ARTICLE

A Strategy to Refine the Phenotyping Approach and Its Implementation to Predict Drug Clearance: A Physiologically Based Pharmacokinetic Simulation Study

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The phenotyping approach to predict drug metabolism activity is often hampered by a lack of correlation between the probe and the drug of interest. In this article, we present a strategy to refine the phenotyping approach based on a physiologically based pharmacokinetic simulation (implemented in Simcyp Simulator version 17) using previously published models. The apparent clearance (CL/F) of erlotinib was better predicted by the sum of caffeine and i.v. midazolam CL/F (r^2 = 0.60) compared to that of either probe drug alone. The clearance of atorvastatin and repaglinide had a strong correlation (r^2 = 0.70 and 0.63, respectively) with that of pitavastatin (a SLCO1B1 probe). Use of multiple probes for drugs that are predominantly metabolized by more than one cytochrome P450 (CYP) enzyme should be considered. In a case in which hepatic uptake transporters play a significant role in the disposition of a drug, the pharmacokinetic of a transporter probe will provide better predictions of the drug clearance.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ The current practice of the phenotyping approach involves assessing the phenotyping metric of a single probe drug to explain the variability in clearance of the drug of interest. Two frequent complicated scenarios are when the drug is cleared by multiple metabolic pathways and transported by hepatic uptake transporters.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This article offers a proof-of-concept study of these common scenarios using a PBPK simulation.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ Use of multiple probes for drugs that are predominantly metabolized by more than one CYP enzyme should be considered. In a case in which hepatic uptake transporters play a significant role, the pharmacokinetic of a transporter probe will be more predictive of the drug clearance.

HOW MIGHT THIS CHANGE DRUG DISCOVERY, DEVELOPMENT, AND/OR THERAPEUTICS?

☑ The strategy proposed in this study is likely to give a higher success rate in the phenotyping approach. The verification of this strategy in a clinical setting would be desirable.

When a drug is administered, individuals vary substantially in their pharmacokinetic (time course of drug concentration) and, thus, the response to treatment.[1] Interindividual variability in pharmacokinetics is often linked to polymorphisms in selected genes encoding drug-metabolizing enzymes (DMEs) and transporters.[2,3] Nevertheless, other intrinsic (sex, age, the abundance of DMEs and transporters, pregnancy, disease state, ethnicity, and epigenetic) as well as extrinsic factors (diet, concomitant administration of interacting drugs, and herbal medicines) may further contribute to this variability.[4,5] For example, cytochrome P450 (CYP)3A isoenzyme activity displays high between-individual variability and is subject to significant alteration by CYP3A modulators.[1,6] However, the level of CYP3A activity is more likely to be the result of multiple genetic and nongenetic determinants, rather than single gene variants.[7] Other CYP isoenzymes, especially CYP2D6, 2C19, and 2C9 are highly polymorphic,[8] but genotype alone does not explain the phenotype variability.[9] To best understand variability in pharmacokinetics it is desirable to assess the activity of DMEs and/or transporters in each individual whenever possible.

Phenotyping is a method to assess *in vivo* DME and transporter activity (phenotype) using the administration of a selective probe drug (or drugs) in order to guide drug dosing in an individual.[6] Different probe drugs for characterizing

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CYP isozyme activity administered either individually or in combination (so called "cocktail" approach) have been proposed.[10,11] There has also been recent interest to identify suitable probe drugs for the main clinically important drug transporters.[12] The ideal metric for a specific probe should be the systemic or apparent clearance, but other alternatives (e.g., area under the plasma-concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) and metabolic ratio in plasma, saliva, or urine and more simplified metrics, such as truncated AUC and single point plasma concentration) have also been widely investigated and implemented.[10]

The current practice involves assessing the phenotyping metric of a single probe drug to explain the variability in clearance of the drug of interest.[10] The phenotyping approach relies on the fundamental premise that the exposure or disposition of a drug can be adequately predicted by the pharmacokinetics of a selective probe drug that shares a common or similar metabolic pathway. However, this may not always be the case, particularly for drugs and probes administered orally. The apparent clearance (CL/F) of a drug also considers the bioavailability (F), which is influenced by determinants unrelated to metabolism (dissolution, permeability, and transporter substrate specificity). Even for i.v. administered drugs, the phenotyping method is not straightforward due to the possible interplay between hepatic uptake transporters and drug metabolizing enzymes. Indeed, a lack of adequate correlation between the pharmacokinetics of a probe drug and the drug of interest has often been reported.[13,14]

The aim of this study was to investigate the factors that determine the suitability of a phenotyping probe drug and to propose a strategy to refine the phenotyping approach for its implementation in predicting the apparent clearance of drugs. Due to the additive nature of clearance from multiple metabolic pathways,[15,16] we hypothesized that the use of pharmacokinetic data from multiple probe drugs will provide improved predictions for drugs that are cleared by multiple CYP enzymes. Based on the extended clearance concept,[17,18] a hepatic uptake transporter probe, instead of probe drugs for specific CYP pathways, is likely to better explain variability in the pharmacokinetics of drugs when there is an interplay between DME and transporter, particularly when hepatic uptake transporters play a significant role. This article offers a proof-of-concept study of selected common scenarios using a physiologically based pharmacokinetic (PBPK) simulation with illustrative examples. The factors that can determine the suitability of a phenotyping probe is also investigated using midazolam as a model probe drug.

METHODS

Physiologically based pharmacokinetic simulation

Population-based PBPK simulations were conducted using the Simcyp Simulator version 17 release 1 (Simcyp, Sheffield, UK). The description of Simcyp Simulator workflow, basic algorithm, structural PBPK model, and ordinary differential equations have been detailed elsewhere.[19,20] The "healthy population" library data within the Simcyp Simulator, which represents typical healthy adult North European white subjects, was used. Virtual individuals aged 20–50 years with a male:female ratio of 1:1 were generated randomly using Correlated Monte Carlo sampling. Ten *in silico* clinical pharmacokinetic trials with 20 subjects each (based on the typical number of participants in an *in vivo* phenotyping study) were conducted in every simulation. Unless otherwise stated, the default models and input parameters of drugs (substrates) supplied by the simulator were used in the simulation.

The CL/F and other relevant pharmacokinetic parameters were assessed following a single administration of the probes or drug of interest. A clinically relevant dose for all probe drugs and the drugs of interest was used throughout the simulation. At a substantially higher dose, drug concentration in the liver potentially exceeds its Michaelis-Menten constant (K_m) value. The PBPK simulation, as implemented in the Simcyp Simulator, is able to capture the saturation of DMEs. Under this circumstance, the correlation between probe drug and drug of interest may deteriorate. The list of substrates and probes used in the simulation are provided in **Table 1** and details on the PBPK model can be found in **Table S1**. The Simcyp Simulator allows the simulations to be generated from the same individuals, thus, the correlation analysis can be done under the conditions of repeated observation (paired study subjects).

Identifying factors that potentially affect the apparent clearance of midazolam

Midazolam is considered to be the optimal probe for CYP3A isoenzyme phenotyping because its exposure (AUC) and clearance to 1-hydroxymidazolam reflect hepatic CYP3A activity.[21] Moreover, midazolam is widely used in clinical studies to phenotype CYP3A activity prior to dosing of the drug of interest and its relationship with other CYP3A probes has been established.[22,23] In this study, midazolam was used as a model phenotyping probe to investigate the factors affecting the CL/F.

