated by scaling $CL_{PD,placenta}$ of midazolam ($CL_{PD,midazolam}$) using eq. 1.

$$CL_{PD,x} = \frac{P_{app,x}}{P_{app,midazolam}} \times CL_{PD,midazolam} (L/h) \quad (1)$$

where $P_{app,midazolam}$ and $CL_{PD,midazolam}$ are 489.9 nm/s and 500 L/h (mean value in CaCo-2 and MDR1-MDCKI cells), respectively (Yamashita *et al.*, 2000; Mahar Doan *et al.*, 2002; Tolle-

Sander et al., 2003; Gertz et al., 2010), and $P_{app,x}$ are the apparent membrane permeability (P_{app}) values (nm/s) of nelfinavir (8.8 in LLC-PK cells, Kim et al., 1998), efavirenz (45.85, mean value of two studies in Caco 2 cells, Takano *et al.*, 2006; Siccardi *et al.*, 2012) and imatinib (6.36 in MDCK II mock cells, Breedveld et al., 2005). Bidirectional unbound intrinsic placental passive diffusion clearance ($CL_{int,PD,placenta}$, $\mu\text{L}/\text{min}/\text{mL}$ placenta volume) at maternal-placenta and placenta-fetal barriers were obtained by dividing $CL_{PD,placenta}$ by placental volume. The placental volume was calculated using eq. 2 (Kapaun *et al.*, 2019).

$$\text{Placental volume} = -1.7646 \times \text{GW} + 0.91775 \times (\text{GW}^2) - 0.011543 \times \text{GW}^3 \quad (2)$$

Where GW is the gestational age (weeks). After incorporating $CL_{int,PD,placenta}$, we predicted the umbilical vein plasma concentrations and estimated the drug $K_{p,uu,fetal}$ (eq. 3) by adjusting the intrinsic placental efflux clearance of the drug at the maternal-placenta barrier ($CL_{int,P-gp,placenta}$ for nelfinavir, $CL_{int,BCRP,placenta}$ for efavirenz, $CL_{int,efflux,placenta}$ for imatinib) until the predicted UV/MP values best matched the observed data (AAFE = 1.0) using the permeability-limited placenta model of Simcyp. The absolute average fold error (AAFE) in the predictions of UV/MP values were calculated as per Eq. 4:

$$K_{p,uu,fetal} = \text{AUC}_{fetal,u} / \text{AUC}_{m,u} \quad (3)$$

$$\text{AAFE} = 10^{\left| \frac{1}{N} \sum \log \frac{\text{predicted}}{\text{observed}} \right|} \quad (4)$$

Where $\text{AUC}_{fetal,u}$ is the area under the curve of the unbound umbilical vein plasma concentration-time profile, $\text{AUC}_{m,u}$ is the area under the curve of the unbound maternal plasma concentration-time profile, N is the number of observed and predicted UV/MP values.

PBPK model prediction of $K_{p,uu,fetal}$ of the drugs at an earlier gestational age (GW15 and GW25)

To predict the $K_{p,uu,fetal}$ of nelfinavir and efavirenz at an earlier gestational age, total placental P-gp and BCRP abundance, previously quantified by us using quantitative targeted proteomics (Anoshchenko *et al.*, 2020) were incorporated into the Simcyp pregnancy module “Sim-Pregnancy”. A second-order polynomial model was fitted to the gestational age-dependent relative abundance of placental P-gp and BCRP (relative to term value which was set as 1.0), respectively (see Eq. 5 and 6; R-square values of the fitted polynomials were 1.0, Fig S1)

$$P - gp \text{ relative abundance} = 0.003 \times (GW^2) - 0.228 \times GW + 5.010 \quad (5)$$

$$BCRP - \text{relative abundance} = 0.001 \times (GW^2) - 0.086 \times GW + 2.899 \quad (6)$$

These equations were used to interpolate the placental abundance of the transporters at GW15 and 25. Then, these interpolated values were used to scale the above estimated (term) placental efflux clearances of nelfinavir and efavirenz ($CL_{int,P-gp,placenta}$: nelfinavir, $CL_{int,BCRP,placenta}$: efavirenz) and incorporated in the Simcyp pregnancy module. Within this module, the above-estimated term $CL_{int,PD,placenta}$ and $CL_{int,efflux,placenta}$ was scaled based on the mean volume of the placenta for the respective gestational age. Then, the maternal-fetal PK profiles of the drugs were predicted at GW15 and GW25 using the same trial design as for term. From these profiles, the $K_{p,uu,fetal}$ of nelfinavir and efavirenz was estimated. Such predictions for imatinib were not possible as the fraction of imatinib transported by P-gp or BCRP is unknown and will need to be determined as we have described previously (Kumar *et al.*, 2021).

