fm estimation using long-term co-cultured human hepatocytes

Full paper:

Estimation of fraction metabolized by cytochrome P450 (CYP) enzymes using long-term co-cultured human hepatocytes

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Abbreviations: AO, aldehyde oxidase; CL_{int}, intrinsic metabolism clearance; CYP, cytochrome P450; DDI, drug-drug interaction; FCS, fetal calf serum; fm,CYP, fraction metabolized by CYP enzymes in liver; FMO, flavin-containing monooxygenase; fu(inc), unbound fraction in incubates; fu(mic), unbound fraction in microsomes; HLM, human liver microsomes; IC₅₀, inhibition constant; K_{i,u}, (unbound) inhibition constant; K_{I,u}, (unbound) concentration that results in half-maximal inactivation of enzymes; LC-MS, liquid chromatography-mass spectrometry; NADPH, reduced β -

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nicotinamide adenine dinucleotide 2'-phosphate; PAPS, 3'-Phosphoadenosine-5'-phosphosulfate; PBPK, physiologically-based pharmacokinetics; PK, pharmacokinetics; UDPGA, uridine 5'-diphosphoglucuronic acid; UGT, glucuronosyltransferase

Abstract

Estimation of the fraction of a drug metabolized by individual hepatic cytochrome P450 (CYP) enzymes relative to hepatic metabolism (f_m ,CYP) or total clearance (f_{CL} ,CYP) has been challenging for low turnover compounds due to insufficient resolution of the intrinsic clearance (CL_{int}) measurement in vitro and difficulties in quantifying the formation of low abundance metabolites. To overcome this gap, inhibition of drug depletion or selective metabolite formation for 7 marker CYP substrates was investigated using chemical inhibitors and a micro-patterned hepatocyte co-culture system (HepatoPac®). The use of 3 μ M itraconazole was successfully validated for estimation of f_m ,CYP3A4 by demonstration of f_m values within a 2-fold of in vivo estimates for 10 out of 13 CYP3A4 substrates in a reference set of marketed drugs. Other CYP3A4 inhibitors (ketoconazole and posaconazole) were not optimal for estimation of f_m ,CYP3A4 for low turnover compounds due to their high CL_{int} . The current study also demonstrated that selective inhibition sufficient for f_m calculation was achieved by inhibitors of CYP1A2 (20 μ M furafylline), CYP2C8 (40 μ M montelukast), CYP2C9 (40 μ M sulfaphenazole), CYP2C19 (3 μ M (-)-N-3-benzyl-phenobarbital) and CYP2D6 (5 μ M quinidine). Good estimation of f_m ,CYP2B6 was not possible in this study due to the poor selectivity of the tested inhibitor (20 μ M ticlopidine). The approach verified in this study can result in an improved f_m estimation which is aligned with the regulatory agencies' guidance and can support a victim drug-drug interaction risk assessment strategy for low clearance discovery and development drug candidates.

Significance Statement

Successful qualification of a chemical inhibition assay for estimation of fraction metabolized requires chemical inhibitors which retain sufficient unbound concentrations over time in the incubates. The current co-cultured hepatocyte assay enabled estimation of fraction metabolized, especially by CYP3A4, during the drug discovery phase where metabolite quantification methods may not be available. The method enables the assessment of PK variability and victim DDI risks due to enzyme polymorphism or inhibition/induction with more confidence, especially for low clearance drug candidates.

Introduction

Determination of the fraction metabolized by cytochrome P450 (CYP) enzymes relative to total hepatic metabolism ($f_{m,CYP}$), or total clearance ($f_{CL,CYP}$), is important to understand the pharmacokinetic (PK) variability and victim drug-drug interaction (DDI) potential of a drug (Bohnert et al., 2016). $f_{CL,CYP}$ values determined in vitro for a molecule can be confirmed by running clinical DDI studies which measure the change of pharmacokinetics when co-administered with specific CYP enzyme modulators. For example, ibrutinib, developed for treating B-lymphocyte cell malignancies, was proven to have f_{CL} by CYP3A4 of >0.95 when the potent and selective CYP3A4 inhibitor ketoconazole increased the oral AUC by 24-fold, while the CYP3A inducer rifampicin decreased the oral AUC by 10-fold in clinical DDI studies (de Zwart et al., 2016). To support clinical development plans efficiently it is necessary to conduct victim DDI risk assessment by first estimating $f_{m,CYP}$ in vitro. Siponimod, which reduces the risk of disability progression in patients with multiple sclerosis, is metabolized by CYP2C9, CYP3A4 and other enzymes with respective f_m values of 0.79, 0.19 and 0.02 based on in vitro reaction phenotyping (Huth et al., 2019). These f_m values, assuming equity with f_{CL} , informed a physiologically-based pharmacokinetic (PBPK) model which was then used to predict PK profiles and victim DDI risks for CYP2C9 genotypes and could later be shown to be in agreement with clinical data.