To identify all possible parameters that have significant impact on the CL/F of midazolam, sensitivity analyses were conducted using the automated sensitivity analysis (ASA) tool built into the Simcyp Simulator.[19] The degree of change in CL/F in a population representative subject as a function of each input variable was examined. The parameters considered in this analysis were blood to plasma ratio (B/P), fraction of drug unbound in plasma and enterocytes (fu_p and fu_G, respectively), maximum rate of metabolite formation (V_{max}), K_m, and the effective intestinal permeability (P_{eff}). As part of the sensitivity analysis, each parameter was varied by two-orders of magnitude spanning the default value, except when there is a biological or physiological constraint. Owing to the use of a population representative subject in the Simcyp ASA tool, the sensitivity analysis to assess the effect of varying the physiological parameter values (such as organ weight and blood flow) could not be conducted.

Correlation of midazolam with other CYP3A probe drugs

The pharmacokinetics of midazolam following 5 mg of oral dosing was simulated and the association of CL/F with the CL/F of alprazolam (0.5 mg), triazolam (0.25 mg), simvastatin (40 mg), and nifedipine (20 mg) generated from *in silico* pharmacokinetic studies (available in the Simcyp simulator) in paired subjects was analyzed. Least squares linear

Table 1 List of substrates and probes used in simulations to illustrate the strategy to refine the phenotyping approach in different scenarios

Substrates	Pathways		Probe drugs
	CYP isoenzymes	Drug transporters	
Correlation between CYP3A probes			
Triazolam	CYP3A		Midazolam
Alprazolam			Midazolam Triazolam
Nifedipine			
Simvastatin			Midazolam
Phenotyping strategy for drugs with multiple metabolic pathways			
Aripiprazole	CYP3A		Midazolam
	CYP2D6		Dextromethorphan
Risperidone	CYP3A		Midazolam
	CYP2D6		Dextromethorphan
Erlotinib	CYP3A		Midazolam (oral)
			Midazolam (i.v.)
	CYP1A2		Caffeine
Phenotyping strategy when hepatic uptake transporters play a significant role			
Atorvastatin	CYP3A		Midazolam
		SLCO1B1	Rosuvastatin
			Pitavastatin
Repaglinide	CYP3A		Midazolam
	CYP2C8		Rosiglitazone
		SLCO1B1	Rosuvastatin
			Pitavastatin
Docetaxel	CYP3A		Midazolam (oral)
			Midazolam (i.v.)
		SLCO1B1	Rosuvastatin
			Pitavastatin

CYP, cytochrome P450.

regression analyses were performed and the coefficient of determination (r^2) was determined for comparison of simulated CL/F values.

Proposing an improved phenotyping strategy for drugs with multiple elimination pathways

Risperidone, aripiprazole, and erlotinib were used as illustrative examples of drugs cleared by multiple CYP enzymes. Risperidone and aripiprazole PBPK models were developed in the simulator based on a report by Vieira *et al.*[24] The simulation results have been validated by comparison to the clinically observed data available from drug–drug (with CYP3A4 and 2D6 inhibitors) and drug–gene (effect of *CYP2D6* polymorphism) interaction studies. The erlotinib PBPK model, on which predictive performance was assessed, was based on a study in healthy subjects with and without the CYP3A4 inhibitor ketoconazole, as described in a previous report.[25]

In the simulation, midazolam and the CYP2D6 probe dextromethorphan (oral doses of 5 and 30 mg, respectively) were used as the probes for the drugs of interest, risperidone (1 mg) and aripiprazole (10 mg), while erlotinib (100 mg) was correlated with midazolam (5 mg orally and 2 mg i.v. bolus) and the CYP1A probe caffeine (150 mg). The assigned fraction of the drug in the body cleared by particular metabolic pathway (f_m) values for risperidone ($f_{m,CYP3A4}$ = 0.37 and $f_{m,CYP2D6}$ = 0.59), aripiprazole ($f_{m,CYP3A4}$ = 0.61 and $f_{m,CYP2D6}$ = 0.38), and erlotinib ($f_{m,CYP3A4}$ = 0.70 and $f_{m,CYP1A2}$ = 0.29) were based on the predictions in the Simcyp Simulator. The correlations of apparent drug clearance with that of a single probe and the sum of CL/F of multiple probes were analyzed. To calculate the sum of multiple-probe CL/F values, while taking into account the contribution of each of the CYP pathways, Eq. 1 was used.

$$(CL/F)_{sum} = f_{m1}.(CL/F)_1 + f_{m2}.(CL/F)_2 + \cdots + f_{m,n}.(CL/F)_n \quad (1)$$

where $f_{m,n}$ indicates the contribution (as a fraction) of metabolic pathway *–n* and $(CL/F)_n$ represents the apparent clearance of the phenotyping probe for metabolic pathway *–n*.

In vitro drug metabolism study from single recombinant CYP enzyme or human liver microsomes (HLMs) with specific inhibitor is a robust approach to be able to quantify f_m. The predictive performance of the PBPK model used in the current study as detailed in the original publications has also been assessed by verifying the simulation prediction results to (published) clinically observed pharmacokinetic data in monotherapy and in a drug-drug interaction scenario (where one or more of the metabolic pathways are perturbed). This approach allows us to assess the reliability and have confidence in the f_m used in the model.[26,27]

Proposing an improved phenotyping strategy when hepatic uptake transporters play a significant role in drug disposition

The proposed strategy to refine the phenotyping approach, accounting for the significant role of hepatic uptake transporters, is based on the extended clearance concept. This hepatic clearance model (Eq. 2) incorporates the interplay between DME and transporters in determining the clearance.[17,18]

$$CL_H = \frac{Q_H fu_p CL_{s,in}(CL_{met} + CL_{c,ef})}{Q_H(CL_{s,ef} + CL_{met} + CL_{c,ef}) + fu_p CL_{s,in}(CL_{met} + CL_{c,ef})} \quad (2)$$

where Q_H, fu_p, CL_{met}, $CL_{s,in}$, $CL_{s,ef}$, and $CL_{c,ef}$ are hepatic blood flow, unbound fraction in plasma, metabolic intrinsic clearance, sinusoidal influx, sinusoidal efflux, and canalicular efflux (or biliary) clearances, respectively. The uptake clearance ($CL_{s,in}$) will be the rate-determining step when $(CL_{met} + CL_{c,ef}) > CL_{s,ef}$[18] and the value of this ratio is close to or > 1.[28] The permeability-limited liver model implemented within the Simcyp Simulator[20] is consistent with the extended clearance concept.

In this study, atorvastatin (40 mg orally), docetaxel (100 mg/m^2 by i.v. infusion over 1 hour) and repaglinide (0.25 mg orally) were used as the substrates of both the CYP3A4 isoenzyme and the SLCO1B1 (organic anion-transporting polypeptide 1B1) transporter. Repaglinide also undergoes CYP2C8-mediated metabolism.[29] Midazolam (5 mg) and rosiglitazone (4 mg) were selected as probes for CYP3A4 and CYP2C8 activities, respectively, and both rosuvastatin (20 mg) and pitavastatin (4 mg) were chosen as SLCO1B1 probes. Pitavastatin and atorvastatin PBPK models in Simcyp have been validated against clinically observed data in healthy subjects.[30] The docetaxel PBPK model was based on that verified with data from patients with cancer.[31] Rosuvastatin, repaglinide, and rosiglitazone PBPK models are available in the simulator. The correlation of parameters between each of the drugs and corresponding probes was constructed. The docetaxel phenotyping study was simulated in the virtual cancer population available in the Simcyp Simulator, with details of the *in silico* trial as described for the healthy population. The virtual cancer population developed in the Simcyp Simulator is based mainly on data from adult patients with solid tumors and is characterized by an increase in serum creatinine and α1-acid glycoprotein concentrations, similar levels of expression of DME[31] and higher abundance of SLCO1B1 but a lower level of SLCO1B3 in liver sinusoid compared to typical healthy people.[32]

RESULTS

Identifying factors that potentially affect the apparent clearance of midazolam

The sensitivity analyses were utilized to identify all parameters that can potentially alter midazolam CL/F, as shown in **Figure 1**. Midazolam CL/F was highly sensitive to CYP3A4 activity with an approximately seven-fold difference caused by a two-order magnitude variation in V_{max} and K_m. When the variation of both V_{max} and K_m were taken into account (thus reflecting the variation of CL_{int}), a two-order magnitude of variation across the default values was accompanied by > 10-fold of change in the CL/F value. Similar relationships were also observed for f_{up} variation from 0.01–1. A twofold difference of simulated midazolam CL/F was observed by the variation of P_{eff} and B/P values across two-orders of magnitude and from 0.55 to 10, respectively. The value of fu_G had minimal impact on midazolam CL/F (i.e., variation from 0.01–1 caused less than twofold change).