Results

PBPK model predictions and validation for the non-pregnant population

Our predictions of nelfinavir PK were successfully validated following IV, single oral dose (fed and fasted), multiple oral dose administration, and coadministration with ritonavir. The observed

concentration-time (C-T) profiles fell within the 95th and 5th percentile of predicted data (Fig. 2A, Fig. S2) and the predicted PK parameters (AUC and $C_{\max}$) also fell within the 0.80 to 1.25-fold of the observed data (Table 2). The PBPK models for efavirenz and imatinib were successfully reproduced and, except for imatinib $C_{\max}$ after coadministration with ketoconazole, their simulated PK profiles were consistent with the reported *in vivo* data (Table 3, Fig. 2B, 2C, and S3).

PBPK model predictions and validation for pregnant women

The PBPK-pregnancy model for nelfinavir and efavirenz successfully predicted the PK of the drugs in postpartum, 2nd trimester and 3rd trimester women (corresponding data for imatinib are not available) (Figs. 3 and 4). Also, the majority of the predicted PK endpoints (AUC and $C_{\max}$) fell within 0.80 to 1.25-fold of the observed data (Table 4).

Estimated human fetal-to-maternal unbound steady-state plasma concentration ratio ($K_{p,uu,fetal}$) at term

Using our acceptance criteria, the predicted MP concentration-time profiles agreed well with the observed data of nelfinavir, efavirenz and imatinib (Fig. 5A, 5D, and 5G). The estimated $CL_{int,PD,placenta}$ of nelfinavir, efavirenz and imatinib at term were 240, 1480 and 170 $\mu\text{L}/\text{min}/\text{mL}$ placenta volume, respectively (Table 5). Without incorporating $CL_{efflux,placenta}$, that is in the presence of only $CL_{PD,placenta}$ of the drug, the UV plasma concentration (Fig. 5B, 5E and 5H) and UV/MP ratio (Fig. 5C, 5F, and 5I) were considerably overpredicted, with AAFE >1, and, as expected the estimated $K_{p,uu,fetal}$ was 1.0 (Table 5).

By adjusting $CL_{int,efflux,placenta}$ of the drugs (nelfinavir: 350, efavirenz: 2200, imatinib: 320 $\mu\text{L}/\text{min}/\text{mL}$ placenta volume), the majority of the observed UV plasma concentrations and the UV/MP ratios fell within the 95th and 5th percentile of the model predicted data (Fig. 5). As these data are steady-state data, the predicted AUC_{fetal}/AUC_m were close to the mean observed UV/MP ratio and AAFE equaled 1.00. $K_{p,uu,fetal}$ values at term estimated from the UV/MP data were 0.41, 0.39 and 0.35 for nelfinavir, efavirenz and imatinib respectively. These data indicate that the fraction of drug transported by placental P-gp and/or BCRP at term ($f_{efflux} = 1 - K_{p,uu,fetal}$) followed the order imatinib (0.65) > efavirenz (0.61) > nelfinavir (0.59).

Prediction of nelfinavir and efavirenz $K_{p,uu,fetal}$ at earlier gestational ages (GW15 and GW25)

The MP plasma concentrations of nelfinavir and efavirenz were marginally affected by gestational age, and the UV plasma concentration, UV/MP ratio and $K_{p,uu,fetal}$ all decreased with gestational age (Fig. 6, Table 5).

Discussion

Nelfinavir and efavirenz are prescribed to prevent the transmission of HIV from the mother to her fetus (Perry et al., 2005; Vrouenraets et al., 2007). However, as we have shown here, they are prevented from distribution into the fetal compartment by extensive placental efflux, thus potentially reducing their efficacy in preventing maternal-fetal HIV transmission. In contrast, imatinib, a selective tyrosine kinase inhibitor, is used to treat cancers (Ali et al., 2009). When administered to a pregnant woman, fetal harm or abortion can occur (Ali et al., 2009). These cases illustrate the importance of estimating fetal drug exposure ($K_{p,uu,fetal}$) at all gestational

ages, to assess the safety and efficacy of drugs administered to pregnant women. In addition, if these safety and efficacy data dictate, these $K_{p,uu,fetal}$ values can be used to design alternative dosing regimens to enhance drug safety and efficacy as we have proposed for antenatal corticosteroids (Anoshchenko, Milad, *et al.*, 2021).

