1521-009X/48/9/778–787$35.00
Drug Metabolism and Disposition

https://doi.org/10.1124/dmd.120.000017
Drug Metab Dispos 48:778–787, September 2020

Application of Azamulin to Determine the Contribution of CYP3A4/5 to Drug Metabolic Clearance Using Human Hepatocytes

Hugues Chanteux,[1] Maria Rosa,[1] Claude Delatour, Johan Nicolaï, Eric Gillent, Sylvie Dell'Aiera, and Anna-Lena Ungell

UCB Biopharma SRL, Braine-l'Alleud, Belgium

Received March 17, 2020; accepted June 1, 2020

ABSTRACT

Early determination of CYP3A4/5 contribution to the clearance of new chemical entities is critical to inform on the risk of drug-drug interactions with CYP3A inhibitors and inducers. Several in vitro approaches (recombinant P450 enzymes, correlation analysis, chemical and antibody inhibition in human liver microsomes) are available, but they are usually labor-intensive and/or suffer from specific limitations. In the present study, we have validated the use of azamulin as a specific CYP3A inhibitor in human hepatocytes. Azamulin (3 μM) was found to significantly inhibit CYP3A4/5 (>90%), whereas other P450 enzymes were not affected (less than 20% inhibition). Because human hepatocytes were used as a test system, the effect of azamulin on other key drug-metabolizing enzymes (aldehyde oxidase, carboxylesterase, UGT, flavin monooxygenase, and sulfotransferase) was also investigated. Apart from some UGTs showing minor inhibition (~20%–30%), none of these non-P450 enzymes were inhibited by azamulin. Use of CYP3A5-genotyped human hepatocyte batches in combination with CYP3cide demonstrated that azamulin (at 3 μM) inhibits both CYP3A4 and CYP3A5 enzymes. Finally, 11 compounds with known in vivo CYP3A4/5 contribution have been evaluated in this human hepatocyte assay. Results showed that the effect of azamulin on the in vitro intrinsic clearance of these known CYP3A4/5 substrates was predictive of the in vivo CYP3A4/5 contribution. Overall, the study showed that human hepatocytes treated with azamulin provide a fast and accurate estimation of CYP3A4/5 contribution in metabolic clearance of new chemical entities.

SIGNIFICANCE STATEMENT

Accurate estimation of CYP3A4/5 contribution in drug clearance is essential to anticipate risk of drug-drug interactions and select the appropriate candidate for clinical development. The present study validated the use of azamulin as selective CYP3A4/5 inhibitor in suspended human hepatocytes and demonstrated that this novel approach provides a direct and accurate determination of the contribution of CYP3A4/5 (fraction metabolized by CYP3A4/5) in the metabolic clearance of new chemical entities.

Introduction

Numerous marketed drugs are metabolized by hepatic cytochrome P450 enzymes. Within the P450 family, CYP3A is the most abundant isozyme present in the liver and is, by far, the main P450 isoform involved in drug metabolism. Indeed, approximately 30% of marketed drugs are known to be substrates of CYP3A4/5 (Wienkers and Heath, 2005; Zanger and Schwab, 2013). The substrates of CYP3A4/5 are chemically diverse, and this broad substrate specificity renders CYP3A4 susceptible to inhibition by a variety of drugs, as demonstrated by the long list of drugs known to be CYP3A4 inhibitors (https://www.fda.gov/drugs/developmentapprovalprocess/developmentresources/druginteractionslabeling/ucm093664.htm). Any new chemical entity (NCE) developed nowadays that is a CYP3A4 substrate is at high risk of being prone to drug-drug interactions (DDIs) in the clinic, which may significantly impact drug safety and efficacy (Desbans et al., 2014). The susceptibility of drugs toward CYP3A4 inhibition/induction is highly dependent on their so-called $F_{CL,metabolism} \times F_{m,CYP3A4}$, which is the fraction of total drug clearance mediated by CYP3A4 (Zhang et al., 2007). Indeed, an NCE with $F_{CL,metabolism} \times F_{m,CYP3A4} \geq 0.5$ is predicted to have an AUC ≥ 2 when coadministered with a strong CYP3A4 inhibitor. Careful assessment of contribution of an individual P450 isoform toward overall metabolism of an NCE is therefore crucial to accurately assess the risk as victim of DDI. Determination of the quantitative contribution of individual P450 enzymes to metabolism of drugs has become standard practice in the development of NCEs (Zientek and Youdim, 2015; Bohnert et al., 2016; Parmentier et al., 2019). It usually involves different approaches: 1) evaluation of the inhibitory effect caused by specific P450 chemical inhibitors or antibodies on drug metabolism using human liver microsomes (HLMs) (Khojasteh et al., 2011); 2) metabolism by individual recombinant P450 enzymes scaled up with relative activity factor/intersystem extrapolation factor (RAF/ISEF) (Emoto et al., 2006); 3) correlation analysis comparing the rate of metabolism to activities of each P450 enzyme in a set of microsomes prepared from individual

[1]H.C. and M.R. contributed equally to this work.
https://doi.org/10.1124/dmd.120.000017.

ABBREVIATIONS: ACN, acetonitrile; AO, aldehyde oxidase; AUC, Area Under the Curve; CES, Carboxylesterase; CL_{int}, Intrinsic clearance; DDI, drug-drug interaction; DME, drug-metabolizing enzyme; FDA, Food and Drug Administration; $F_{CL,metabolism}$, Fraction of total drug clearance mediated by metabolism; $F_{m,\ CYP3A4}$, Fraction of metabolic clearance mediated by CYP3A4; FMO, flavin monooxygenase; HLM, human liver microsome; HRMS, High resolution mass spectrometry; IS, internal standard; ISEF, intersystem extrapolation factor; LC-MS/MS, liquid chromatography tandem mass spectrometry; m/z, mass-to-charge transition; NCE, new chemical entity; P450, cytochrome P450; RAF, relative activity factor; SULT, sulfotransferase; UGT, uridine glucuronyltransferases; UPLC, ultraperformance liquid chromatography.

donor livers (Bohnert et al., 2016). A full assessment of P450 contribution is labor-intensive and is usually not performed before a candidate is selected for clinical development. In addition, the aforementioned in vitro tests suffer from specific limitations (Ogilvie et al., 2008).

Given the high risk of DDI for CYP3A4 substrates, an early identification of this DDI liability is desirable to avoid any surprises once the compound is selected for further development. Typically, HLM incubated with ketoconazole is used to determine the contribution of CYP3A4 to the overall metabolism, as ketoconazole has many advantages, i.e., potency, ease of use (no preincubation step), and commercial availability (Maurice et al., 1992; Zhang et al., 2002). However, ketoconazole is not as specific as it should be for use as a CYP3A4 inhibitor in phenotyping experiments. Indeed, in HLM, at concentrations required to inhibit ~95% of CYP3A4, other P450 enzymes, such as CYP1B1, CYP2B6, and CYP2C8/9/19, are also significantly inhibited (~20% to ~60%) (Newton et al., 1995; Moody et al., 1999; Stresser et al., 2004). Hence, this results in a potential overestimation of the contribution of CYP3A4 to overall drug clearance. In addition, HLMs are not the optimal test system to measure metabolic clearance of NCE, as they are lacking different key drug-metabolizing enzymes, such as aldehyde oxidase (AO), xanthine oxidase, and sulfotransferase (SULT), or they require addition of a specific uridine glucuronyltransferase (UGT) cofactor necessary to activate UGTs. Therefore, the information obtained by assays utilizing HLM should be considered carefully, as it may not represent the true $F_{CL,metabolism} \times F_{m,CYP3A4}$ of the NCE because the importance of other metabolic routes may have been overlooked.

Human hepatocytes are considered a gold-standard test system for metabolic stability studies (Soars et al., 2007). However, their use is not common for reaction phenotyping in drug discovery, although they offer a major advantage over HLM with the presence of the full machinery of drug-metabolizing enzymes, which is essential to more accurately assign F_m.

The present study investigates the use of azamulin as a CYP3A4/5 inhibitor (Sevrioukova, 2019) to determine the $F_{m,CYP3A4/5}$ of NCEs by using cryopreserved human hepatocytes. Since selectivity is a key criterion to assign a $F_{m,CYP3A4/5}$ value to NCE, a huge variety of specific substrates [P450, UGT, SULTs, flavin monooxygenase (FMO), AO, carboxylesterase 1] were used to assess azamulin's selectivity. CYP3A4 selectivity over CYP3A5 was evaluated by using human hepatocyte batches with high CYP3A5 activity treated with CYP3cide, a known specific CYP3A4 inhibitor (Walsky et al., 2012; Tseng et al., 2014). Finally, compounds with a wide range of reported $F_{m,CYP3A4/5}$ values were used to validate the assay conditions and demonstrate that this assay can be used for direct determination of $F_{CL,metabolism} \times F_{m,CYP3A4/5}$ of NCEs.