Correlation of midazolam with other CYP3A probes

The statistical testing for the significance of the correlation was not reported here due to its high dependence on sample size.[33] In each simulation, a total of 200 subjects was used. These pooled analyses were likely to capture the true underlying relationship by providing an excellent accuracy and precision on the r^2 value. This results in a very low P value ($P < 0.001$) even when there was only a weak predictive value (i.e., very low r or r^2), which can be potentially misleading, as illustrated in **Figure S1**. Therefore, the extent of association was assessed semiquantitatively using $r^2 = 0.60$ as the cutoff for what is considered a strong correlation, as has been proposed previously.[6,34] The use of r^2, as suggested by Harmatz and Greenblatt,[35] is preferred over a specific P value, especially for data with a large sample size.

The CL/F of midazolam was strongly correlated with that of triazolam CL/F ($r^2 = 0.87$) and to a lesser extent with nifedipine CL/F ($r^2 = 0.56$), as depicted in **Figure 2**. Conversely, midazolam CL/F had a poor relationship with alprazolam and simvastatin CL/F ($r^2 \leq 0.40$). The relevant physicochemical properties, blood binding characteristics, and pharmacokinetic parameters of midazolam and other CYP3A probes are summarized in **Table 2**.

Proposing an improved phenotyping strategy for drugs with multiple elimination pathways

There was a weak association between CL/F values for midazolam and risperidone and aripiprazole. Dextromethorphan CL/F better predicted risperidone and aripiprazole CL/F with r^2 of 0.78 and 0.52, respectively. Using the sum of midazolam and dextromethorphan CL/F increased the correlation further with a slightly improved r^2 compared to dextromethorphan alone (**Figure 3**). The apparent clearance of erlotinib was correlated with that of i.v. midazolam (CL), but not with the orally administered probe (CL/F). Caffeine CL/F was modestly associated with erlotinib CL/F ($r^2 = 0.39$). The simulation showed that the sum of caffeine and i.v. midazolam CL/F adequately describes the variability in erlotinib CL/F in both healthy people and patients with cancer (r^2 of 0.60 and 0.59, respectively).

Proposing an improved phenotyping strategy when hepatic uptake transporters play a significant role

The ratio of simulated $CL_{met} + CL_{c,ef}$ to $CL_{s,ef}$ of atorvastatin, repaglinide, and docetaxel based on the permeability-limited liver model in the Simcyp Simulator were 23, 0.8, and 0.3, respectively. It was assumed that $CL_{s,ef}$ equals the passive diffusion clearance (CL_{PD}) when there is no active transport by sinusoidal efflux transporters. Atorvastatin and repaglinide CL/F were poorly predicted by midazolam

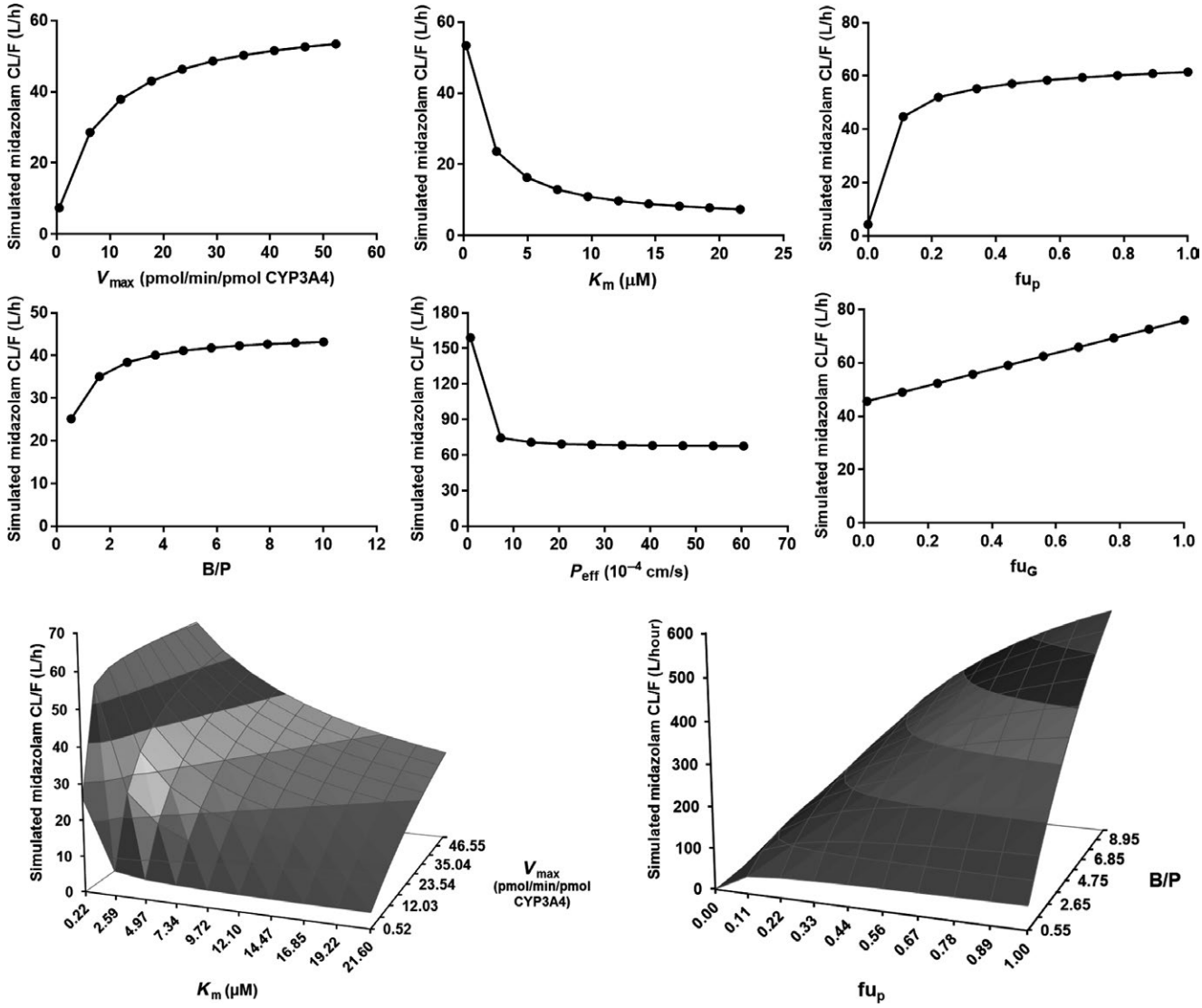

Figure 1 Sensitivity analyses depicting the impact of V_{max}, K_m, CL_{int}, fu_p, B/P, P_{eff}, and fu_G variation on apparent midazolam clearance. B/P, blood to plasma ratio; CL_{int}, hepatic intrinsic clearance; CYP, cytochrome P450; fu_G, fraction of drug unbound in enterocytes; fu_p, fraction of drug unbound in plasma; K_m, Michaelis-Menten constant; P_{eff}, the effective intestinal permeability; V_{max}, the maximum rate of metabolite formation.