While $K_{p,uu,fetal}$ can be estimated at term from UV/MP values, sampling UV blood is not possible at earlier gestational age. Therefore, to estimate drug $K_{p,uu,fetal}$ at earlier gestational ages, the only recourse is PBPK modeling and simulation. For all the above reasons, we estimated $K_{p,uu,fetal}$ of nelfinavir, efavirenz and imatinib at term and earlier in gestation (nelfinavir and efavirenz only). In addition, though drugs are frequently taken by pregnant women, no UV/MP data are available for the majority of these drugs. Because obtaining such data is extremely challenging, the only recourse is to estimate $K_{p,uu,fetal}$ for these drugs. We have previously shown that this is possible through *in vitro* transport studies combined with m-f PBPK modeling and simulation and the quantitative targeted proteomics-informed relative expression factor (REF) approach (Anoshchenko, Storelli, *et al.*, 2021). However, such predictive methods need to be validated. Thus, another reason for estimating term nelfinavir, efavirenz and imatinib $K_{p,uu,fetal}$, was to use them in the future to validate predictions made by our m-f PBPK model (Anoshchenko, Storelli, *et al.*, 2021).

$K_{p,uu,fetal}$ is determined by several factors, namely placental transport (efflux or influx), placental metabolism, and fetal clearance of the drug. Since the placenta is not endowed with the CYP enzymes found in adult livers, the metabolism of most drugs within this organ is negligible (Unadkat *et al.*, 2005). The fetal liver size is small. In addition, except for CYP3A7, it also does not express many of the CYP enzymes found in the adult liver until about one year after birth (Thakur *et al.*, 2021). For both these reasons, the fetal liver plays a miniscule role in the CYP

clearance of drugs. Therefore, for the drugs studied here, we assumed that the placental and fetal metabolism of these drugs was negligible. Consequently, as we have shown before, $K_{p,uu,fetal}$ of these drugs will be determined solely by passive diffusion and transport across the placenta (Zhang *et al.*, 2017).

To estimate $K_{p,uu,fetal}$ we deliberately used the UV/MP values as our endpoint rather than just the UV unbound plasma AUC profile. This is because the latter is determined by maternal unbound plasma concentrations that are highly variable (see Fig. 5), resulting in highly variable UV plasma concentrations (total and unbound). This high variability is due to pooling UV and MP values from multiple maternal-fetal dyads. Using UV/MP values as an endpoint mitigates the variability observed when using the UV values as endpoints.

In the present study, the PK parameters of three drugs, effluxed by the placental transporters, were successfully predicted and validated after PBPK modeling and simulation of PK data in non-pregnant adults and pregnant women (Tables 2-4). Then, the $K_{p,uu,fetal}$ of these drugs, at term, were estimated to be 0.41, 0.39 and 0.35 for nelfinavir, efavirenz and imatinib, respectively. The fraction of these drugs effluxed by the placenta, f_{efflux} ($=1 - K_{p,uu,fetal}$) was 0.59, 0.61 and 0.65, respectively, demonstrating that placental P-gp and/or BCRP significantly prevent their distribution into the fetal compartment. To our knowledge, this is the first time that the $K_{p,uu,fetal}$ of a placental BCRP substrate as well as that of a dual P-gp/BCRP substrate has been estimated. Furthermore, this is the first study to construct and validate a PBPK model for the disposition of nelfinavir in non-pregnant adults and pregnant women.