Materials and Methods

Reagents

Cryopreserved human hepatocytes (pools of 10 or 20 donors) were obtained from Xenotech (Lenexa, KS) or BioIVT (Brussels, Belgium). CYP3A5-genotyped cryopreserved human hepatocytes were obtained from BioIVT. Azamulin was purchased from Sigma-Aldrich (St Louis, MO) and Toronto Research Chemicals (Toronto, ON, Canada). Midazolam was obtained from Apin chemicals (Milton, Abingdon, UK). All other compounds [P450 probe substrates (phenacetin, bupropion, rosiglitazone, diclofenac, (*S*)-(+)-mephenytoin, and dextromethorphan] and their respective metabolites (acetaminophen, hydroxybupropion, 5-hydroxyrosiglitazone, 4′-hydroxydiclofenac, 4′-hydroxymephenytoin, dextrorphan, and 1′-hydroxymidazolam), phase II enzyme substrates (1-hydroxymidazolam, 4-hydroxycoumarin, imipramine, 5-hydroxytryptophol, naloxone, and *S*-oxazepam), aldehyde oxidase substrate (phthalazine), FMO substrates (cimetidine and ranitidine), CYP3cide (a reported specific CYP3A4 inhibitor), CYP3A4 substrates used for the validation of the assay (felodipine, simvastatin, quinidine, sildenafil, nifedipine, cerivastatin, metoprolol, loperamide, mirtazapine, zolpidem, and pimozide), and various internal standards (acetaminophen-D_4, hydroxybupropion-D_6, 1′-hydroxymidazolam-D_4, 4′-hydroxydiclofenac-$^{13}C_6$, 4′-hydroxymephenytoin-D_3, dextrorphan-D_3, 5-hydroxyrosiglitazone-D4, and ketoconazole) were purchased from Sigma-Aldrich (Bornem, Belgium). CDP323 was synthetized at UCB Biopharma (Braine-l'Alleud, Belgium). Glucuronidase/arylsulfatase were obtained from Roche (Vilvoorde, Belgium). Biosolve UPLC-grade (Valkenswaard, The Netherlands) water, acetonitrile, methanol, trifluoracetic acid, and formic acid were used without further purification on the Microbore UPLC systems. Ammonium acetate solution at 5 M and acetic acid glacial, ≥99.85%, were purchased from Sigma-Aldrich.

In Vitro Incubations to Evaluate the Inhibitory Effect of Azamulin on CYP3A4/5 in Suspensions of Pooled Human Hepatocytes with and without Preincubation

Cryopreserved human hepatocytes (pool of five male and five female donors from Xenotech, batch 1110211) were thawed according to the provider's information. Viability (trypan blue exclusion 0.2%) was higher than 73%. Incubations (0.5×10^6 cells/ml) were carried out in triplicate in a 48-well plate (final volume 400 μl) placed on a rotor agitator (450 rpm) at 37°C in CO_2 environment with WGH medium (Williams' E medium without phenol red, containing 2 mM glutamine and 15 mM Hepes). After a 5-minute preincubation of 200 μl of suspended hepatocytes (1 million hepatocytes/ml) at 37°C, a second preincubation was initiated by the addition of 200 μl of a mix containing various concentrations of azamulin in WGH medium. After 0, 10, 100, and 220 minutes of preincubation, reactions were initiated by the addition of 2 μl of midazolam at 1 mM (final concentration 5 μM) and further incubated for 20 minutes to respect initial rate conditions of 1′-hydroxymidazolam formation. Final solvent concentration in incubates was ≤1% v/v. All reactions were stopped by addition of two volumes of ice-cold acetonitrile (ACN). The tubes were then thoroughly mixed by using a vortex mixer and centrifuged (14,000*g*, at 4°C) for 5 minutes. Clear supernatants were aspirated, diluted two times with water, and directly analyzed by using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Determination of the Inhibition Potential of Azamulin Against CYP1A2, 2B6, 2C8, 2C9, 2C19, and 2D6 in Suspensions of Pooled Human Hepatocytes

The inhibitory effect of azamulin on various P450 enzymes was evaluated at 3 μM with and without a 3-hour preincubation to determine its selectivity toward CYP3A4/5. Phenacetin *O*-deethylation, bupropion 4′-hydroxylation, rosiglitazone 5-hydroxylation, diclofenac 4′-hydroxylation, (*S*)-(+)-mephenytoin 4′-hydroxylation, dextromethorphan *O*-demethylation, and midazolam 1′-hydroxylation were used as probe reactions of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A, respectively. Probe substrates were incubated at a final concentration of 2, 7, and 20 μM for phenacetin, bupropion, and diclofenac, respectively, and 5 μM for other substrates [these concentrations are at or below K_m values previously determined in our laboratory (data not shown)]. Probe substrates were added to the in vitro incubates as solutions in ACN. Azamulin (0 and 3 μM, final concentration in incubates) was solubilized in H_2O/ACN (50:50, v/v). Final solvent concentration in incubates was ≤1%.

Viability of cryopreserved human hepatocytes (pool of 10 mixed-sex donors from Xenotech, batch 1110211) was higher than 71% (trypan blue exclusion). Incubations were performed as described above. Incubation times [60 minutes for (*S*)-(+)-mephenytoin, 20 minutes for midazolam, and 30 minutes for other probe substrates] were selected in order that initial rate conditions for each P450 activity were respected. For rosiglitazone, before analysis, supernatants were first evaporated under N_2 and then further incubated with glucuronidase (0.358 U/ml) and arylsulfatase (0.48 U/ml) in 50 mM Na-Acetate buffer (pH 5) at 37°C for 1 hour to convert glucuronide and sulfate metabolites back into 5-hydroxyrosiglitazone.

Determination of the Inhibition Potential of Azamulin Against CYP3A5 and Non-P450 Enzymes in Suspensions of Pooled Human Hepatocytes

1′-Hydroxymidazolam (substrate of UGTs), 4-hydroxycoumarin and acetaminophen (substrates of UGTs and SULTs), phthalazine (substrate of AO), cimetidine and ranitidine (substrates of FMO), CDP323 (substrate of CES1),

and several specific UGT substrates (imipramine for UGT1A4, 5-OH tryptophol for UGT1A6, *S*-oxazepam for UGT2B15, propofol for UGT1A9, and naloxone for UGT2B7) were incubated with suspensions of human hepatocytes (pool of 10 male and 10 female donors from BioIVT, BSU batch) in presence and in absence of azamulin (3 μM). All substrates were dissolved in DMSO and azamulin in H_2O/ACN (50:50, v/v). Final DMSO and ACN concentration in incubates were ≤0.01% and ≤1%, respectively. Cryopreserved human hepatocytes were thawed according to the provider's information. Viability was higher than 85% (trypan blue exclusion). Incubations (1×10^6 cells/ml) were carried out in singular in a 48-well plate placed on a vibrating agitator (Titramax 100, at 450 rpm) in an incubator (5% CO_2:95% air, humidified atmosphere at 37°C) with WGH medium.

After a 30-minute preincubation of 250 μl of suspended hepatocytes (2 million hepatocytes/ml) at 37°C, reactions were initiated by the addition of an equivalent volume of a mix containing azamulin (or not) and the probe substrate in WGH medium. Final concentrations of azamulin and probe substrate in the incubation medium were 3 and 0.5 μM, respectively, except for cimetidine (2 μM) and acetaminophen (10 μM). At selected time points, samples were taken, and reactions were stopped by using one volume of ice-cold ACN containing ketoconazole (0.5 μM) as internal standard (IS). Samples were subsequently centrifuged at 3700 rpm (4°C) for 15 minutes. Clear supernatants were transferred to 96-well deep-well plates, diluted two times with water, and directly analyzed by LC-MS/MS or UPLC-HRMS.

To evaluate the selectivity of azamulin for CYP3A4, CYP3A5-genotyped human hepatocytes and CYP3cide (a specific CYP3A4 inhibitor) (Walsky et al., 2012) were used.

Single-donor cryopreserved human hepatocytes (from BioreclamationIVT) were genotyped for CYP3A5*3 by using extracted genomic DNA in a real-time PCR assay. Two different types of genotypes were used in this assay: CYP3A5*3/*3 (nonexpressers of CYP3A5) and CYP3A5*1/*1 (expressing two copies of CYP3A5 and known as high expressers of CYP3A5).

Viability of hepatocytes was higher than 74% (trypan blue exclusion). CYP3cide was dissolved in H_2O/ACN (50:50, v/v). Final DMSO and ACN concentrations in incubates were ≤0.01% and ≤1%, respectively. Incubations were performed as described in the paragraph above.

Validation of the Phenotyping Assay with a Set of Known CYP3A4/5 Substrates

Compounds (midazolam, felodipine, simvastatin, quinidine, sildenafil, nifedipine, loperamide, cerivastatin, metoprolol, mirtazapine, zolpidem, and pimozide) were all dissolved in ACN. Final solvent concentration in incubates was ≤1%.

Incubations (substrate concentration at 0.5 μM) were carried out in presence and absence of azamulin (3 μM) as explained above for the substrates of non-P450 enzymes and are detailed in Table 3.

LC-MS/MS Analysis of Metabolites Formed from P450 Probes in the In Vitro Samples

The metabolites of P450 probe substrates (acetaminophen, 4-hydroxybupropion, 4′-hydroxydiclofenac, and 1′-hydroxymidazolam on one hand and 4′-hydroxymephenytoin, 5-hydroxyrosiglitazone, and dextrorphan on the other hand) were quantified by separate LC-MS/MS methods (cocktail 1 and cocktail 2, respectively).