CL/F ($r^2 < 0.1$). Rosiglitazone CL/F could only modestly explain repaglinide CL/F ($r^2 = 0.27$). Rosuvastatin and pitavastatin CL/F values had a better correlation ($r^2 \geq 0.5$) with both atorvastatin and repaglinide CL/F data, as shown in **Figure 4**. Docetaxel clearance was better predicted by CL after i.v. midazolam than the corresponding predictions for either pitavastatin or rosuvastatin CL/F values (r^2 of 0.67 vs. 0.02 and 0.11, respectively).

DISCUSSION

This study utilized the capability of a PBPK platform in conjunction with the *in vitro* to *in vivo* extrapolation technique[36] to predict the apparent clearance (CL/F) of drugs (and probe drugs) in different scenarios. As may be expected in the sensitivity analyses, the CL/F of midazolam was most sensitive to CYP3A4-mediated CL_{int} (and, thus, the V_{max} and K_m) and fu_p with > 10-fold variation across a two-order magnitude of change in the underlying parameters. The B/P ratio and intestinal P_{eff} modestly affected midazolam CL/F. The latter will have a greater impact on CL/F when $CLu_{int,G}$ is low relative to CL_{perm}.[37] The value of fu_G had minimal impact on midazolam CL/F and, indeed in many cases, fu_G is assumed to be 1 to give a better accuracy of F_G prediction.[37] The equation for the apparent clearance was dissected and the corresponding influential parameters are shown in **Figure 5**. It is clear that both metabolic and nonmetabolic determinants[33] work in concert to maintain a particular CL/F value. Physiological factors, including CYP enzymes abundance, liver size, and the hepatic (Q_H) and villous blood flow (Q_{villi}), are also important. The last two parameters will make a significant contribution for high

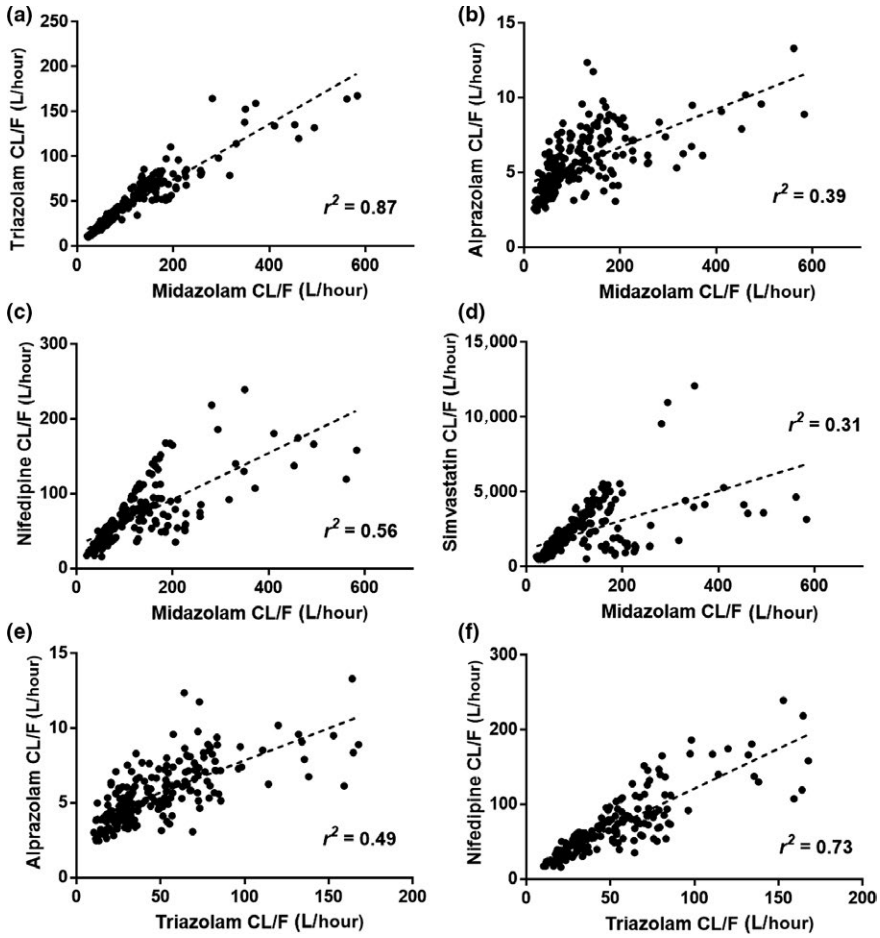

Figure 2 Relationship between simulated apparent midazolam clearance (CL/F) with those of triazolam (**a**), alprazolam (**b**), nifedipine (**c**), and simvastatin (**d**). The association of triazolam-alprazolam (**e**) and triazolam-nifedipine CL/F (**f**) were also shown. Each simulation included 10 *in silico* trials with 20 typical healthy subjects in each trial.

Table 2 Summary of physicochemical properties, blood binding characteristics and pharmacokinetic parameters of midazolam and other CYP3A probes

	CYP3A probes				
Parameters	**Midazolam**	**Triazolam**	**Alprazolam**	**Simvastatin**	**Nifedipine**
Physicochemical and blood-binding properties					
Log P	3.53	2.42	2.12	4.68	2.69
B/P	0.60	0.62	0.83	1.00	0.74
fu_p	0.03	0.18	0.29	0.02	0.04
Pharmacokinetic parameters[a]					
f_a	0.88	0.88	0.88	0.88	0.99
F_G	0.58	0.66	1.00	0.12	0.65
F_H	0.55	0.71	0.96	0.35	0.63
F	0.28	0.41	0.84	0.03	0.41
V_{ss} (L/kg)	0.88	0.48	0.76	1.50	0.69
CL/F (L/h)	118.71	49.23	5.66	2,317.77	67.00
$f_{m,CYP3A4}$	0.86	0.90	0.71	0.89	0.96
$f_{m,CYP3A5}$	0.10	0.08	0.05		0.04
$f_{m,others}$	0.04 (UGT1A4 and renal)	0.02 (renal)	0.24 (renal)	0.11 (other CYPs)	

B/P, blood to plasma ratio; CL/F, apparent clearance; CYP, cytochrome P450; F, bioavailability; f_a, fraction of drug absorbed from the intestinal tract; F_G and F_H, fraction that escapes the intestinal and hepatic first-pass metabolism, respectively; f_m, fraction of drug metabolized through particular metabolic pathway; fu_p, fraction of drug unbound in plasma; Log P, the partition coefficient; V_{ss}, volume of distribution at steady-state based on total tissue volumes.
[a]The pharmacokinetic parameters are presented as the mean value from the simulation (10 *in silico* trials in typical healthy people with 20 subjects each).