Based on the above term pregnancy data, because we have quantified the abundance of placental transporters at various gestational ages (Anoshchenko *et al.*, 2020), we were able to predict the $K_{p,uu,fetal}$ of nelfinavir and efavirenz earlier in gestation (GW15 and GW25). The

Simcyp pregnancy module does not allow predictions any earlier (< GW15) as physiological data at these earlier gestational ages are not currently available. In addition, we could not make these predictions for imatinib as the f_{efflux} of this drug by placental P-gp and BCRP is currently not known. However, these values can be predicted in the future from *in vitro* transport data and REF as we have done before for other drugs (Kumar *et al.*, 2021). Consistent with our expectations and previous publication (Anoshchenko, Milad, *et al.*, 2021), due to a decrease in placental size, both $CL_{\text{efflux,placenta}}$ and $CL_{\text{PD,placenta}}$ decreased with gestational age, but the decrease in the latter was greater than the former. Therefore, the $K_{\text{p,uu,fetal}}$ of both nelfinavir and efavirenz at GW15 (=0.23, 0.27) and GW25 (=0.34, 0.33) was lower than at term (=0.41, 0.39). These data can inform the fetal efficacy and toxicity of these drugs at earlier gestational ages.

There are a few limitations to our study. First, the PBPK model of imatinib was not validated for pregnant women due to a lack of such *in vivo* data. Second, imatinib may be transported by human organic anion transporting polypeptide 1A2 (OATP1A2) and multidrug resistance protein 4 (MRP4) (Hu *et al.*, 2008; Yamakawa *et al.*, 2011). However, data on pregnancy-induced changes in OATP1A2 and MRP4 activity are not available and therefore were not included in our model based on Adiwidjaja's model (Adiwidjaja *et al.*, 2020). Third, for our nelfinavir PBPK model, f_m by each CYP isoform were based on CYP inhibition of nelfinavir metabolism in HLMs, and enzyme cross-inhibition by these inhibitors was not taken into consideration (Patilea-Vrana *et al.*, 2019). However, none of the above limitations detracts from correctly estimating $K_{\text{p,uu,fetal}}$ provided the maternal plasma concentrations are predicted well. Fourth, we assumed that nelfinavir solely binds to AAG rather than albumin (HSA), as the association constant of nelfinavir for AAG ($7.25 \times 10^7/\text{M}$) is 70 times higher than that for HSA ($1.11 \times 10^6/\text{M}$) (Motoya *et al.*, 2006). Fifth, the fraction unbound of the drugs in fetal plasma was the Simcyp predicted value (Table S2), because the corresponding experimentally measured values are not available

in the literature. Any inaccuracy in our estimate of the fraction of drug bound in the maternal and fetal compartment will result in inaccuracy in our $K_{p,uu,fetal}$ estimate. Sixth, the potential effects of HIV or cancer comorbidity on the placental drug permeability or transporters is unknown and therefore was not incorporated in the model. Again, this does not detract from our estimate of $K_{p,uu,fetal}$ as it was based on the observed data from women who had these clinical conditions. Seventh, the Simcyp model does not allow passage of drug from the placenta directly into the amniotic fluid which can be swallowed by the fetus. Irrespective of the route of drug passage, our $K_{p,uu,fetal}$ values will be unaffected as they are based on the observed UV/MP values.

In summary, we estimated the *in vivo* $K_{p,uu,fetal}$ of nelfinavir, efavirenz and imatinib through PBPK modeling and simulation. Prospectively, these $K_{p,uu,fetal}$ of these drugs could be used to design dosing regimens of these drugs for pregnant women, throughout pregnancy, to maximize their efficacy and minimize their fetal toxicity. Furthermore, in the future, these $K_{p,uu,fetal}$ could be used to validate their predictions made through *in vitro* studies using the proteomics-informed REF approach. Once validated, these m-f-PBPK models, in combination with *in vitro* studies, could be used in the future to predict fetal exposure, throughout pregnancy, to any drug that is actively effluxed by placental P-gp and/or BCRP.

Authorship Contributions

Participated in research design: Peng, Ladumor and Unadkat.

Conducted experiments: Peng and Ladumor.

Performed data analysis: Peng, Ladumor and Unadkat.

Wrote or contributed to the writing of the manuscript: Peng, Ladumor and Unadkat.