Cocktail 1 LC-MS/MS Analytical Method. The qualification and description of this analytical method is available from Gerin et al. (2013). Briefly, five volumes of sample supernatant were mixed with one volume of IS solution (containing acetaminophen-D_4, hydroxybupropion-D_6, 1′-hydroxymidazolam-D_4, and 4′-hydroxydiclofenac-$^{13}C_6$), evaporated to dryness under N_2 at 40°C, and reconstituted in six volumes of water containing 0.1% trifluoracetic acid adjusted to pH 2.4. The UPLC system used was an Agilent 1290 series instrument (Agilent Technologies, Santa Clara, CA) coupled with a Sciex API 5000 mass spectrometer (Applied Biosystems, Mississauga, Canada). The analytical column was a Zorbax Eclipse XDB-C18 (5 μm, 50.0 × 2.1 mm) operated at 40°C. Eluent A was water containing 0.1% trifluoracetic acid adjusted to pH 2.4, and eluent B was 100% ACN. The flow rate was 0.412 ml/min. A gradient elution was started at 95% A (2.16 minutes) and was then ramped successively to 75% A in 0.24 minutes, 60% A in 1.46 minutes, 50% A in 0.01 minutes, and 20% A in 0.47 minutes and held at 20% A for 0.49 minutes and then at 10% A for 0.72 minutes before returning to the initial condition (95% A for 0.93 minutes). Data acquisition was performed, and the analytical parameters were determined by the application software Analyst version 1.5.2 (Applied Biosystems). The mass-to-charge transitions (m/z) of precursor ions and product ions for each compound were identified as follows: m/z 152 → 110 for acetaminophen, m/z 156 → 114 for acetaminophen-D_4, m/z 256 → 139 for hydroxybupropion, m/z 262 → 139 for hydroxybupropion-D_6, m/z 312 → 230 for 4′-hydroxydiclofenac, m/z 318 → 236 for 4′-hydroxydiclofenac-$^{13}C_6$, m/z 342 → 203 for 1′-hydroxymidazolam, and m/z 346 → 203 for 1′-hydroxymidazolam-D_4. The concentrations were determined by the peak area ratio method by using a calibration curve made up of eight concentrations. In addition, three levels of quality control (QC) samples (low, medium, and high) were included in each analytical run to validate the results.

Cocktail 2 LC-MS/MS Analytical Method. One volume of sample supernatant was mixed with two volumes of IS solution (containing 4′-hydroxymephenytoin-D_3, 5-hydroxyrosiglitazone-D_4, and dextrorphan-D_3) and then evaporated to dryness under a gentle stream of nitrogen at 50°C and reconstituted in one volume of H_2O/ACN/formic acid (90:10:0.1, v/v/v). The UPLC system used was an Agilent 1290 series instrument (Agilent Technologies) coupled with a Sciex API 5000 mass spectrometer (Applied Biosystems). The analytical column was a Zorbax Eclipse plus XDB C18 (1.8 μm, 50 × 2.1 mm) operated at 30°C. Eluent A was water containing 0.1% formic acid, and eluent B was methanol containing 0.1% formic acid. The flow rate was 0.350 ml/min. A gradient elution was started at 95% A (1 minute), ramped to 80% A in 1.5 minutes, held at 80% A for 0.5 minutes, and then ramped to 20% A in 3 minutes and held at 10% A for 1 minute before returning to the initial condition (95% A). Data acquisition was performed, and the analytical parameters were determined by the application software Analyst version 1.5.2 (Applied Biosystems). The mass-to-charge transitions of precursor ions and product ions for each compound were identified as follows: m/z 235 → 150 for 4′-hydroxymephenytoin, m/z 238 → 150 for 4′-hydroxymephenytoin-D_3, m/z 258 → 157 for dextrorphan, and m/z 261 → 157 for dextrorphan-D_3. The concentrations were determined by the peak area ratio method by using a calibration curve made up of eight concentrations. In addition, three levels of QC samples (low, medium, and high) were included in each analytical run to validate the results.

LC-MS/MS Analysis for Monitoring Parent Drug Disappearance

The disappearance (peak area) of CYP3A4 substrates used for the validation of the assay (felodipine, simvastatin, quinidine, sildenafil, nifedipine, cerivastatin, metoprolol, zolpidem, loperamide, mirtazapine, and pimozide), as well as of the various phase II enzymes (1-hydroxymidazolam, 4-hydroxycoumarin, and 5-hydroxytryptophol) and AO (phthalazine), was measured by the following UPLC-MS/MS method. Samples were diluted 4-fold with water before analysis. The UPLC system used was an Agilent 1290 series instrument (Agilent technologies) coupled with a Sciex API 5000 mass spectrometer (Applied Biosystems). The analytical column was an Acquity UPLC HSS T3 (1.8 μm, 2.1 × 30 mm) operated at 40°C. Eluent A was water containing 0.1% formic acid, and eluent B was ACN containing 0.1% formic acid. The flow rate was 1 ml/min. A gradient elution was started at 95% A, ramped to 30% A in 1 minute, and held at 10% A for 0.3 minutes before returning to the initial condition (95% A for 0.44 minutes). Analytical parameters were determined by the software Discovery Quant Analyze version 2.1.1 (Applied Biosystems). Data acquisition was performed by the application software Analyst version 1.5.2 (Applied Biosystems).

UPLC–HRMS Analysis for Monitoring Specific Metabolite Formation

For cimetidine, ranitidine, CDP323, imipramine, propofol, naloxone, and *S*-oxazepam, the metabolite formed by the enzyme of interest was monitored by UPLC-HRMS. Samples were diluted 2-fold with water before analysis. An Acquity UPLC instrument (Waters Corporation, Manchester, UK) coupled to a XevoG2S Qtof high-resolution mass spectrometer (Waters) was used to perform the analysis. The analytical column was an Acquity UPLC HSS C18 (1.7 μm, 2.1 × 100 mm), operated at 40°C. Eluent A was water containing 10 mM ammonium acetate with 0.1% acetic acid, and eluent B was ACN. The flow rate was 0.400 ml/min. A gradient elution was started at 90% A (for 1 minute), ramped to 10% A in 8 minutes, and held at 10% A for 1.5 minutes before returning to the initial condition (90% A for 1.49 minutes). Data acquisition was performed by the application software MassLynx version 4.1, SCN884, and MetaboLynx version 4.1, SCN871 (Waters Corporation, Mildford, MA).

Data Analysis

The P450 probe substrate metabolite formation rates were calculated by using the following equation:

$$v = \frac{[metabolite]}{t*[hepatocytes]}$$

where [*metabolite*] is the concentration of P450 probe substrate metabolite produced during the incubation, *t* is the incubation time, and [*hepatocytes*] is the cell density of hepatocytes in incubates. Percentage of inhibition caused by azamulin was calculated by using zero-azamulin samples as control values.

The in vitro intrinsic clearance determined from parent drug disappearance was calculated by using the half-life of compound disappearance by using the following equation:

$$CL_{int} = \frac{\lambda_z}{[hepatocytes]}$$

where λ_z was the slope obtained from linear regression of the natural logarithm of the compound peak area ratio in relation with time, and [*hepatocytes*] was the cell density of hepatocytes expressed in million hepatocytes per milliliter.

For cimetidine, ranitidine, CDP323, imipramine, propofol, naloxone, and *S*-oxazepam, no reference standards were available to quantify the metabolite formation rate. Therefore, the peak area of the metabolite of interest was used to measure the slope (peak area over time) as a marker of the formation rate. This slope was determined for both conditions (presence and absence of azamulin) and used to determine the effect of azamulin on the metabolic pathway of interest.

Fraction metabolized by CYP3A4/5 ($F_{m,CYP3A4/5}$) was calculated for each compound from the ratio between CL_{int} (based on parent drug disappearance) or metabolite formation rate [*v*] (or slope corresponding to metabolite formation rate) in absence and in presence of azamulin, by using the following equation:

$$Fm_{CYP3A4/5} = 1 - \frac{CL_{intwithazamulin}}{CL_{intwithoutazamulin}} = 1 - \frac{v_{with\ \ azamulin}}{v_{without\ \ azamulin}}$$

IC_{50} values were determined by nonlinear regression analysis by using a four-parameter equation in GraphPad Prism (version 7.02; GraphPad Software Inc., La Jolla, CA) with top and bottom parameters set to 100 and 0, respectively.

Statistical Analysis

The accuracy of the prediction was assessed and compared from root mean-squared error (*RMSE*) and average fold error (*AFE*), calculated with the following equations (Sheiner and Beal, 1981; Obach et al., 1997).

$$RMSE = \sqrt{\frac{1}{N}\sum(predicted - Observed)^2}$$

$$AFE = 10^{\frac{1}{N}\sum\left[log\left(\frac{Predicted}{Observed}\right)\right]}$$

Results

Evaluation of the Time-Dependent Inhibitory Effect of Azamulin on CYP3A4/5 Activity in Suspensions of Pooled Human Hepatocytes. Because azamulin was known to be a time-dependent inhibitor in HLM, we first evaluated the effect of the preincubation time on the IC_{50} of azamulin. As shown in Fig. 1A and Table 1, even without preincubation, azamulin was a potent CYP3A4/5 inhibitor, with an IC_{50} value of 1.0 μM. A preincubation step as short as 10 minutes already increased its potency by 2-fold (IC_{50} of 0.46 μM). Longer preincubation steps did not significantly increase its inhibitory potency. Indeed, a 22-fold increase of the preincubation time (220 minutes) only led to an additional 3-fold increase in potency (0.13 μM) compared with the 10-minute preincubation. During these preliminary investigations, the metabolic stability of azamulin in human hepatocytes (over 4 hours of incubation) was also determined and showed that over 40% of azamulin remained present after 4 hours (Fig. 1B). The major metabolic pathway of azamulin was found to be mediated by direct glucuronidation (data not shown).