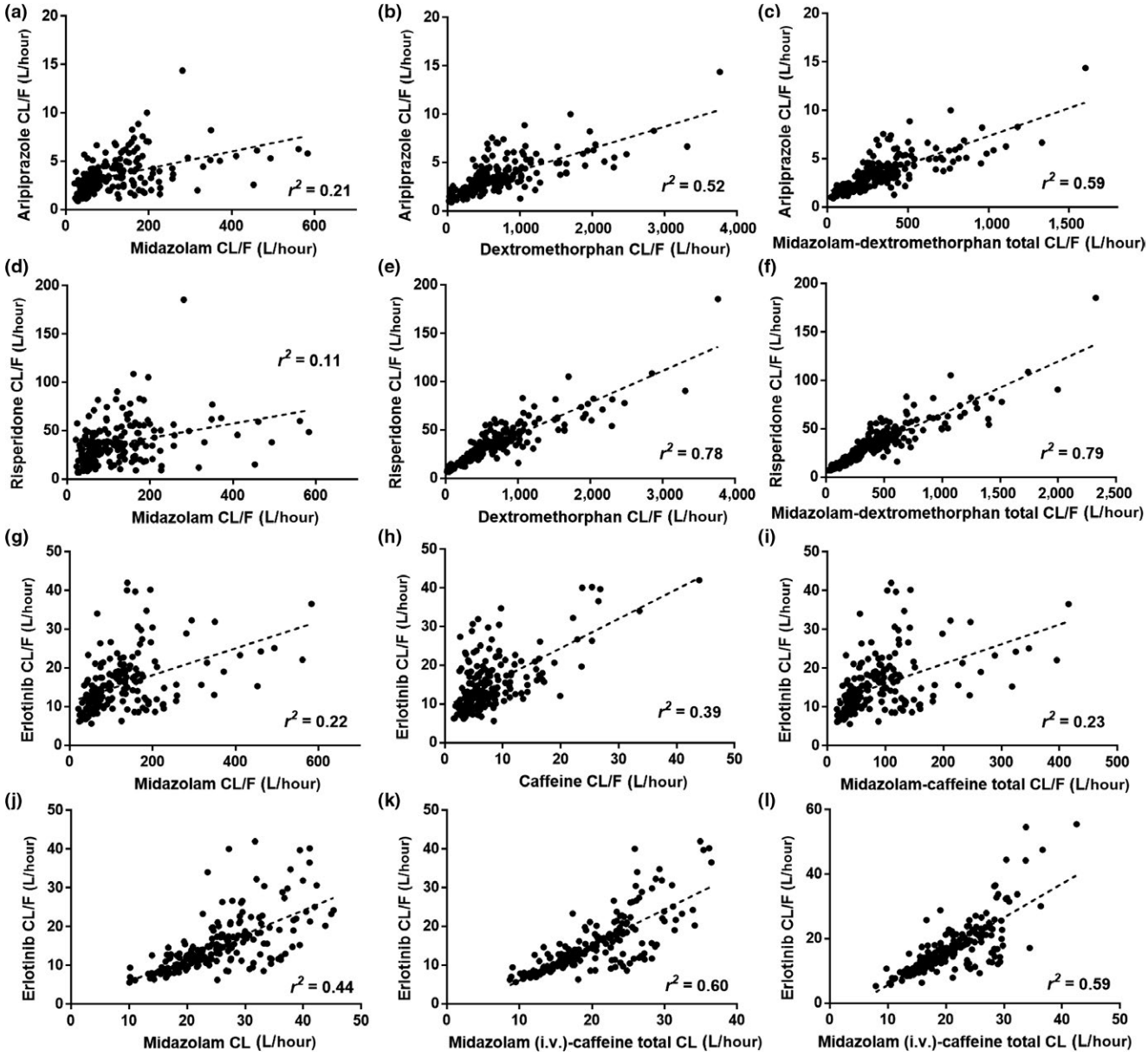

Figure 3 Correlation analyses between aripiprazole and risperidone apparent clearance (CL/F) with midazolam (**a** and **d**) and dextromethorphan CL/F (**b** and **e**) and the sum of midazolam and dextromethorphan CL/F based on the corresponding f_m value (**c** and **f**). The simulated erlotinib CL/F was predicted by oral midazolam (**g**), caffeine (**h**), i.v. midazolam (**j**), and the sum of oral midazolam and caffeine CL/F (**i**) in healthy people as well as the sum of simulated i.v. midazolam and caffeine CL/F in healthy people (**k**) and patients with cancer (**l**). Each simulation was generated using 10 *in silico* trials with 20 subjects each.

hepatic extraction ratio drugs given intravenously (Q_H)[38] and for drugs with high intestinal permeability (Q_{villi}).

Midazolam, alprazolam, triazolam, nifedipine, and simvastatin are almost exclusively metabolized by CYP3A4, suggesting a strong correlation between midazolam CL/F and the CL/F of these other drugs. However, this was true only for the correlation with triazolam, consistent with a study in healthy people.[34] Based on the F_G and F_H value, midazolam and triazolam share similarity in terms of the absolute and relative magnitude of gut and hepatic clearance (**Table 2**). Conversely, alprazolam has a considerably lower extraction ratio (low $CLu_{int,H}$ and negligible $CLu_{int,G}$) and a prominent contribution of renal clearance (fe = 0.24), and, hence, lacks correlation of CL/F with either midazolam or triazolam. Despite similar F_G, F_H, and fu_p, nifedipine CL/F was only modestly correlated with that of midazolam, possibly due to a substantially lower contribution of CYP3A5 (4% vs. 10%). The lack of correlation between midazolam and nifedipine CL/F has been addressed in previous reports.[22,23]

In the simulations, there is only a weak correlation between the midazolam and simvastatin CL/F, reasonably explained by a lower bioavailability and a greater contribution of hepatic than intestinal clearance for the latter. However, a study in healthy participants found that dose adjustment