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Footnotes

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Figure Legends

Fig. 1. Workflow for estimation of *in vivo* $K_{p,uu,fetal}$ using the Simcyp m-f-PBPK model. A PBPK model for each drug was developed for the nonpregnant population using the Simcyp simulator (v20) and the predicted PK profiles of these drugs were validated with data after intravenous (IV) and oral administration, as well as drug-drug interaction studies (step 1). Systemic maternal PK of drugs in the second, third trimester and postpartum were predicted using the pregnant population of the Simcyp simulator and validated with the observed data (step 2). Then, using the estimated passive diffusion clearance (CL_{PD}) of the drugs, the magnitude of the placental efflux clearance ($CL_{efflux,placenta}$) and the $K_{p,uu,fetal}$ was estimated by adjusting the $CL_{efflux,placenta}$ until the predicted UV/MP values best matched the observed data (step 3).

Fig. 2. Predicted and observed plasma concentration-time (C-T) profiles of nelfinavir, efavirenz and imatinib in the non-pregnant adults. A) Observed (geometric mean) and predicted plasma C-T profile after single oral dose of nelfinavir (1250 mg) in non-pregnant adults (Sarapa *et al.*, 2005; Damle *et al.*, 2006); B) Observed (mean) and predicted plasma C-T profile of 600 mg efavirenz (p.o., once daily) at steady-state in non-pregnant adults (Villani *et al.*, 1999); C) Observed (median) and predicted plasma C-T profile after single-dose of 100 mg imatinib in non-pregnant adults (Ostrowicz *et al.*, 2014). The observed data (open circles) fell within the 95th and 5th percentile (dashed lines) of the predicted data (black continuous line). The predicted PK endpoints (AUC and C_{max}) also fell within the 0.80 to 1.25-fold of the observed data (Table 2-3).

Fig. 3. Predicted and observed plasma concentration-time (C-T) profiles of nelfinavir in pregnant women throughout pregnancy for several studies. Observed (geometric) (Fang *et al.*, 2012) and predicted steady-state plasma C-T profile of nelfinavir (1250 mg, p.o., twice daily) in postpartum (A), 2nd trimester (B) and 3rd trimester (C) women; observed (median) (Read *et al.*, 2008) and predicted steady-state plasma C-T profile of nelfinavir (1250 mg, p.o., twice daily) in postpartum (D), 2nd (E) and 3rd trimester (F) women; observed (geometric mean) (Van Heeswijk *et al.*, 2004) and predicted steady-state plasma C-T profile of nelfinavir (1250 mg, p.o., twice daily) in postpartum (G) and 3rd trimester (H) women (2nd trimester data are not available). The observed data (open circles) fell within the 95th and 5th percentile (dashed lines) of the

predicted data (black continuous line). The predicted PK endpoints (AUC and $C_{\max}$) also fell within the 0.80 to 1.25-fold of the observed data (Table 4).

Fig. 4. Predicted and observed plasma concentration-time (C-T) profile of efavirenz in pregnant women throughout pregnancy for several studies. Observed (median) (Kreitchmann *et al.*, 2019) and predicted plasma C-T profile of efavirenz (600 mg, p.o., once daily) at steady-state in postpartum (A), 2nd trimester (B) and 3rd trimester (C), respectively; Observed (geometric mean) (Lamorde *et al.*, 2018) and predicted plasma C-T profile of efavirenz (400mg, p.o., once daily) at steady-state in postpartum (D) and 3rd trimester (E) (2nd trimester data are not available), respectively; Observed (median) (Cressey *et al.*, 2012) and predicted plasma C-T profile of efavirenz (600 mg, p.o., once daily) in postpartum (F) and 3rd trimester (G) (2nd trimester data are not available), respectively. The observed data (open circles) fell within the 95th and 5th percentile (dashed lines) of the predicted data (black continuous line). The predicted PK endpoints (AUC and $C_{\max}$) also fell within the 0.80 to 1.25-fold of the observed data (Table 4).

Fig. 5. Predicted and observed (pooled) steady-state maternal plasma concentration-time profiles (MP) (A, D, G), umbilical vein (UV) plasma concentration-time profiles (B, E, H) and UV/MP (C, F, I) profiles of the drugs with (black line) or without (blue line) *in vivo* placental efflux clearance. Pregnant women (between 31 and 41 gestation weeks) were administered 1250 mg nelfinavir (p.o., fasted) on the day of delivery after receiving 1250 mg twice daily (p.o., fed) for at least 15 days (Hirt *et al.*, 2007) (A, B, C); Efavirenz (600 mg, once daily) was administered between 37- and 41-week of gestation (Cressey *et al.*, 2012) (D, E, F); Imatinib (400 mg daily) was administered between 35–41 weeks of gestation (Chelysheva *et al.*, 2018) (G, H, I). The x-axis is the time between the last dose and delivery. Dashed lines - 95th and 5th percentile of the predicted data in the presence of placental CL_{efflux} , open circles - observed data. $K_{p, \text{uu, fetal}}$ values for nelfinavir, efavirenz and imatinib estimated from the UV/MP data were 0.41, 0.39 and 0.35, respectively.