Evaluation of the Selectivity of Azamulin Toward CYP3A4/5 in Suspensions of Pooled Human Hepatocytes. The purpose of the present work was to set up an assay in suspensions of pooled human hepatocytes to determine the $F_{m,CYP3A4/5}$ of NCEs by measuring the effect of azamulin on parent drug disappearance, which is usually measured over a 4-hour incubation time. It was therefore deemed critical to ensure CYP3A4/5 selectivity of azamulin over this 4-hour incubation period. To check this, the inhibitory effect of azamulin on major P450 enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, and 2D6) was investigated by using formation of a selective metabolite for each P450. However, to respect initial rate conditions for metabolite formation, the incubations with each probe substrate had to be performed within a maximum time frame of 1 hour. The 4-hour incubation period could not be directly covered. Therefore, to overcome this limitation and allow the evaluation of the CYP3A4/5 selectivity of azamulin over a 4-hour period, a dedicated assay design was applied. The inhibition potential of azamulin toward other P450s was determined by using two different conditions: 1) without preincubation of azamulin (azamulin and selective probe substrate were coincubated for 1 hour at maximum) and 2) with a 3-hour preincubation with azamulin (probe substrate is added at the end of 3-hour preincubation for an incubation of 1 hour at

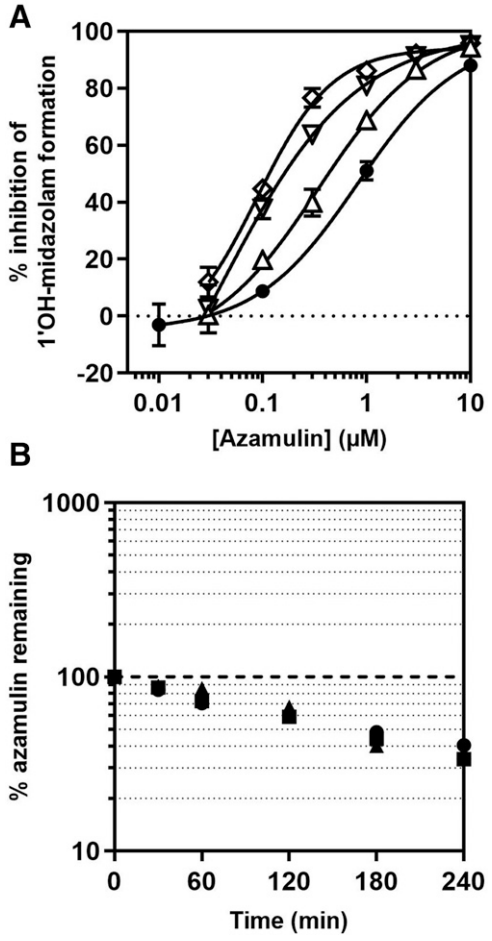

Fig. 1. (A) IC_{50} curves for the effect of azamulin on the formation of 1′-hydroxymidazolam from midazolam (5 μM) in suspended human hepatocytes (0.5×10^6 cells/ml) at different preincubation times: 0 (•), 10 (Δ), 100 (∇), and 220 minutes (◇). Points represent mean values ± S.D. ($n = 3$). IC_{50} values are shown in Table 1. (B) Stability of azamulin (3 μM) in suspended human hepatocytes (1×10^6 cells/ml). Points represent singular values of three independent experiments.

TABLE 1

Time dependence of the inhibitory effect of azamulin on CYP3A4/5

Preincubation Time (min)	IC_{50} ± S.E. (μM)
0	1.0 ± 0.1
10	0.46 ± 0.02
100	0.20 ± 0.02
220	0.13 ± 0.01

maximum in presence of remaining azamulin). As shown in Fig. 2A, azamulin (3 μM) caused less than 20% of inhibition of all P450 enzymes tested, with and without 3 hours of preincubation (for most of the P450s even less than 10%). These data clearly demonstrate that azamulin is very specific to CYP3A over a 4-hour incubation period. In contrast, ketoconazole (3 μM–Fig. 2B) showed a lack of CYP3A4 selectivity, as it significantly inhibited (>20% inhibition) CYP2C9, CYP2C19, and CYP2B6.

The main advantage of using a hepatocyte assay is the presence of a full array of drug-metabolizing enzymes (DMEs). It was therefore deemed critical to assess as much as possible the potential inhibition of azamulin toward non-P450 enzymes. To this aim, the inhibitory effect of azamulin on the metabolism of 1′hydroxymidazolam (UGT1A4, 2B4, 2B7) (Seo et al., 2010), hydroxycoumarin (UGTs and SULTs substrate)(Wang et al., 2006), phthalazine (aldehyde oxidase substrate) (Barr and Jones, 2011), cimetidine and ranitidine (FMO substrate) (Cashman et al., 1993), CDP323 (CES1 substrate) (Chanteux et al., 2014), and several specific UGT substrates (imipramine for UGT1A4, 5-hydroxytryptophol for UGT1A6, *S*-oxazepam for UGT2B15, propofol for UGT1A9, and naloxone for UGT2B7) (Court et al., 2002; Krishnaswamy et al., 2004; Mukai et al., 2014) was also evaluated. The effect of azamulin was measured either by using parent drug disappearance or specific metabolite formation for compounds known to have other major metabolic pathways not representative of the target enzyme. The percentages of inhibition caused by azamulin are presented in Table 2. Azamulin caused a very modest inhibition (20%–30%) of some UGTs (1′-hydroxymidazolam, naloxone, and oxazepam) and FMO (ranitidine) substrates, but it had no significant effect (<15% inhibition) on the other metabolic pathways evaluated (glucuronidation and sulfation of hydroxycoumarin, glucuronidation of 5-hydroxytryptophol and propofol, phthalazine oxidation by aldehyde oxidase, cimetidine oxidation by FMO). Additional data with recombinant UGT enzymes expressed in the liver (UGT1A1, 1A3, 1A4, 1A9, 2B4, 2B7, 2B10, 2B15, and 2B17) were generated to complement the findings observed with 1′-hydroxymidazolam and *S*-oxazepam in suspended human hepatocytes. Although UGT2B15 was confirmed to be the primary UGT isoform involved in *S*-oxazepam glucuronidation, UGT1A9, UGT2B4, and UGT2B7 were also found to contribute to a lesser extent to the formation of *S*-oxazepam glucuronide. Regarding glucuronidation of 1′-hydroxymidazolam, only UGT1A4, 2B4, and 2B7 were identified as UGT isoforms involved in this reaction. In this experimental setting (recombinant UGT incubated in presence or absence of azamulin at 3 μM), azamulin also demonstrated weak inhibition potential of UGT1A9 (24% with *S*-oxazepam), UGT2B4 (23% with *S*-oxazepam and 30% with 1′-hydroxymidazolam), UGT2B7 (23% with 1′-hydroxymidazolam), and UGT2B15 (35% with *S*-oxazepam). No significant inhibition (<10%) was observed in UGT1A4 (1′-hydroxymidazolam) and UGT2B7 (*S*-oxazepam) (data not shown).

Evaluation of the Inhibition of Midazolam 1′-Hydroxylation in CYP3A5-Genotyped Human Hepatocytes: Comparison with CYP3cide. In the work of Stresser et al., azamulin demonstrated a 10-fold more potent inhibition against CYP3A4 versus CYP3A5 by using recombinant enzymes and 7-benzyloxy-4-trifluoromethylcoumarin as probe substrate for both CYP enzymes. More recently, Parmentier et al. (2017) demonstrated that azamulin specifically inhibited CYP3A4 and not CYP3A5 in HLM. In the present study, we have also looked at the CYP3A selectivity of azamulin. To this aim, we used an indirect approach to determine whether azamulin, in the conditions of our assay (incubation at 3 μM and midazolam as CYP3A4/5 probe substrate), would still demonstrate an apparent selectivity for CYP3A4. To achieve this, the effect of azamulin on the 1′-hydroxylation of midazolam was investigated in CYP3A5-genotyped human hepatocytes. Incubations were performed in parallel with CYP3cide, which is a well known selective CYP3A4 inhibitor (Walsky et al., 2012). The inhibition obtained in presence of azamulin and CYP3cide is shown in Fig. 3. In both batches of hepatocytes with the CYP3A5*3/*3 genotype (non-expressers of CYP3A5, Fig. 3A), a high inhibition (~90%) was observed, as expected, with both CYP3cide and azamulin. On the other hand, in batches of human hepatocytes with the CYP3A5*1/*1 genotype (high CYP3A5 expressers, Fig. 3B), the 1′-hydroxylation of midazolam

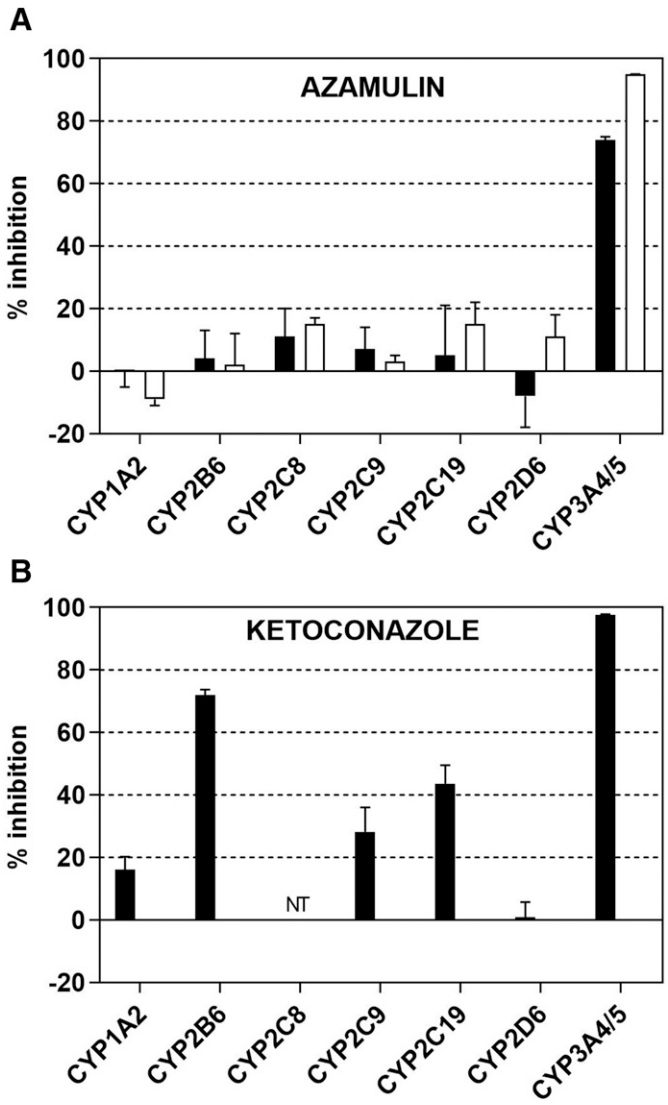

Fig. 2. Inhibitory effect of azamulin (3 μM) (A) and ketoconazole (3 μM) (B) on major P450 enzymes (CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4) in suspended human hepatocytes (1×10^6 cells/ml) after 0 minutes (closed bars) or 3 hours (open bars, azamulin only) of preincubation in the presence of inhibitor. Bars represent mean values ± S.D. ($n = 3$).