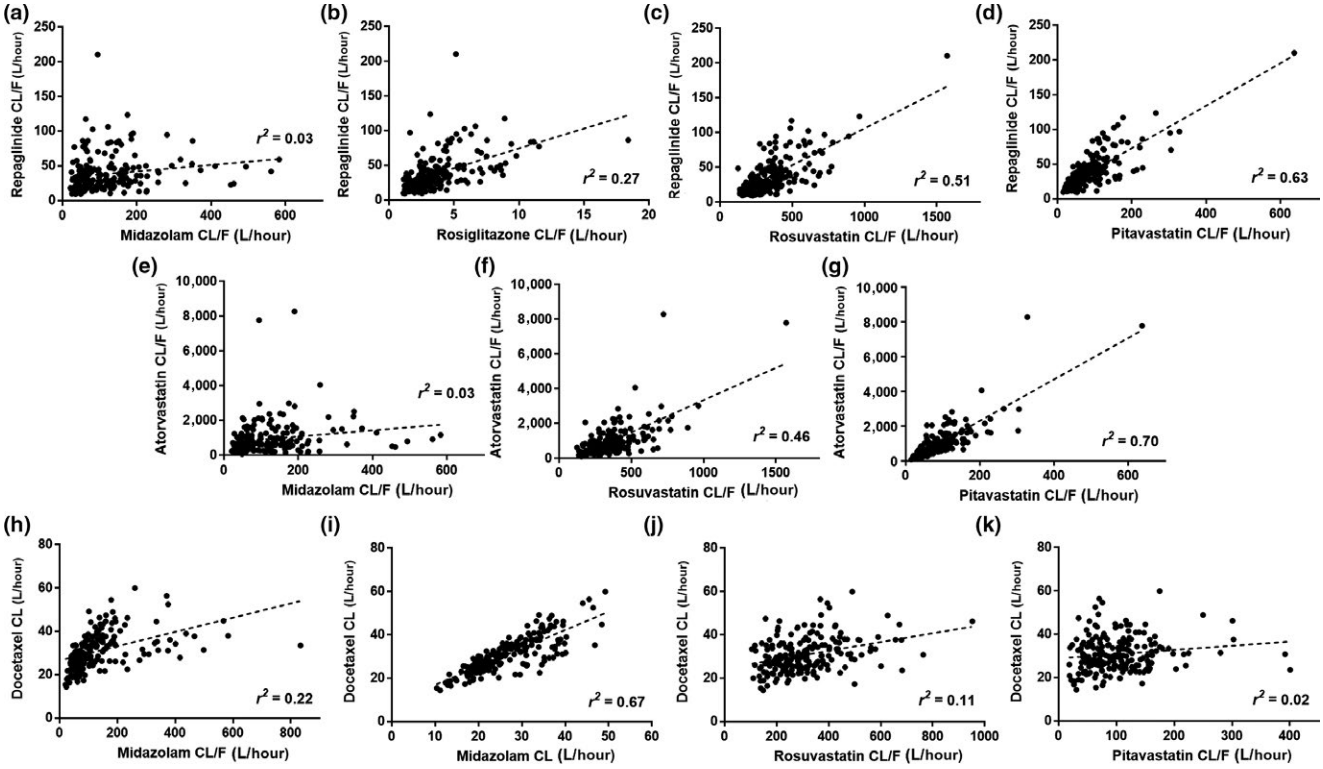

Figure 4 Association of cytochrome P450 (CYP)3A probe represented by oral midazolam (**a** and **e**) and rosuvastatin (**c** and **f**) and pitavastatin (**d** and **g**) as SLCO1B1 probes with atorvastatin and repaglinide. Relationship of the latter and rosiglitazone apparent clearance (CL/F) was also analyzed (**b**). The simulations were carried out in virtual healthy volunteers (10 trials with 20 subjects each). The correlation of oral (**h**) and i.v. midazolam (**i**), rosuvastatin (**j**), and pitavastatin (**k**) with that of docetaxel clearance in virtual patients with cancer (10 trials with 20 subjects each) were shown.

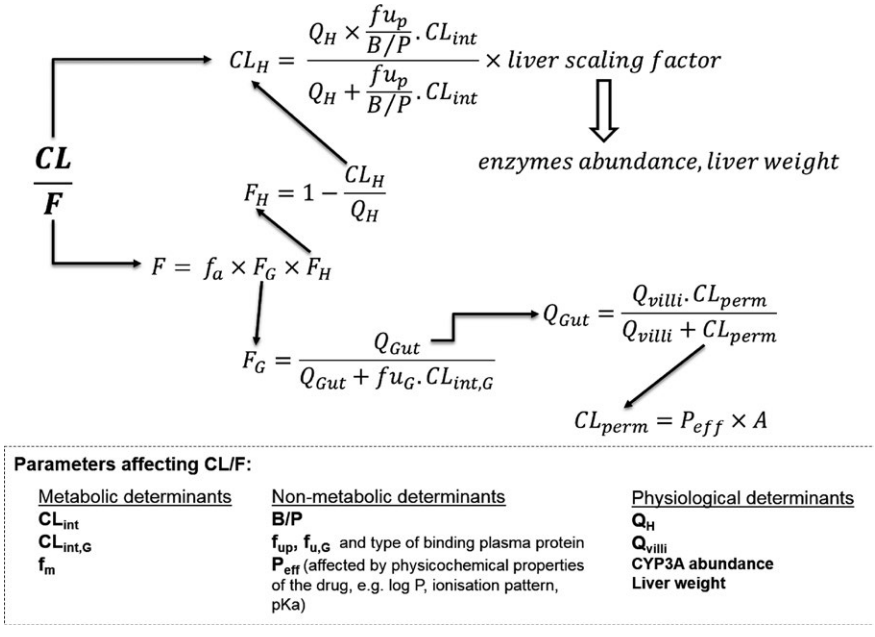

Figure 5 Dissection of the apparent clearance (CL/F) formula to describe all possible influencing parameters. The CL_{int} equation is based on the well-stirred hepatic liver model and F_G is estimated using the Q_{Gut} model. A, surface area of the small intestine; B/P, blood to plasma ratio; CL_{int}, hepatic intrinsic clearance; $CL_{int,G}$, intestinal intrinsic clearance; f_a, fraction of drug absorbed from the intestinal tract; f_m, fraction of drug metabolized through particular metabolic pathway; fu_G, fraction of drug unbound in enterocytes; fu_p, fraction of drug unbound in plasma; F_G and F_H, fraction that escapes the intestinal and hepatic first-pass metabolism, respectively; P_{eff}, the effective intestinal permeability; Q_{Gut}, the gut blood flow rate; Q_H, hepatic blood flow rate; Q_{villi}, the villous blood flow rate.

based on CYP3A phenotyping by midazolam reduced the variability of simvastatin exposure[39] and a significant, although lower, correlation of midazolam and simvastatin apparent clearance was found in another healthy volunteer study.[40] This disparity might partly be related to the role of SLCO1B1 transporter in the hepatic uptake of simvastatin.[41] In individuals with midazolam CL/F at or above the mean value, the associated simvastatin CL/F tended to be more clustered above the regression line (**Figure 2**).

Risperidone and aripiprazole are predominantly metabolized by both CYP3A4 and 2D6.[42,43] Phenotyping of drugs, which are substrates of multiple DMEs, has generally been hindered by the lack of significant relationship with a single probe. Here, we proposed the combined use of CYP3A4 and 2D6 probes (midazolam and dextromethorphan, respectively) to phenotype the CL/F of risperidone and aripiprazole. Due to the additive nature of clearance from multiple metabolic pathways,[15,16] the parameter can be calculated from the sum of all contributing metabolic pathways. Use of the sum of midazolam and dextromethorphan CL/F indeed improved the correlation to CL/F of both risperidone and aripiprazole compared to either probe alone.

Midazolam CL calculated after i.v. administration correlated better to erlotinib CL/F than did the corresponding oral parameter. This was due to the limited contribution of erlotinib intestinal metabolism ($F_G = 0.9$) as intestinal and hepatic CYP3A4 are regulated independently.[44] Hence, the i.v. administration of midazolam, which assesses hepatic CYP3A, is more suitable as opposed to overall CYP3A activity after oral administration of midazolam,[10] contrary to the theory that the probe and the drug of interest should be administered by the same route.[6] For drugs with a low extraction ratio (E_H) and $CLu_{int,G}$, the use of i.v. probe drugs seems more appealing. We deliberately limited this hypothesis to low E_H drugs because the hepatic clearance of an i.v. probe with the characteristic of high E_H is dictated only by hepatic blood flow (assuming negligible role of hepatic uptake transporters).[38] Our results are consistent with the observation in patients with advanced non-small cell lung cancer that apparent caffeine clearance, and not oral midazolam clearance, was significantly correlated with erlotinib CL/F.[14] The use of i.v. administered midazolam with caffeine is likely to be a better option to describe variability in erlotinib CL/F both in healthy subjects and in patients with cancer (**Figure 3**). Use of multiple probes for drugs that are mainly metabolized, with significant contributions from two or more CYP isoenzymes, should be considered because more variability in CL/F can be accounted for.