Fig. 6. Simulated steady-state maternal plasma (MP) concentrations (A, D), umbilical vein (UV) plasma concentrations (B, E) and the UV/MP (C, F) profiles of nelfinavir (A, B, C) or efavirenz (D, E, F) at varying gestational ages. Profiles were simulated after administration of nelfinavir (1250 mg twice daily regimen in fed state for 15 days) (A, B, C); efavirenz (600 mg,

once daily for 15 days) (D, E, F). $K_{p,uu,fetal}$ values for nelfinavir were 0.41, 0.34 and 0.23 at GW 38, 25 and 15, respectively. $K_{p,uu,fetal}$ values for efavirenz were 0.39, 0.33 and 0.27 at GW 39, 25 and 15, respectively.

Table 1. Nelfinavir drug-related parameters

Parameter	Unit	Value	Reference
Physicochemical and blood-binding properties			
Molecular weight	g/mol	567.80	ChEMBL, Drugbank
Log Po:w		4.07	
Ionization pattern		Diprotic base	(Longer <i>et al.</i> , 1995)
pKa		6,11.06	
B/P		1.00	(Zhang <i>et al.</i> , 2001)
fu		0.014	
Plasma binding component		AAG	(Motoya <i>et al.</i> , 2006)
Absorption phase			
Model		ADAM	
Papp	10^{-6} cm/s, Caco2	7.11	(Kim <i>et al.</i> , 1998)
Solubility	mg/mL	4.50	(Longer <i>et al.</i> , 1995)
Distribution phase			
Prediction method		Full PBPK model Method 1	
Vss	L/kg	2.00 for healthy 5.20 for pregnancy	Predicted by Simcyp
Elimination phase			
CL _{iv}	L/h	37.70	(Sarapa <i>et al.</i> , 2005)
CL _{int,CYP3A} ($f_{m,CYP}$)	μ L/min/pmol CYP	1.30 (25.19%)	(US FDA, 2003)
CL _{int,CYP2C19} ($f_{m,CYP}$)	μ L/min/pmol CYP	29.62 (15.99%)	
CL _{int,CYP2C9} ($f_{m,CYP}$)	μ L/min/pmol CYP	0.90 (8.72%)	
CL _{int,CYP1A2} ($f_{m,CYP}$)	μ L/min/pmol CYP	0.99 (6.30%)	
CL _{int,CYP2E1} ($f_{m,CYP}$)	μ L/min/pmol CYP	1.43 (11.63%)	
CL _{int,CYP2D6} ($f_{m,CYP}$)	μ L/min/pmol CYP	8.19 (10.17%)	
Additional HLM CL _{int} (f_m)	μ L/min/mg protein	145.24 (12.00%)	
CL _{int,bile} (f_{CLbile})	μ L/min/million cells	26.35 (10.00%)	
CL _R (f_e)	L/h	0.57 (2.00%)	
Drug interactions			
Inhibition			
$k_{inact,CYP3A}$	min ⁻¹	0.16	(Kirby <i>et al.</i> , 2011)
$K_{app,CYP3A}$	μ mol/L	1.82	
$k_{i,CYP3A}$	μ mol/L	4.80	(Lillibridge <i>et al.</i> , 1998)
$k_{i,CYP2C19}$	μ mol/L	126.00	
$k_{i,CYP2C19}$	μ mol/L	192.00	
Induction			
$E_{max,CYP3A}$		11.20	(Kirby <i>et al.</i> , 2011)
EC50 _{CYP3A}	μ mol/L	6.50	