Different techniques are applied for in vitro CYP enzyme reaction phenotyping in the pharmaceutical industry (Harper and Brassil 2008). One approach involves determination of metabolic clearance of the test drug with specific chemical inhibitors of the major hepatic CYP enzymes (FDA, 2020). An assay format deploying only one type of biological material, such as HLM or human hepatocytes, can be a simple and

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attractive option to predict fm,CYPs for drug candidates at the compound selection stage. However, since metabolically stable compounds are increasingly preferred as drug candidates due to longer retention in the body, difficulties in measuring drug turnover or production of small quantities of multiple metabolites may prevent accurate fm estimation. For accurate determination of low metabolic clearance in the absence and presence of selective CYP inhibitors, systems allowing for longer incubation times than primary hepatocytes in suspension (limited to four to six hours) are beneficial (Docci et al., 2019). HepatoPac® has been shown to provide improved prediction accuracy and precision for hepatic CYP intrinsic metabolic clearance compared to hepatocytes in monoculture (Umehara et al., 2020), and other approaches are also available such as Hurel microliver platform® and the relay method (Hultman et al., 2016; Murgasova, 2019). Recently, a method to estimate fm,CYP3A4 by incubating low clearance compounds with high concentrations of erythromycin (a moderate time-dependent CYP3A4 inhibitor) in human HepatoPac® was reported (Chan et al., 2020). This was then expanded to estimation of fm,CYP1A2, fm,CYP2C9, fm,CYP2C19 and fm,CYP2D6 by Smith et al (2021). In addition, the human hepatocyte relay method for CYP enzyme reaction phenotyping has been successfully characterized for metabolically stable compounds (Yang et al., 2016). We wanted to build on these approaches by establishing in vitro to in vivo translation for a wider panel of CYP3A4 substrates and to explore further opportunities for these methodologies to be employed for other CYP enzymes.

In this study, human HepatoPac® (multi-donor pooled long term co-cultured hepatocytes) were comprehensively investigated as a tool to estimate fm,CYP by measuring metabolic depletion or selective metabolite formation of probe substrates for CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4 with co-incubation of selective and

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non-selective CYP inhibitors. A drawback of the chemical inhibition method is that compounds established as specific inhibitors for use in short-term human liver microsomal studies may exhibit some degree of off-target inhibition when used at concentrations needed to ensure inhibition effect over extended incubation periods using human hepatocytes. Therefore, optimization of the inhibitor concentrations suitable for fm,CYP estimation using long-term hepatocyte cultures was performed for all tested CYPs. This was further expanded to investigate the effect of the selective CYP inhibitors on non-CYP enzymes since some evidence of this was previously observed (Kratochwil et al., 2017). Finally, the method was applied to estimate fm,CYP3A4 for 13 drugs, followed by a comparison with data collected from the literature. The verification exercise should enable estimation of fm, especially for CYP3A4, for drug discovery compounds including those with low turnover, leading to early assessment of PK variability and victim DDI risks.

Materials and Methods

Chemicals and biological materials. 1-Aminobenzotriazole (ABZ), 17 β -estradiol, carbazeran, bupropion, buspirone, dextromethorphan, diclofenac, furafylline, 4-hydroxymephenytoin, ketoconazole, nifedipine, prednisolone, quinidine, sulfaphenazole, ticlopidine, tienilic acid, tolbutamide and zidovudine were purchased from Sigma-Aldrich (St. Louis, MO). S-Mephenytoin, posaconazole, 2-phenyl-2-(1-piperidynil) propane (PPP) and repaglinide were obtained from Toronto Research Chemicals (Toronto, Canada). Atorvastatin, benzydamine and rosuvastatin were purchased from ChemPacific (Baltimore, MD) and Honeywell Research Chemicals (Charlotte, NC), respectively. Montelukast, simvastatin and tacrine were purchased from Cayman, LKT Laboratories (St. Paul, MN), respectively. Itraconazole was purchased from Spectrum chemical (Wellingborough, UK). (-)N-3-Benzylphenobarbital (NBPB) was purchase from CYPEX (Dundee, UK). Alectinib, alprazolam, idasanutlin, midazolam, mefloquine, triazolam, troglitazone, zolpidem, zopiclone and oxazepam [as internal standard for liquid chromatography-mass spectrometry (LC-MS/MS) analysis] were synthesized at F. Hoffmann-La Roche Ltd. (Basel, Switzerland). Acetonitrile and phosphate-buffered saline (PBS) were obtained from Biosolve Chimie (Dieuze, France) and Thermo Fisher Scientific (Waltham, MA), respectively. Dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used to prepare stock solutions of the test drugs, resulting in the designated DMSO concentrations (% v/v) in the final incubation samples.