TABLE 2

Evaluation of the selectivity of azamulin toward CYP3A4 in suspended human hepatocytes: determination of its inhibitory effect on phase II enzymes, aldehyde oxidase, FMO, and CES1

Substrate	Enzyme	Reaction Monitored	Percentage of Inhibition[a]
1′-Hydroxymidazolam	UGT1A4, 2B4, 2B7	Parent disappearance	26 (14)
4′-Hydroxycoumarin	UGTs, SULTs	Parent disappearance	4 (8)
Phthalazine	Aldehyde oxidase	Parent disappearance	−0.6 (6)
Cimetidine	FMO	Metabolite formation	13 (13)
Ranitidine	FMO	Metabolite formation	24 (4)
CDP323	CES1	Metabolite formation	−7 (23)
Imipramine	UGT1A4	Metabolite formation	−6 (13)
5-Hydroxytryptophol	UGT1A6	Parent disappearance	−2 (13)
Propofol	UGT1A9	Metabolite formation	−3 (0.1)
Naloxone	UGT2B7	Metabolite formation	29 (5.4)
S-Oxazepam	UGT2B15	Metabolite formation	31 (8.4)

[a]Mean of three independent values (S.E.M.).

was still significantly inhibited (~85%) by azamulin, whereas the inhibition observed in presence of CYP3cide was significantly lower (29% and 61%). Overall, these data demonstrated that azamulin (3 μM) is not as selective as CYP3cide for CYP3A4, as it also inhibits the CYP3A5 isoform.

Validation of the Phenotyping Assay with a Set of Well Known CYP3A4/5 Substrates. To validate the use of this assay as a new tool to determine $F_{m,CYP3A4/5}$ of NCEs, the inhibitory effect of azamulin (3 μM) on intrinsic clearance of a set of 11 compounds, which are reported to be highly, moderately, poorly, or not metabolized by CYP3A4/5 (midazolam, felodipine, simvastatin > quinidine, sildenafil, nifedipine, loperamide > zolpidem, pimozide, cerivastatin, mirtazapine > metoprolol), was evaluated in suspended human hepatocytes according to the experimental conditions described in Table 3. The CL_{int} values obtained for each compound in absence and in presence of azamulin are presented in Table 4 together with the predicted and observed $F_{m,CYP3A4/5}$. In addition, the graphs showing the disappearance of each compound in presence and in absence of azamulin are also presented in Fig. 4. As illustrated in Fig. 5, the correlation between the predicted and observed $F_{m,CYP3A4/5}$ is significant ($r^2 = 0.77$). In addition, the average fold error (0.97) is close to 1, and the root mean-squared error is only 0.12, supporting the idea that the experimental conditions used in the present study are adequate for determining the in vivo CYP3A contribution in drug's metabolic clearance. Prediction of $F_{m,CYP}$ is quite challenging, especially when the $F_{m,CYP}$ is in the range of 0.3–0.7, in which a huge interindividual variability is observed because of the presence of multiple pathways, all of which may vary among individuals (Desbans et al., 2014). Therefore, it is not surprising to see that the four compounds (loperamide, nifedipine, pimozide, and zolpidem), being slightly outside the 10% boundaries, have an observed $F_{m,CYP3A4/5}$ in this range (0.3–0.7), in which high interindividual variability occurred. It should not be forgotten that in vitro determination of $F_{m,CYP3A4/5}$ is highly dependent on the intrinsic CYP3A4/5 activity in the batch of hepatocytes used. Therefore, the selection of the human hepatocytes batch is primordial to ensure an appropriate prediction of the in vivo $F_{m,CYP3A4/5}$.

Discussion

Nowadays, it is well recognized that an early evaluation of the contribution of individual P450 isoforms to the total metabolic clearance of an NCE is very important to predict the risk of victim DDI, which could occur by coadministration with other drugs affecting P450 activity. This can lead to a loss or increase of the pharmacodynamic effects and/or serious side effects. In addition to DDI risk, knowledge of DME in clearance of NCE is primordial to inform on risks associated with modulation of DME in certain disease states (Harvey and Morgan, 2014), in specific populations (pediatrics), or because of inherent variability associated with abundance or the polymorphic nature of

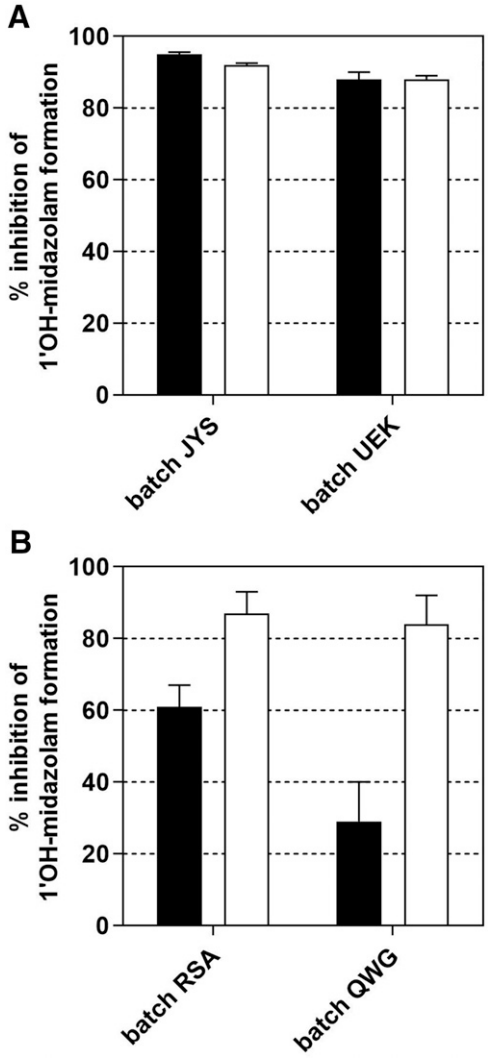

Fig. 3. Comparison of midazolam 1′-hydroxylation inhibition by azamulin (open bars) and CYP3cide (closed bars) in CYP3A5-genotyped hepatocytes. Two different genotypes were tested: CYP3A5*3/*3 [(A) batch JYS and UEK, nonexpressers of CYP3A5] and CYP3A5*1/*1 [(B) batch RSA and QWG, high expressers of CYP3A5]. Bars represent mean values ± S.D. ($n = 3$).

TABLE 3

Incubation conditions for routine assessment of $F_{mCYP3A4/5}$ of NCE

Culture Plate	48-Well
Culture medium	Williams' E medium without phenol red, containing 2 mM glutamine and 15 mM Hepes
Incubation	37°C in 5% CO_2/95% air, humidified atmosphere under agitation (450 rpm)
Hepatocytes suspension prewarming	30 min at 2 million/ml in similar conditions as incubation
Final incubation volume	500 μl
Final hepatocyte concentration	1 million/ml
Substrate concentration	0.5 μM
Azamulin concentration	3 μM
Final organic solvent concentration	1% ACN
Incubation time	4 h
Sampling time	0, 30, 60, 120, 180, and 240 min
Sampling volume	50 μl/time point

specific DME (e.g., CYP2D6, UGT1A1). DDIs not only affect patient safety but also add to the cost of drug development because of the high costs of failure in clinical development (Xu et al., 2009). Consequently, identifying the risks associated with DDI plays an increasingly important role in the pharmaceutical industry, contributing to the selection of suitable candidates for (pre)clinical development and to support clinical pharmacologists to establish a suitable clinical DDI trial design (Desbans et al., 2014).

Therefore, it is critical to understand early in drug development the contribution of individual P450 enzymes to metabolism of NCE ($F_{CL,metabolism} \times F_{m,CYP}$) to anticipate and mitigate the risk of DDI as victim, since DDI clinical trials are usually conducted in later stages of clinical development (post proof of concept). Although the RAF/ISEF approach is widely used to determine the fractional CL_{int} (Bohnert et al., 2016), this method suffers from one major limitation. Indeed, it is assumed that drug candidates interact with the P450 binding site in the same manner as the probe substrate used for RAF/ISEF determination and, therefore, that RAF/ISEF are similar between substrates. This main assumption may not be valid, knowing the presence of multiple binding sites with different substrate selectivities described for several P450 isoforms (Venkatakrishnan et al., 2000; Crewe et al., 2011; Siu and Lai, 2017). Therefore, the use of a specific inhibitor is still an interesting alternative. However, for this approach to be successful, the inhibitor should be potent, selective, and metabolically stable to be able to attain >90% inhibition of the targeted enzyme over the incubation period. Based on the limited literature data available so far (Stresser et al., 2004; Parmentier et al., 2017), the potent CYP3A inhibitor azamulin has potentially better properties to meet these criteria compared with the gold-standard CYP3A inhibitor ketoconazole, which mainly suffers from a lack of selectivity (Liu et al., 2007), high nonspecific binding (Tran et al., 2002; Quinney et al., 2013), and metabolic instability (Stresser et al., 2004). Therefore, in the present study we aimed to fully characterize azamulin as a potential tool for the estimation of CYP3A contribution to drug metabolism. Human hepatocytes were selected as a test system to directly quantify $F_{CL,metabolism} \times F_{m,CYP3A}$, since they contain the whole enzymatic inventory involved in drug metabolism. The first objective of the study was to confirm the inhibitory properties of azamulin in human hepatocytes. As demonstrated previously in human liver microsomes (Stresser et al., 2004; Parmentier et al., 2017), azamulin also showed in human hepatocytes a time-dependent inhibition profile, as its IC_{50} decreased with an increase of the preincubation time. An optimal concentration (3 μM) was selected to achieve >90% CYP3A inhibition for incubations lasting up to 4 hours.