AAG: α 1-acid glycoprotein; ADAM: Advanced Dissolution, Absorption and Metabolism model; B/P: blood-to-plasma partition ratio; CL_{iv}: intravenous clearance; CL_{int,CYPx}: intrinsic clearance via the listed CYP isozyme; CL_{int,bile}: intrinsic biliary clearance; CL_R: renal clearance; $E_{max,CYP3A}$: maximal fold induction of CYP3A relative to control; EC50_{CYP3A}: nelfinavir concentration that produces half-maximal induction of CYP3A; f_{CLbile} : fraction of drug excreted in the bile; f_e : fraction of drug excreted in the urine; fu: unbound fractions in plasma; f_m : fraction of drug metabolized; $f_{m,CYP}$: fraction metabolized by CYP enzymes;

$K_{app,CYP3A}$: concentration of mechanism-based inhibitor associated with half-maximal inactivation rate of CYP3A enzymes; $k_{i,CYPx}$: concentration of inhibitor that produces half-maximal inhibition of CYPx isozyme; $k_{inact,CYP3A}$: maximum inactivation rate of CYP3A; P_{app} : apparent permeability coefficients, at 5 μ M; pK_a : acid dissociation constant; $P_{o:w}$: Octanol-water partition coefficient; V_{ss} : steady-state volume of distribution

Table 2. Observed and PBPK model-predicted plasma pharmacokinetics of nelfinavir in non-pregnant adults

	IV Infusion (day 1)*			IV Infusion (day 11) #			Single Oral 1250 mg (day 1)			Oral 1250 mg twice daily (day 15)			Reference
Parameters	Observed	Predicted	ratio	Observed	Predicted	ratio	Observed	Predicted	ratio	Observed	Predicted	ratio	
N	6			6			6			12			
AUC_{last} (mg·h/L)	23.60	26.74	1.13	29.20	31.88	1.09	26.20	26.94	1.03	33.70	35.06	1.04	(Sarapa <i>et al.</i> , 2005; Damle <i>et al.</i> , 2006)
C_{max} (mg/L)	24.30	19.33	0.80	24.40	20.19	0.83	4.18	4.25	1.02	5.13	5.55	1.08	
	Single Oral 1250mg (fed)			Single Oral 1250mg (fast)			1250mg nelfinavir + 100 mg Ritonavir oral bid 14 days						(Kurowski <i>et al.</i> , 2002; Kaeser <i>et al.</i> , 2005)
	Observed	Predicted	ratio	Observed	Predicted	ratio	Observed	Predicted	ratio				
N	52			52			12						
AUC_{last} (mg·h/L)	32.90	26.41	0.80	4.84	4.91	1.01	31.80	28.91	0.91				
C_{max} (mg/L)	4.30	4.36	1.01	0.81	0.87	1.07	4.09	4.67	1.14				

IV: intravenous; N: number of subjects of observed data; 100 virtual subjects (10 trials × 10 subjects) were simulated for each study; Ratio - Predicted/Observed values of AUC_{last} (area under the plasma concentration-time curve from time 0 to time of last measurable concentration) or C_{max} , the maximum plasma drug concentration

* A single 30-minute IV infusion of 1 mg of nelfinavir

11-days oral 1250-mg dose of nelfinavir with food followed by a single 30-minute IV infusion of 1 mg nelfinavir

Table 3. PK profiles of efavirenz and imatinib in non-pregnant population

Efavirenz	400mg once daily			600mg once daily			600mg once daily			Reference (Villani <i>et al.</i> , 1999; Dickinson <i>et al.</i> , 2016)		
	Parameters	Observed	Predicted	Ratio	Observed	Predicted	Ratio	Observed	Predicted		Ratio	
	N	311			295				11			
	AUC _{last} (mg.h/L)	49.20	51.81	1.05	67.20	70.97	1.06	57.15	70.84		1.24	
	C _{max} (mg/L)	2.52	3.00	1.19	3.66	4.20	1.15	4.00	4.21		1.05	
Imatinib	IV Infusion 60 min (100 mg)			Capsule (400 mg)			Oral Solution (400 mg)			(Peng <i>et al.</i> , 2004) 		

IV: intravenous; N: number of subjects of observed data; 100 virtual subjects (10 trials × 10 subjects) were simulated for each study. Ratio - Predicted/Observed values of AUC_{last} (area under the plasma concentration-time curve from time 0 to time of last measurable concentration), AUC_{inf} (area under the plasma concentration-time curve from time 0 extrapolated to infinity) or C_{max}, the maximum plasma drug concentration

Table 4. Predicted and observed pharmacokinetics of nelfinavir and efavirenz in pregnant women