Human liver cytosols (150-donor pool; mixed gender), recombinant UGT1A1, UGT2B7 and FMO3 were obtained from Corning Inc (One Riverfront Plaza Corning, NY). Ready-to-use Human HepatoPac® cultures (long-term hepatocyte co-cultures; mixed, n=5 for male and n=5 for female) and stromal mouse fibroblasts (lots/donors:

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8305/YFA and 9177/ACR; pooled) with the plates for incubations, application medium and maintenance medium were acquired from BioIVT.

Metabolism by human co-cultured hepatocytes in the absence of chemical inhibitors of CYPs. After delivery of the cell culture plates, the medium will be exchanged with maintenance medium and kept at 37°C with 10% CO₂ until the experiment start. Before the experiment starts, the cells were pre-incubated with application medium at 37°C and 5%CO₂ for at least two hours. Incubations for the test compounds (0.1, 1 or 3 μM, 0.1% v/v DMSO; except for 40 μM S-mephenytoin and 10 μM idasanutlin) were performed in 96-well plates containing either a co-culture of adherent hepatocytes with mouse fibroblast control cells or control cells alone (5% CO₂ atmosphere and 37°C). HepatoPac® kits contained 5000 human hepatocytes per well in a 96-well plate. At defined time points (2, 18, 26, 48, 72 and 96 h), whole wells were quenched with two-volumes of ice-cold acetonitrile containing an internal standard. Samples were then centrifuged appropriately and the supernatant analyzed by LC-MS/MS.

Measured concentrations were plotted against incubation time (min) and a linear fit made to the natural logarithm transformed data with emphasis upon the initial rate of compound disappearance, except for incubating with S-mephenytoin where formation rates of the selective metabolite (4'-hydroxymephenytoin) were monitored. This calculation was performed for samples prepared from HepatoPac® and fibroblast control cells (stromal cells). The slope of the fit was then used to calculate the apparent intrinsic clearance:

$$CL_{int,app} (\mu\text{L}/\text{min}/\text{mg protein}) = [\text{slope of depletion or metabolite formation (min}^{-1}) \times 1000] / \text{protein concentration (mg protein/mL)} \quad (1)$$

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The apparent intrinsic clearance was corrected for fibroblast metabolism. Each well in HepatoPac® plates has 75% surface area as fibroblasts and 25% surface area as hepatocytes (Khetani and Bhatia, 2008; Chan et al., 2019). The *in vitro* intrinsic clearance determined from the fibroblast lysate according to equation (1) were multiplied by 0.75 and then subtracted from the respective value determined in HepatoPac® plates, to exclude metabolic clearance by fibroblasts. Hence, the hepatocyte-specific intrinsic clearance (CL_{int}) was calculated using equation (2):

$$CL_{int} (\mu\text{L}/\text{min}/\text{mg protein}) = \text{HepatoPac}^{\circledR} CL_{int,app} (\mu\text{L}/\text{min}/\text{mg protein}) - (0.75 \times \text{Fibroblasts (stromal cells)} CL_{int} (\mu\text{L}/\text{min}/\text{mg protein})) \quad (2)$$

It was assumed that intrinsic clearance values in the current *in vitro* assays were measured under linear conditions: substrate concentration (S) < K_m and therefore fm estimation unaffected by any small variations in drug concentration, e.g. due to protein binding or change of drug concentration with time. Based on the high quality and reproducibility of the data for depletion slopes previously measured in our laboratory for a large number of test substances in human HepatoPac® cells (Docci et al., 2019), which was irrespective of the clearance classification (low to high from 0.2 to 100 $\mu\text{L}/\text{min}/\text{mg protein}$), incubations could be efficiently carried out on a single occasion using metabolism time-course profiles made up of 6 independently measured incubation wells. There was high experiment to experiment consistency as illustrated by our quinidine CL_{int} data collected over many experiments (Supplementary Figure S2). HepatoPac® cells were prepared from pooled donor livers throughout the study.

Assessment of metabolic depletion of chemical inhibitors of CYP(3A4).