Secondly, azamulin was tested for its CYP3A4/5 selectivity toward other P450 enzymes and a wide panel of DMEs. The present data clearly demonstrated the superiority of azamulin compared with ketoconazole regarding P450 selectivity. Indeed, less than 20% inhibition of CYP1A2, 2B6, 2C8, 2C9, 2C19, and 2D6 was observed in presence of 3 μM azamulin, whereas ketoconazole significantly inhibited CYP2B6 and CYP2C19 at concentrations required to achieve >90% CYP3A4/5 inhibition. These findings obtained in human hepatocytes are similar to those described by Stresser et al. (2004), advocating for the higher selectivity of azamulin over ketoconazole in human liver microsomes and recombinant enzymes.

These encouraging data triggered additional investigations on the potential impact of azamulin on other drug-metabolizing enzymes (FMO, carboxylesterase, UGTs, SULTs, AO). This was deemed very important to assess, since human hepatocytes were used as a test system. Again, azamulin demonstrated no or minimal inhibition (<15%) of these enzymes, with the exception of some UGTs, including UGT2B7, UGT2B4, and UGT2B15, which demonstrated modest inhibition (~20%–30%). This is most likely due to competitive inhibition, as glucuronidation was found to be the main metabolic pathway of azamulin. It should be noted that some key hepatic UGTs, such as UGT1A1 and UGT1A3, were not evaluated in the current work. The present data showed a trend of weak inhibition on some UGTs, which needs to be considered in the interpretation of the data when

TABLE 4

Determination of CYP3A4/5 contribution to the metabolic clearance obtained in human hepatocytes for 12 marketed drugs

Data are means ± S.D. of three independent experiments.

Compounds	CL_{int} (μl/min per 10^6 hepatocytes)		Predicted In Vitro $F_{m,CYP3A4/5}$	Observed In Vivo $F_{m,CYP3A4/5}$	References
	Control	With Azamulin			
Midazolam	56 ± 4.6	3.4 ± 0.6	0.94 ± 0.01	0.84–0.99	Galetin et al., 2006; Shou et al., 2008; Xu et al., 2011
Felodipine	69 ± 5.4	9.9 ± 1.0	0.86 ± 0.01	0.81–0.99	Galetin et al., 2006
Simvastatin	153 ± 16	27 ± 1.0	0.82 ± 0.01	0.83–0.99	Shou et al., 2008; Xu et al., 2011
Quinidine	2.9 ± 0.5	0.81 ± 0.3	0.73 ± 0.04	0.61–0.76	Galetin et al., 2006; Shou et al., 2008
Sildenafil	29 ± 2.4	8.3 ± 1.3	0.71 ± 0.02	0.66–0.82	Shou et al., 2008; Xu et al., 2009
Nifedipine	56 ± 5.5	10 ± 1.7	0.82 ± 0.02	0.63–0.71	Galetin et al., 2006; Shou et al., 2008; Xu et al., 2011
Loperamide	15 ± 0.4	7.6 ± 0.7	0.48 ± 0.06	0.62	Tayrouz et al., 2001
Zolpidem	2.7 ± 0.5	0.97 ± 0.3	0.63 ± 0.11	0.41	Xu et al., 2011
Pimozide	7.6 ± 0.7	2.7 ± 0.5	0.65 ± 0.07	0.40	Galetin et al., 2006
Cerivastatin	7.2 ± 0.7	5.8 ± 3.4	0.46 ± 0.03	0.37	Galetin et al., 2006
Mirtazapine	5.6 ± 0.2	4.2 ± 0.2	0.26 ± 0.03	0.33	Notice FDA
Metoprolol	2.1 ± 0.6	1.8 ± 0.4	0.10 ± 0.13	0.15	Xu et al., 2009

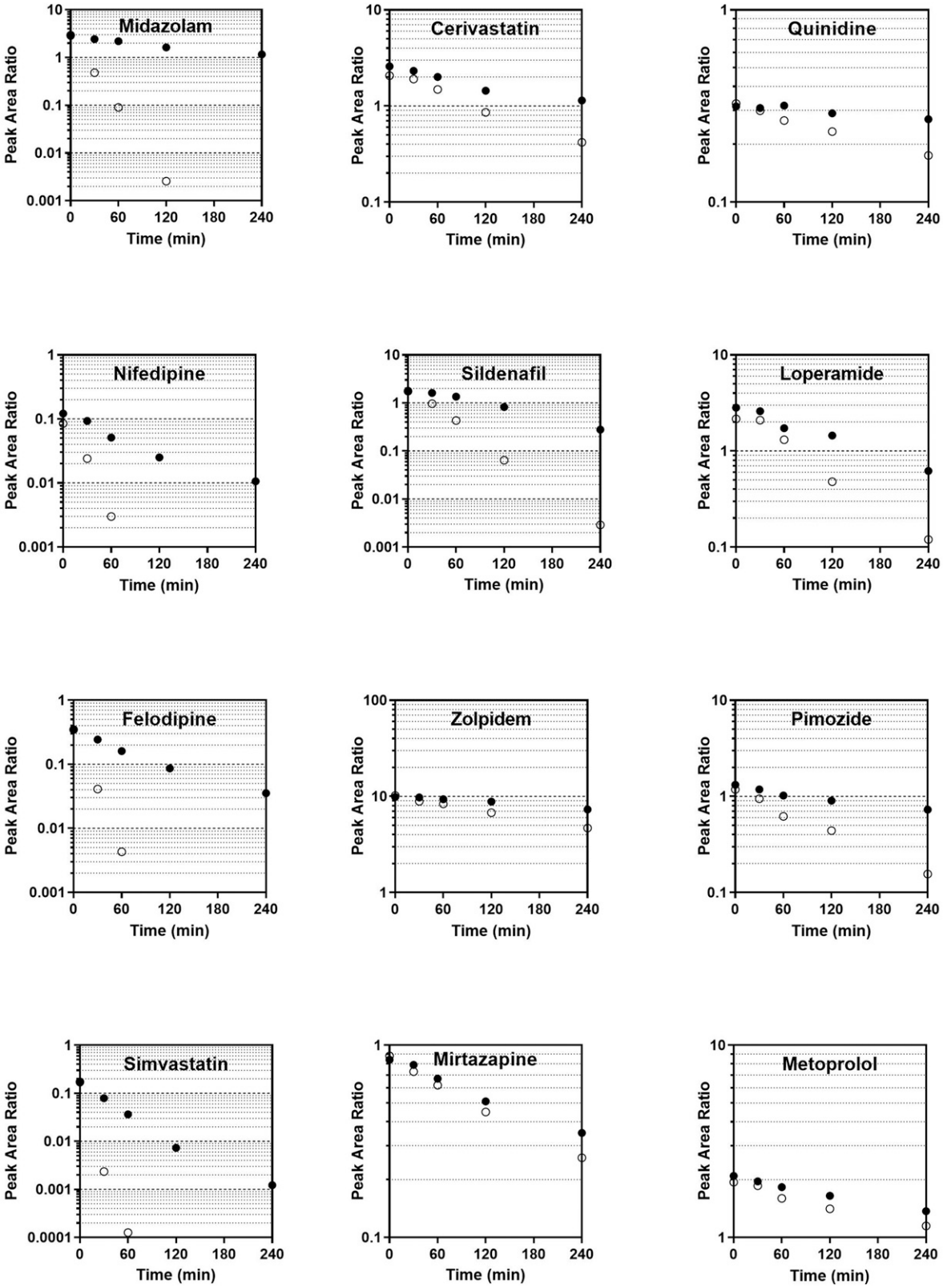

Fig. 4. Metabolic disappearance of CYP3A4/5 substrates with various $F_{m,CYP3A4/5}$ in suspended human hepatocytes (1×10^6 cells/ml) in absence (○) or presence (●) of azamulin (3 μM). Results of one single experiment are shown and are representative of three independent experiments.

direct glucuronidation of parent drug is expected or known. However, the impact on the CYP3A determination is unlikely to be significant, owing to the minor inhibition observed. Overall, these data support the use of azamulin as a selective CYP3A inhibitor in human hepatocytes without impacting significantly other major DMEs.