Atorvastatin is metabolized almost exclusively by CYP3A4. However, it is also a substrate of SLCO1B1 (organic anion-transporting polypeptide 1B1), a sinusoidal uptake transporter. Uptake into the liver is rate-limiting and, thus, hepatic clearance of atorvastatin is determined by the sinusoidal influx clearance ($CL_{s,in}$) and not by the metabolic intrinsic clearance ($CLu_{int,H}$ or CL_{met}).[18] In line with this, the midazolam CL/F in an individual subject was predicted to have a poor correlation with atorvastatin CL/F. Rosuvastatin and pitavastatin, both of which are SLCO1B1 substrates, better predict atorvastatin clearance (**Figure 4**). Pitavastatin is a more sensitive and selective substrate of SLCO1B1 than rosuvastatin,[45] confirmed by a superior r^2 value in the simulated data. Repaglinide is a substrate of both CYP3A4 and 2C8, as well as the SLCO1B1 transporter, which governs uptake into the hepatocytes. Repaglinide clinical pharmacokinetic and pharmacodynamic are influenced by *SLCO1B1* genetics.[46,47] In terms of CL/F, midazolam and rosiglitazone were poorly correlated with repaglinide and much less predictive than rosuvastatin and pitavastatin. Conversely, the metabolic clearance of docetaxel is the rate-determining step in elimination, even though it is also a substrate of SLCO1B1. From the ratio of $CL_{met} + CL_{c,ef}$ to $CL_{s,ef}$, which is far < 1, it can be predicted that hepatic clearance of docetaxel is not limited by $CL_{s,in}$.[28] Therefore, i.v. midazolam is predicted to be a better probe to assess docetaxel clearance, closely in agreement with *in vivo* findings in patients with cancer.[48]

A limitation of this *in silico* study is the reliance on CL/F or CL, instead of the specific metrics for each probe. The parameter CL/F was used in the correlation analyses for simplicity and as the ideal metric for a phenotyping study. The main difficulty to estimate CL/F is the requirement for frequent blood sampling, which is often not feasible in clinical practice. A significant relationship between probes and substrates may not always be recapitulated across different phenotyping metrics.[10,49] It is also noteworthy that in these simulations (conducted in the Simcyp Simulator) the variability (or coefficient variation) was not directly assigned to the V_{max} and K_m value. The interindividual variability in V_{max} (and, thus, CL_{int}) was represented by the variance in the level of expression of corresponding CYP enzyme. This holds true for *in vitro* metabolic data derived from HLMs, assuming that the HLM samples were from adequately large pools of individuals.[15] The variability of hepatic scaling factors (i.e., microsomal protein per gram of liver and liver weight) further contributes to interindividual variability of hepatic clearance of the simulated drugs. This study covers only two common scenarios: a scenario involving drug metabolism mediated by multiple CYP enzymes and a scenario in which hepatic uptake transporters play a significant role in the pharmacokinetics of a drug. Other possible scenarios are beyond the scope of this simulation study. Although the simulation provided a proof-of-concept of the proposed strategy, verification in a clinical setting would be desirable.

The use of probe drugs has a continuing role in phenotyping for drug elimination. There are many parameters, including nonmetabolic determinants that must be considered in selecting suitable phenotyping probes, creating additional potential difficulties. However, we have demonstrated that PBPK in conjunction with an *in vitro-in vivo* extrapolation approach, as implemented in the Simcyp Simulator,[50] may guide the selection of suitable probes and improves the design of *in vivo* phenotyping studies. The use of multiple probes for drugs that are predominantly metabolized by more than one CYP enzyme should be considered, and where hepatic uptake transporters play a significant role, a specific transporter probe will be more predictive of drug clearance.

Supporting Information. Supplementary information accompanies this paper on the *CPT: Pharmacometrics & Systems Pharmacology* website (www.psp-journal.com).

Figure S1. Illustration of the effect of subject size (n) on P value in the correlation analyses for three different level of coefficient of determination (r^2).
Table S1. Input parameter values for PBPK model of probes and drugs of interest used in the simulation study.