	Parameters	Observed Postpartum	Predicted Postpartum	Ratio	Observed 2 nd trimester	Predicted 2 nd trimester	Ratio	Observed 3 rd trimester	Predicted 3 rd trimester	Ratio	
Nelfinavir	N	10			16			14			
	AUC _{last} (mg.h/L)	38.50	33.84	0.88	21.60	26.98	1.25	20.70	24.50	1.18	(Fang <i>et al.</i> , 2012)
	C _{max} (mg/L)	5.02	4.32	0.86	3.32	3.58	1.08	3.18	3.30	1.04	
	N	22			4			27			
	AUC _{last} (mg.h/L)	30.80	32.52	1.06	27.30	27.77	1.02	18.90	25.06	1.33	(Read <i>et al.</i> , 2008)
	C _{max} (mg/L)	4.60	4.24	0.92	4.70	3.62	0.77	3.20	3.37	1.05	
	N	11						11			(Van Heeswijk <i>et al.</i> , 2004)
	AUC _{last} (mg.h/L)	33.50	33.46	1.00				25.20	23.67	0.94	
	C _{max} (mg/L)	5.80	4.28	0.74				4.51	3.21	0.71	
Efavirenz	N	40			15			42			
	AUC _{last} (mg.h/L)	62.70	73.87	1.18	47.3	55.13	1.17	60.02	48.18	0.80	(Kreitchmann <i>et al.</i> , 2019)
	C _{max} (mg/L)	4.41	4.41	1.00	3.87	3.61	0.93	5.13	3.26	0.64	
	N	26						26			
	AUC _{last} (mg.h/L)	44.11	54.14	1.23				39.94	36.33	0.91	(Lamorde <i>et al.</i> , 2018)
	C _{max} (mg/L)	2.77	3.18	1.15				2.75	2.40	0.87	
	N	25						26			
	AUC _{last} (mg.h/L)	58.30	74.63	1.28				55.40	52.18	0.94	(Cressey <i>et al.</i> , 2012)
	C _{max} (mg/L)	5.10	4.48	0.88				5.44	3.38	0.62	

N - number of subjects of observed data; 100 virtual subjects (10 trials × 10 subjects) were simulated for each study. Ratio - Predicted/Observed values of AUC_{last} (area under the plasma concentration-time curve from time 0 to time of last measurable concentration) or C_{max}, the maximum plasma drug concentration; Nelfinavir dosing regimen: 1250-mg bid with food at least 2 weeks; Efavirenz dosing regimen: 400/600 mg once daily at least 2 weeks

Table 5. Estimated and predicted $K_{p,uu,fetal}$ with and without $CL_{efflux,placenta}$

	$CL_{int,PD,placenta}$ ($\mu\text{L}/\text{min}/\text{mL}$ placenta volume)	$CL_{int,efflux,placenta}$ ($\mu\text{L}/\text{min}/\text{mL}$ placenta volume)	AAFE	Predicted AUC_{fetal}/AUC_m	Average observed UV/MP ratio (range)	$K_{p,uu,fetal}$			Reference
						At term	GW 25	GW 15	
Nelfinavir	240	0.00	2.39	0.61	0.25 (0.05–5.18)	1.00	1.00	1.00	(Hirt <i>et al.</i> , 2007)
		350.00	1.00	0.25		0.41	0.34	0.23	
Efavirenz	1480	0.00	2.21	0.95	0.49 (0.37–0.74)	1.00	1.00	1.00	(Cressey <i>et al.</i> , 2012)
		2200.00	1.00	0.43		0.39	0.33	0.27	
Imatinib	170	0.00	2.91	0.27	0.11 (0.05 -0.22)	1.00	NA	NA	(Chelysheva <i>et al.</i> , 2018)
		320.00	1.00	0.09		0.35			

AAFE: absolute average fold error; AUC_{fetal} : area under the curve of umbilical vein total plasma concentration-time profile; AUC_m : area under the curve of total maternal plasma concentration-time profile; $CL_{int,efflux,placenta}$: intrinsic placental efflux clearance; $CL_{int,PD,placenta}$: intrinsic placental passive diffusion clearance; GW: gestational weeks; MP: maternal plasma; NA: data not available; UV: Umbilical vein

Fig. 1

Fig. 2

Fig. 3

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Fig. 4

Fig. 5

Fig. 6