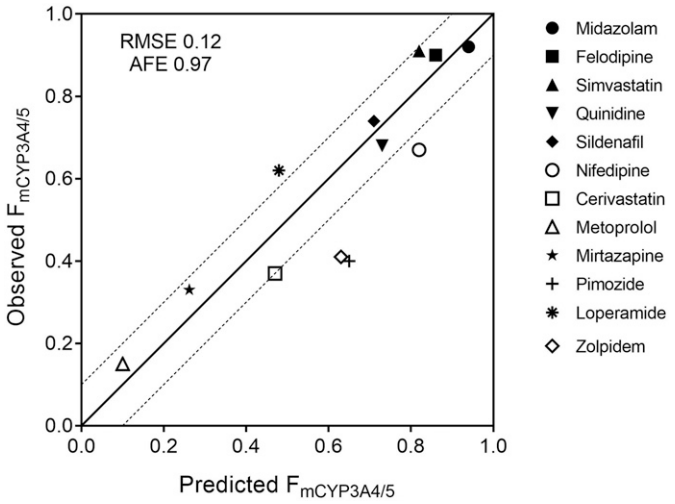

Fig. 5. Correlation between observed in vivo and predicted $F_{m,CYP3A4/5}$. All $F_{m,CYP3A4/5}$ values are shown in Table 4. The solid line represents the line of unity, and the dotted line represents the ±10% error interval. Points represent $F_{m,CYP3A4/5}$ calculated from three independent experiments.

Finally, we have assessed the selectivity of azamulin for CYP3A4 over CYP3A5. Indeed, understanding the relative contribution of CYP3A4 and CYP3A5 to drug metabolism is important primarily because expression of the CYP3A5 enzyme is subject to a genetic polymorphism that most humans do not express (Lamba et al., 2002), and some CYP3A substrates showed different disposition profiles in extensive and poor CYP3A5 metabolizers (Haufroid et al., 2006; Jin et al., 2007). In the absence of specific CYP3A4 and CYP3A5 probe substrates, we used genotyped human hepatocytes (non-CYP3A5 expressers and high CYP3A5 expressers) together with CYP3cide, a well known specific CYP3A4 inhibitor (Tseng et al., 2014), to characterize the CYP3A4/CYP3A5 selectivity of azamulin in our assay conditions. Our data suggest that azamulin, under our conditions (3 μM in human hepatocytes), is a CYP3A4/5 inhibitor and cannot be used to determine the relative CYP3A4/5 contribution. Although CYP3cide is a useful inhibitor for that purpose, azamulin is more appropriate for DDI prediction with CYP3A inhibitors, as most CYP3A4 inhibitors also inhibit CYP3A5 enzymes.

Our observations on the lack of CYP3A4/5 selectivity of azamulin are in contradiction to the conclusions drawn recently by Parmentier et al (2017). Although test systems (recombinant enzymes and HLM) were different, azamulin concentration (5 μM) was in the same range (Parmentier et al., 2017). In their experiment with CYP3A4-silensomes (HLM prepared with pretreatment with azamulin), they showed that an additional reduction of midazolam and nifedipine clearance (15%–20%) was observed in the CYP3A4-silensomes in presence of ketoconazole (0.3 μM, pan-CYP3A inhibitor). Authors have ascribed the remaining CYP3A activities to CYP3A5 only. Although this interpretation may be a plausible explanation of the data, it should have been supported by stronger evidence. Indeed, it cannot be excluded that the additional effect of ketoconazole is due to remaining CYP3A4 activities, since full CYP3A4 inhibition in CYP3A4-silensomes has not been demonstrated. In addition, we cannot rule out that a significant proportion of CYP3A5 has already been inhibited during preparation of CYP3A4-silensomes, since no information on the CYP3A5 genotype and/or activity present in their HLM batch has been provided. Finally, the evidence of an additional effect of ketoconazole was based on suboptimal experimental conditions. Indeed, the effect of ketoconazole was determined in conditions in which metabolic clearance of midazolam and nifedipine was already very low (more than 95% parent drug remaining at the end of the assay in the control conditions). Under such conditions (very low turnover of parent drug), any intrinsic clearance determination based on parent drug consumption is expected to be inaccurate and should not be used to determine a potential additional effect of ketoconazole.

Stresser et al. (2004) suggested that azamulin was 10-fold more selective for CYP3A4 over CYP3A5 by using recombinant CYP3A4 and CYP3A5 enzymes. However, this has been assessed in a competitive inhibition model with 7-benzyloxy-4-trifluoromethylcoumarin as probe substrate. Results obtained with recombinant P450 always need to be handled with caution, as they are difficult to extrapolate. Differences in the expression levels of NADPH-P450 reductase and cytochrome b5 (Emoto and Iwasaki, 2007) or differences in activity due to different lipidic environment, pH, or oxidative stress may have an impact on the translation of data from recombinant P450 to liver microsomes or hepatocytes (Marheineke et al., 1998; Brignac-Huber et al., 2016). Also, a 10-fold difference is clearly not enough to ensure full inhibition of CYP3A4 (>90%) on one hand and no or very little inhibition of CYP3A5 on the other hand.

In summary, azamulin (3 μM) is a highly potent and selective inhibitor of CYP3A in human hepatocytes. The assay presented in the current study can be used to accurately assess the in vivo contribution of CYP3A in the metabolic clearance of any NCE and inform on the risk of DDI with CYP3A inhibitors. However, it should be acknowledged that one clear limitation of the current assay is the determination of the $F_{m,CYP3A4/5}$ when NCEs have very low turnover in human hepatocytes. Further work is required to validate the use of relative metabolite formation (in absence of authentic reference standards) to determine the CYP3A4/5 contribution to NCE metabolic clearance.

Acknowledgments

We would like to thank Julie Maron and Oriana Laforgia for dedicated technical support for in vitro assays.

Authorship Contributions

Participated in research design: Chanteux, Rosa, Nicolaï, Ungell.
Conducted experiments: Rosa, Delatour, Nicolaï, Gillent, Dell'Aiera.
Contributed new reagents or analytic tools: Delatour, Gillent, Dell'Aiera.
Performed data analysis: Chanteux, Rosa, Delatour, Nicolaï, Ungell.
Wrote or contributed to the writing of the manuscript: Chanteux, Rosa.

References

Barr JT and Jones JP (2011) Inhibition of human liver aldehyde oxidase: implications for potential drug-drug interactions. *Drug Metab Dispos* **39**:2381–2386.
Bohnert T, Patel A, Templeton I, Chen Y, Lu C, Lai G, Leung L, Tse S, Einolf HJ, Wang YH, et al.; International Consortium for Innovation and Quality in Pharmaceutical Development (IQ) Victim Drug-Drug Interactions Working Group (2016) Evaluation of a new molecular entity as a victim of metabolic drug-drug interactions-an industry perspective. *Drug Metab Dispos* **44**: 1399–1423.
Brignac-Huber LM, Park JW, Reed JR, and Backes WL (2016) Cytochrome P450 organization and function are modulated by endoplasmic reticulum phospholipid heterogeneity. *Drug Metab Dispos* **44**:1859–1866.
Cashman JR, Park SB, Yang ZC, Washington CB, Gomez DY, Giacomini KM, and Brett CM (1993) Chemical, enzymatic, and human enantioselective S-oxygenation of cimetidine. *Drug Metab Dispos* **21**:587–597.
Chanteux H, Rosa M, Delatour C, Prakash C, Smith S, and Nicolas JM (2014) In vitro hydrolysis and transesterification of CDP323, an α4β1/α4β7 integrin antagonist ester prodrug. *Drug Metab Dispos* **42**:153–161.
Court MH, Duan SX, Guillemette C, Journault K, Krishnaswamy S, Von Moltke LL, and Greenblatt DJ (2002) Stereoselective conjugation of oxazepam by human UDP-glucuronosyltransferases (UGTs): S-oxazepam is glucuronidated by UGT2B15, while R-oxazepam is glucuronidated by UGT2B7 and UGT1A9. *Drug Metab Dispos* **30**:1257–1265.
Crewe HK, Barter ZE, Yeo KR, and Rostami-Hodjegan A (2011) Are there differences in the catalytic activity per unit enzyme of recombinantly expressed and human liver microsomal cytochrome P450 2C9? A systematic investigation into inter-system extrapolation factors. *Biopharm Drug Dispos* **32**:303–318.
Desbans C, Hilgendorf C, Lutz M, Bachellier P, Zacharias T, Weber JC, Dolgos H, Richert L, and Ungell AL (2014) Prediction of fraction metabolized via CYP3A in humans utilizing cryopreserved human hepatocytes from a set of 12 single donors. *Xenobiotica* **44**:17–27.
Emoto C and Iwasaki K (2007) Approach to predict the contribution of cytochrome P450 enzymes to drug metabolism in the early drug-discovery stage: the effect of the expression of cytochrome b(5) with recombinant P450 enzymes. *Xenobiotica* **37**:986–999.

Emoto C, Murase S, and Iwasaki K (2006) Approach to the prediction of the contribution of major cytochrome P450 enzymes to drug metabolism in the early drug-discovery stage. *Xenobiotica* **36**:671–683.

Galetin A, Burt H, Gibbons L, and Houston JB (2006) Prediction of time-dependent CYP3A4 drug-drug interactions: impact of enzyme degradation, parallel elimination pathways, and intestinal inhibition. *Drug Metab Dispos* **34**:166–175.

Gerin B, Dell'Aiera S, Richert L, Smith S, and Chanteux H (2013) Assessment of cytochrome P450 (1A2, 2B6, 2C9 and 3A4) induction in cryopreserved human hepatocytes cultured in 48-well plates using the cocktail strategy. *Xenobiotica* **43**:320–335.

Harvey RD and Morgan ET (2014) Cancer, inflammation, and therapy: effects on cytochrome p450-mediated drug metabolism and implications for novel immunotherapeutic agents. *Clin Pharmacol Ther* **96**:449–457.

Haufroid V, Wallemacq P, VanKerckhove V, Elens L, De Meyer M, Eddour DC, Malaise J, Lison D, and Mourad M (2006) CYP3A5 and ABCB1 polymorphisms and tacrolimus pharmacokinetics in renal transplant candidates: guidelines from an experimental study. *Am J Transplant* **6**: 2706–2713.