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1. Wilkinson, G.R. Drug metabolism and variability among patients in drug response. *N. Engl. J. Med.* **352**, 2211–2221 (2005).
2. Zanger, U.M. & Schwab, M. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol. Ther.* **138**, 103–141 (2013).
3. Yee, S.W. *et al.* Influence of transporter polymorphisms on drug disposition and response: a perspective from the International Transporter Consortium. *Clin. Pharmacol. Ther.*; e-pub ahead of print 2018. https://doi.org/10.1002/cpt.1082
4. Thummel, K.E. & Lin, Y.S. Sources of interindividual variability. *Methods Mol. Biol.* **1113**, 363–415 (2014).
5. Zanger, U.M. *et al.* Genetics, epigenetics, and regulation of drug-metabolizing cytochrome p450 enzymes. *Clin. Pharmacol. Ther.* **95**, 258–261 (2014).
6. Hohmann, N., Haefeli, W.E. & Mikus, G. CYP3A activity: towards dose adaptation to the individual. *Expert Opin. Drug Metab. Toxicol.* **12**, 479–497 (2016).
7. Werk, A.N. & Cascorbi, I. Functional gene variants of CYP3A4. *Clin. Pharmacol. Ther.* **96**, 340–348 (2014).
8. Zhou, Y., Ingelman-Sundberg, M. & Lauschke, V.M. Worldwide distribution of cytochrome P450 alleles: a meta-analysis of population-scale sequencing projects. *Clin. Pharmacol. Ther.* **102**, 688–700 (2017).
9. Tracy, T.S. *et al.* Interindividual variability in cytochrome P450-mediated drug metabolism. *Drug Metab. Dispos.* **44**, 343–351 (2016).
10. Fuhr, U., Jetter, A. & Kirchheiner, J. Appropriate phenotyping procedures for drug metabolizing enzymes and transporters in humans and their simultaneous use in the "cocktail" approach. *Clin. Pharmacol. Ther.* **81**, 270–283 (2007).
11. de Andres, F. & Llerena, A. Simultaneous determination of cytochrome P450 oxidation capacity in humans: a review on the phenotyping cocktail approach. *Curr. Pharm. Biotechnol.* **17**, 1159–1180 (2016).
12. Stopfer, P. *et al.* Pharmacokinetic evaluation of a drug transporter cocktail consisting of digoxin, furosemide, metformin, and rosuvastatin. *Clin. Pharmacol. Ther.* **100**, 259–267 (2016).
13. Wong, M. *et al.* Predictors of vinorelbine pharmacokinetics and pharmacodynamics in patients with cancer. *J. Clin. Oncol.* **24**, 2448–2455 (2006).
14. Parra-Guillen, Z.P. *et al.* Role of cytochrome P450 3A4 and 1A2 phenotyping in patients with advanced non-small-cell lung cancer receiving erlotinib treatment. *Basic Clin. Pharmacol. Toxicol.* **121**, 309–315 (2017).
15. Howgate, E.M., Rowland Yeo, K., Proctor, N.J., Tucker, G.T. & Rostami-Hodjegan, A. Prediction of in vivo drug clearance from in vitro data. I: impact of inter-individual variability. *Xenobiotica* **36**, 473–497 (2006).
16. Cubitt, H.E., Houston, J.B. & Galetin, A. Prediction of human drug clearance by multiple metabolic pathways: integration of hepatic and intestinal microsomal and cytosolic data. *Drug Metab. Dispos.* **39**, 864–873 (2011).
17. Shitara, Y., Horie, T. & Sugiyama, Y. Transporters as a determinant of drug clearance and tissue distribution. *Eur. J. Pharm. Sci.* **27**, 425–446 (2006).
18. Patilea-Vrana, G. & Unadkat, J.D. Transport vs. metabolism: what determines the pharmacokinetics and pharmacodynamics of drugs? Insights from the extended clearance model. *Clin. Pharmacol. Ther.* **100**, 413–418 (2016).
19. Jamei, M. *et al.* The Simcyp population based simulator: architecture, implementation, and quality assurance. *In Silico Pharmacol.* **1**, 9 (2013).
20. Jamei, M. *et al.* A mechanistic framework for in vitro-in vivo extrapolation of liver membrane transporters: prediction of drug-drug interaction between rosuvastatin and cyclosporine. *Clin. Pharmacokinet.* **53**, 73–87 (2014).
21. Thummel, K.E. *et al.* Use of midazolam as a human cytochrome P450 3A probe: I. In vitro-in vivo correlations in liver transplant patients. *J. Pharmacol. Exp. Ther.* **271**, 549–556 (1994).
22. Foti, R.S., Rock, D.A., Wienkers, L.C. & Wahlstrom, J.L. Selection of alternative CYP3A4 probe substrates for clinical drug interaction studies using in vitro data and in vivo simulation. *Drug Metab. Dispos.* **38**, 981–987 (2010).
23. Kenworthy, K.E., Bloomer, J.C., Clarke, S.E. & Houston, J.B. CYP3A4 drug interactions: correlation of 10 in vitro probe substrates. *Br. J. Clin. Pharmacol.* **48**, 716–727 (1999).
24. Vieira, M.D. *et al.* PBPK model describes the effects of comedication and genetic polymorphism on systemic exposure of drugs that undergo multiple clearance pathways. *Clin. Pharmacol. Ther.* **95**, 550–557 (2014).
25. Rakhit, A. *et al.* The effects of CYP3A4 inhibition on erlotinib pharmacokinetics: computer-based simulation (SimCYP) predicts in vivo metabolic inhibition. *Eur. J. Clin. Pharmacol.* **64**, 31–41 (2008).
26. Tsamandouras, N., Rostami-Hodjegan, A. & Aarons, L. Combining the 'bottom up' and 'top down' approaches in pharmacokinetic modelling: fitting PBPK models to observed clinical data. *Br. J. Clin. Pharmacol.* **79**, 48–55 (2013).
27. Shebley, M. *et al.* Physiologically based pharmacokinetic model qualification and reporting procedures for regulatory submissions: a consortium perspective. *Clin. Pharmacol. Ther.* **104**, 88–110 (2018).
28. Patilea-Vrana, G. & Unadkat, J.D. When does the rate-determining step in hepatic clearance of a drug switch from sinusoidal uptake clearance to all hepatobiliary clearances? *Drug Metab. Pharmacokinet.* **33**, S28 (2018).
29. Sall, C., Houston, J.B. & Galetin, A. A comprehensive assessment of repaglinide metabolic pathways: impact of choice of in vitro system and relative enzyme contribution to in vitro clearance. *Drug Metab. Dispos.* **40**, 1279–1289 (2012).
30. Duan, P., Zhao, P. & Zhang, L. Physiologically based pharmacokinetic (PBPK) modeling of pitavastatin and atorvastatin to predict drug-drug interactions (DDIs). *Eur. J. Drug Metab. Pharmacokinet.* **42**, 689–705 (2017).
31. Thai, H.T., Mazuir, F., Cartot-Cotton, S. & Veyrat-Follet, C. Optimizing pharmacokinetic bridging studies in paediatric oncology using physiologically-based pharmacokinetic modelling: application to docetaxel. *Br. J. Clin. Pharmacol.* **80**, 534–547 (2015).
32. Burt, H.J. *et al.* Abundance of hepatic transporters in Caucasians: a meta-analysis. *Drug Metab. Dispos.* **44**, 1550–1561 (2016).
33. Kharasch, E.D., Thummel, K.E. & Watkins, P.B. CYP3A probes can quantitatively predict the in vivo kinetics of other CYP3A substrates and can accurately assess CYP3A induction and inhibition. *Mol. Interv.* **5**, 151–153 (2005).
34. Masica, A.L., Mayo, G. & Wilkinson, G.R. In vivo comparisons of constitutive cytochrome P450 3A activity assessed by alprazolam, triazolam, and midazolam. *Clin. Pharmacol. Ther.* **76**, 341–349 (2004).
35. Harmatz, J.S. & Greenblatt, D.J. Regression and correlation. *Clin. Pharmacol. Drug Dev.* **4**, 161–162 (2015).
36. Rostami-Hodjegan, A. Physiologically based pharmacokinetics joined with in vitro-in vivo extrapolation of ADME: a marriage under the arch of systems pharmacology. *Clin. Pharmacol. Ther.* **92**, 50–61 (2012).
37. Yang, J., Jamei, M., Yeo, K.R., Tucker, G.T. & Rostami-Hodjegan, A. Prediction of intestinal first-pass drug metabolism. *Curr. Drug Metab.* **8**, 676–684 (2007).
38. Benet, L.Z. & Hoener, B.A. Changes in plasma protein binding have little clinical relevance. *Clin. Pharmacol. Ther.* **71**, 115–121 (2002).
39. Stoll, F. *et al.* Reduced exposure variability of the CYP3A substrate simvastatin by dose individualization to CYP3A activity. *J. Clin. Pharmacol.* **53**, 1199–1204 (2013).
40. Chung, E., Nafziger, A.N., Kazierad, D.J. & Bertino Jr, J.S.. Comparison of midazolam and simvastatin as cytochrome P450 3A probes. *Clin. Pharmacol. Ther.* **79**, 350–361 (2006).
41. Noe, J., Portmann, R., Brun, M.E. & Funk, C. Substrate-dependent drug-drug interactions between gemfibrozil, fluvastatin and other organic anion-transporting peptide (OATP) substrates on OATP1B1, OATP2B1, and OATP1B3. *Drug Metab. Dispos.* **35**, 1308–1314 (2007).
42. Fang, J., Bourin, M. & Baker, G.B. Metabolism of risperidone to 9-hydroxyrisperidone by human cytochromes P450 2D6 and 3A4. *Naunyn Schmiedebergs Arch. Pharmacol.* **359**, 147–151 (1999).
43. Molden, E., Lunde, H., Lunder, N. & Refsum, H. Pharmacokinetic variability of aripiprazole and the active metabolite dehydroaripiprazole in psychiatric patients. *Ther. Drug Monit.* **28**, 744–749 (2006).
44. von Richter, O. *et al.* Cytochrome P450 3A4 and P-glycoprotein expression in human small intestinal enterocytes and hepatocytes: a comparative analysis in paired tissue specimens. *Clin. Pharmacol. Ther.* **75**, 172–183 (2004).

45. Prueksaritanont, T. *et al.* Pitavastatin is a more sensitive and selective organic anion-transporting polypeptide 1B clinical probe than rosuvastatin. *Br. J. Clin. Pharmacol.* **78**, 587–598 (2014).
46. He, J. *et al.* Effects of SLCO1B1 polymorphisms on the pharmacokinetics and pharmacodynamics of repaglinide in healthy Chinese volunteers. *Eur. J. Clin. Pharmacol.* **67**, 701–707 (2011).
47. Kalliokoski, A., Backman, J.T., Neuvonen, P.J. & Niemi, M. Effects of the SLCO1B1*1B haplotype on the pharmacokinetics and pharmacodynamics of repaglinide and nateglinide. *Pharmacogenet. Genomics* **18**, 937–942 (2008).
48. Goh, B.C. *et al.* Explaining interindividual variability of docetaxel pharmacokinetics and pharmacodynamics in Asians through phenotyping and genotyping strategies. *J. Clin. Oncol.* **20**, 3683–3690 (2002).
49. Perera, V., Gross, A.S. & McLachlan, A.J. Measurement of CYP1A2 activity: a focus on caffeine as a probe. *Curr. Drug Metab.* **13**, 667–678 (2012).
50. Rowland, A., Mangoni, A.A., Hopkins, A., Sorich, M.J. & Rowland, A. Optimized cocktail phenotyping study protocol using physiological based pharmacokinetic modeling and in silico assessment of metabolic drug-drug interactions involving modafinil. *Front. Pharmacol.* **7**, 517 (2016).