For assay validation, the retention of the chemical inhibitors in the incubate medium

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was assessed via determinations of their intrinsic hepatic unbound clearance ($CL_{int,u}$) [equation (3)]:

$$CL_{int,u} (\mu L/min/mg \text{ protein}) = CL_{int} (\mu L/min/mg \text{ protein})/f_u(inc) \quad (3)$$

where $f_u(inc)$ is the unbound fraction in incubates. Furthermore, it was assumed that the unbound concentration in the medium represents the intracellular hepatocyte concentration (i.e. no active transport was also assumed). Then a one-compartment model was applied (software: Phoenix 64; Certara Inc., Princeton, NJ), using the measured total CL_{int} values for the four CYP3A4 inhibitors to simulate unbound concentrations for four days after single dosing at total concentrations of 0.3, 1 and 3 μM . The $f_u(inc)$ in the samples supplemented with 10% FCS were 0.23 for ketoconazole, 0.13 for ritonavir, 0.01 for itraconazole and 0.17 for posaconazole. These were estimated with the dilution method (Schuhmacher et al., 2000) using reported plasma unbound fractions: ketoconazole: 0.029 (SimCYP Version 18), ritonavir: 0.015 (SimCYP Version 18), itraconazole: 0.0015 (Chen et al, 2019a) and posaconazole: 0.02 (Cristofolletti et al., 2017). The minimum achieved ratio of simulated unbound inhibitor concentrations to the respective inhibition constants was also calculated.

Inhibition of CYP-mediated metabolism by chemical inhibitors. The chemical inhibitors of CYPs were used at the following concentrations: furafylline (CYP1A2, 20 μM), PPP (25 and/or 100 μM ; CYP2B6), ticlopidine (20 μM ; CYP2B6), montelukast (40 μM ; CYP2C8), sulfaphenazole (40 or 100 μM ; CYP2C9), NBPB (3 μM ; CYP2C19), quinidine (5 μM ; CYP2D6), ketoconazole (0.1, 1 or 3 μM ; CYP3A4), itraconazole (0.1, 1 or 3 μM ; CYP3A4), posaconazole (0.1, 1 or 3 μM ; CYP3A4), ritonavir (0.1, 1 or 3 μM ; CYP3A4), and ABZ (1 mM; non-selective CYP inhibitor) supplemented with tienilic acid (15 μM ; CYP2C9). The reactions were started by

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adding the substrate and inhibitor solutions, terminated by addition of two volumes of ice-cold acetonitrile containing an internal standard for LC-MS measurement. Incubations served as vehicle controls representing maximal substrate metabolism under the conditions used. The final concentration of the organic solvent in the incubation mixtures was equal to 0.1%. All incubations were carried out in n=1 or 2.

Inhibitory effects of the chemical inhibitors on non-CYP enzyme activities in the liver. The effects of chemical inhibitors [furafylline (20 μ M), PPP (100 μ M), montelukast (1 or 40 μ M), sulfaphnazole (100 μ M), NBPB (3 μ M), quinidine (5 μ M) and itraconazole (0.1 or 3 μ M)] on direct glucuronidation of 17 β -estradiol (10 μ M, a substrate of UGT1A1) and zidovudine (10 μ M, a substrate of UGT2B7) were evaluated using the human recombinant enzymes pre-treated with 10 μ g/mL alamethicin. The oxidative metabolism of 10 μ M benzydamine by human recombinant FMO3 were also measured in the presence of the chemical inhibitors of CYPs. Individual enzyme preparations (0.5 mg/mL for UGT1A1, UGT2B7 and FMO3) were pre-incubated with a designated substrate in 100 mM Tris HCl buffer (pH7.5) or 100 mM sodium phosphate buffer (pH7.4), with 5 mM magnesium chloride in the absence and presence of inhibitors for 30 min at 37°C. The reactions were started by adding uridine 5'-diphosphoglucuronic acid (UDPGA, 4.5 mM) for UGTs and reduced β -nicotinamide adenine dinucleotide 2'-phosphate solution (NADPH, 1 mM) for FMO3, and then terminated after 2, 5, 10, 15, 30 and 45 min by addition of two volumes of ice-cold acetonitrile containing an internal standard for LC-MS measurement. The final concentration of the organic solvent DMSO in the incubation mixtures was 0.1%. All incubations were carried out in n=1 or 2.

Incubations of 1 μ M carbazeran (a substrate of aldehyde oxidase AO) and 1 μ M troglitazone (a substrate of sulfotransferase SULT) with human liver cytosols (0.5

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mg protein/mL) were performed in 96-well plates in the presence of the chemical inhibitors of CYPs stated as above. The reactions were started by adding 3'-phosphoadenosine-5'-phosphosulfate (PAPS, 5 mM) for SULT reactions, and then terminated after 2, 5, 10, 15, 30 and 45 min. The final concentration of the organic solvent DMSO in the incubation mixtures was 0.1%. All incubations were carried out in n= 1 or 2.