Jin Y, Wang YH, Miao J, Li L, Kovacs RJ, Marunde R, Hamman MA, Philips S, Hilligoss J, and Hall SD (2007) Cytochrome P450 3A5 genotype is associated with verapamil response in healthy subjects [published correction appears in *Clin Pharmacol Ther* (2010) 88:138]. *Clin Pharmacol Ther* **82**:579–585.

Khojasteh SC, Prabhu S, Kenny JR, Halladay JS, and Lu AY (2011) Chemical inhibitors of cytochrome P450 isoforms in human liver microsomes: a re-evaluation of P450 isoform selectivity. *Eur J Drug Metab Pharmacokinet* **36**:1–16.

Krishnaswamy S, Hao Q, Von Moltke LL, Greenblatt DJ, and Court MH (2004) Evaluation of 5-hydroxytryptophol and other endogenous serotonin (5-hydroxytryptamine) analogs as substrates for UDP-glucuronosyltransferase 1A6. *Drug Metab Dispos* **32**:862–869.

Lamba JK, Lin YS, Schuetz EG, and Thummel KE (2002) Genetic contribution to variable human CYP3A-mediated metabolism. *Adv Drug Deliv Rev* **54**:1271–1294.

Liu YT, Hao HP, Liu CX, Wang GJ, and Xie HG (2007) Drugs as CYP3A probes, inducers, and inhibitors. *Drug Metab Rev* **39**:699–721.

Marheineke K, Grünewald S, Christie W, and Reiländer H (1998) Lipid composition of Spodoptera frugiperda (Sf9) and Trichoplusia ni (Tn) insect cells used for baculovirus infection. *FEBS Lett* **441**:49–52.

Maurice M, Pichard L, Daujat M, Fabre I, Joyeux H, Domergue J, and Maurel P (1992) Effects of imidazole derivatives on cytochromes P450 from human hepatocytes in primary culture. *FASEB J* **6**:752–758.

Moody GC, Griffin SJ, Mather AN, McGinnity DF, and Riley RJ (1999) Fully automated analysis of activities catalysed by the major human liver cytochrome P450 (CYP) enzymes: assessment of human CYP inhibition potential. *Xenobiotica* **29**:53–75.

Mukai M, Tanaka S, Yamamoto K, Murata M, Okada K, Isobe T, Shigeyama M, Hichiya H, and Hanioka N (2014) In vitro glucuronidation of propofol in microsomal fractions from human liver, intestine and kidney: tissue distribution and physiological role of UGT1A9. *Pharmazie* **69**: 829–832.

Newton DJ, Wang RW, and Lu AY (1995) Cytochrome P450 inhibitors. Evaluation of specificities in the in vitrometabolism of therapeutic agents by human liver microsomes. *Drug Metab Dispos* **23**:154–158.

Obach RS, Baxter JG, Liston TE, Silber BM, Jones BC, MacIntyre F, Rance DJ, and Wastall P (1997) The prediction of human pharmacokinetic parameters from preclinical and in vitro metabolism data. *J Pharmacol Exp Ther* **283**:46–58.

Ogilvie B, Usuki E, Yerino P, and Parkinson A (2008) In vitro approaches for studying the inhibition of drug-metabolizing enzymes and identifying the drug-metabolizing enzymes responsible for the metabolism of drugs (reaction phenotyping) with emphasis on cytochrome P450, in *Drug-Drug Interactions* (Rodrigues D 213–358, Informa Healthcare, London.

Parmentier Y, Pothier C, Delmas A, Caradec F, Trancart MM, Guillet F, Bouaita B, Chesne C, Brian Houston J, and Walther B (2017) Direct and quantitative evaluation of the human CYP3A4 contribution (f_m) to drug clearance using the in vitro SILENSOMES model. *Xenobiotica* **47**:562–575.

Parmentier Y, Pothier C, Hewitt N, Vincent L, Caradec F, Liu J, Lin F, Trancart MM, Guillet F, Bouaita B, et al. (2019) Direct and quantitative evaluation of the major human CYP contribution (fmCYP) to drug clearance using the in vitro Silensomes™ model. *Xenobiotica* **49**:22–35.

Quinney SK, Knopp S, Chang C, Hall SD, and Li L (2013) Integration of in vitro binding mechanism into the semiphysiologically based pharmacokinetic interaction model between ketoconazole and midazolam. *CPT Pharmacometrics Syst Pharmacol* **2**:e75.

Seo KA, Bae SK, Choi YK, Choi CS, Liu KH, and Shin JG (2010) Metabolism of 1′- and 4-hydroxymidazolam by glucuronide conjugation is largely mediated by UDP-glucuronosyltransferases 1A4, 2B4, and 2B7. *Drug Metab Dispos* **38**:2007–2013.

Sevrioukova IF (2019) Structural insights into the interaction of cytochrome P450 3A4 with suicide substrates: mibefradil, azamulin and 6′,7′-dihydroxybergamottin. *Int J Mol Sci* **20**:4245.

Sheiner LB and Beal SL (1981) Some suggestions for measuring predictive performance. *J Pharmacokinet Biopharm* **9**:503–512.

Shou M, Hayashi M, Pan Y, Xu Y, Morrissey K, Xu L, and Skiles GL (2008) Modeling, prediction, and in vitro in vivo correlation of CYP3A4 induction. *Drug Metab Dispos* **36**: 2355–2370.

Siu YA and Lai WG (2017) Impact of probe substrate selection on cytochrome P450 reaction phenotyping using the relative activity factor. *Drug Metab Dispos* **45**:183–189.

Soars MG, McGinnity DF, Grime K, and Riley RJ (2007) The pivotal role of hepatocytes in drug discovery. *Chem Biol Interact* **168**:2–15.

Stresser DM, Broudy MI, Ho T, Cargill CE, Blanchard AP, Sharma R, Dandeneau AA, Goodwin JJ, Turner SD, Erve JC, et al. (2004) Highly selective inhibition of human CYP3Aa in vitro by azamulin and evidence that inhibition is irreversible. *Drug Metab Dispos* **32**:105–112.

Tayrouz Y, Ganssmann B, Ding R, Klingmann A, Aderjan R, Burhenne J, Haefeli WE, and Mikus G (2001) Ritonavir increases loperamide plasma concentrations without evidence for P-glycoprotein involvement. *Clin Pharmacol Ther* **70**:405–414.

Tran TH, Von Moltke LL, Venkatakrishnan K, Granda BW, Gibbs MA, Obach RS, Harmatz JS, and Greenblatt DJ (2002) Microsomal protein concentration modifies the apparent inhibitory potency of CYP3A inhibitors. *Drug Metab Dispos* **30**:1441–1445.

Tseng E, Walsky RL, Luzietti RA Jr., Harris JJ, Kosa RE, Goosen TC, Zientek MA, and Obach RS (2014) Relative contributions of cytochrome CYP3A4 versus CYP3A5 for CYP3A-cleared drugs assessed in vitro using a CYP3A4-selective inactivator (CYP3cide). *Drug Metab Dispos* **42**:1163–1173.

Venkatakrishnan K, von Moltke LL, Court MH, Harmatz JS, Crespi CL, and Greenblatt DJ (2000) Comparison between cytochrome P450 (CYP) content and relative activity approaches to scaling from cDNA-expressed CYPs to human liver microsomes: ratios of accessory proteins as sources of discrepancies between the approaches. *Drug Metab Dispos* **28**:1493–1504.

Walsky RL, Obach RS, Hyland R, Kang P, Zhou S, West M, Geoghegan KF, Helal CJ, Walker GS, Goosen TC, et al. (2012) Selective mechanism-based inactivation of CYP3A4 by CYP3cide (PF-04981517) and its utility as an in vitro tool for delineating the relative roles of CYP3A4 versus CYP3A5 in the metabolism of drugs. *Drug Metab Dispos* **40**:1686–1697.

Wang Q, Ye C, Jia R, Owen AJ, Hidalgo IJ, and Li J (2006) Inter-species comparison of 7-hydroxycoumarin glucuronidation and sulfation in liver S9 fractions. *In Vitro Cell Dev Biol Anim* **42**:8–12.

Wienkers LC and Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Discov* **4**:825–833.

Xu L, Chen Y, Pan Y, Skiles GL, and Shou M (2009) Prediction of human drug-drug interactions from time-dependent inactivation of CYP3A4 in primary hepatocytes using a population-based simulator. *Drug Metab Dispos* **37**:2330–2339.

Xu Y, Zhou Y, Hayashi M, Shou M, and Skiles GL (2011) Simulation of clinical drug-drug interactions from hepatocyte CYP3A4 induction data and its potential utility in trial designs. *Drug Metab Dispos* **39**:1139–1148.

Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Ther* **138**: 103–141.

Zhang H, Davis CD, Sinz MW, and Rodrigues AD (2007) Cytochrome P450 reaction-phenotyping: an industrial perspective. *Expert Opin Drug Metab Toxicol* **3**:667–687.

Zhang W, Ramamoorthy Y, Kilicarslan T, Nolte H, Tyndale RF, and Sellers EM (2002) Inhibition of cytochromes P450 by antifungal imidazole derivatives. *Drug Metab Dispos* **30**: 314–318.

Zientek MA and Youdim K (2015) Reaction phenotyping: advances in the experimental strategies used to characterize the contribution of drug-metabolizing enzymes. *Drug Metab Dispos* **43**: 163–181.

Address correspondence to: Hugues Chanteux, UCB Biopharma, Chemin du Foriest, Braine-l'Alleud B-1420, Belgium. E-mail: Hugues.Chanteux@UCB.com