All absolute activities for metabolism by UGT1A1, UGT2B7, FMO3, AO and SULTs in the absence and presence of the chemical inhibitors were converted into relative activities by defining the enzymatic activity without addition of inhibitor as 100% using Microsoft Excel Version 2016 (Microsoft Corporation, Redmond, WA).

LC-MS/MS analysis. The HPLC system consisted of LC-30AD pumps, a CBM-20A controller, a CTO-20AC oven, a DGU-20A5R degasser from Shimadzu (Kyoto, Japan) and a HTS CTC PAL auto-sampler (CTC Analytics AG; Zwingen, Switzerland). Sample solutions (5 μ L) were injected into the analytical column heated to 60°C [Ascentis Express C18; 2 cm $\times$ 2.1 mm, particle size: 2.7 μ m; and Supelco, Triart or YMC (for troglitazone) C18 plus; 33 x 2.1 mm, particle size: 3 μ m]. To elute the compounds, the following mobile phases used were: A – Water + formic acid 0.1% or formic acid 0.5% (v/v) for troglitazone; and B – acetonitrile + formic acid 0.1% or acetonitrile for troglitazone. A high pressure linear gradient from 0% to 95% B in 40 s was applied at a flow rate of 600 μ L/min. MS detection with multiple reaction monitoring was operated in the positive ion mode with an API6500 mass spectrometer equipped with a TurbolonSpray source (IonSpray Voltage 5,500V; Sciex, Framingham, MA). Analyst 1.6.3 software (Sciex) was used for data processing using linear regression with $1/x^2$ weighting on peak area ratio. The

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precision and accuracy of linear regression of the standard curve samples was between 80% and 120%.

Calculation of $f_{m,CYP}$ or $f_{CL,CYP}$. The CL_{int} (given in $\mu\text{L}/\text{min}/\text{mg}$ protein) of the test drug in human HepatoPac® was calculated by dividing decrease rate of the parent drug or the formation rate of the oxidation product by the initial substrate concentration and the protein concentration [equations (1) and (2)]. The $f_{m,CYP}$ or $f_{CL,CYP}$ was determined as $[1 - (\text{ratio of } CL_{int} \text{ in the presence vs. absence of the chemical inhibitor})]$ using Microsoft Excel Version 2016 (Microsoft Corporation, Redmond, WA).

Static calculation of AUC changes in the absence and presence of strong CYP3A4 inhibition effects. The systemic exposure change of the 13 reference drugs with co-medications of a strong CYP3A4 inhibitor was calculated using measured and observed $f_{m,CYP3A4}$ values in this study according to equation (4):

$$AUCR = \frac{1}{1 - f_{m,CYP3A4}} \quad (4)$$

where AUCR is an AUC ratio of a victim drug in the presence and absence of co-administration of a perpetrator drug. These extrapolated results are due to the complete inhibition effect on the hepatic metabolism and represent the situation in a preclinical research and development setting where further information related to oral bioavailability and non-metabolic clearance pathways are not yet available. Therefore, only the predicted categories of victim DDI risks based on the AUCR values were reported in this study according to FDA (2020): no ($AUCR < 1.25$), weak ($1.25 \leq AUCR < 2$), moderate ($2 \leq AUCR < 5$), and strong ($5 \leq AUCR$).

ChanResults

Selection of a CYP3A4 inhibitor to estimate fm,CYP3A4. Depletion of CYP3A4 inhibitors (ketoconazole, itraconazole, ritonavir and posaconazole) over four days of incubation time in long-term co-cultured hepatocytes is shown in Figure 1, with in vitro metabolic clearance (CL_{int}) values at 1 μ M of 24.1, 6.50, 3.50 and 6.50 μ L/min/mg determined, respectively. Effects of the inhibitors at 0.3, 1 and 3 μ M on the metabolism of midazolam (1 μ M) are summarized in Table 1.

Estimated fm,CYP3A4 of midazolam with co-incubation of itraconazole at 3 μ M (0.88) was comparable to a reported fm,CYP3A4 of ~0.9 in HLM (Njuguna et al., 2016). This magnitude of inhibition was not achieved at a lower concentration of itraconazole (0.3 μ M). Both ketoconazole and midazolam showed moderate to high metabolic CL_{int}: 24.1 and 33.4 μ L/min/mg, respectively (Table 1). Although a strong inhibition was demonstrated by ketoconazole over the first day of incubation (e.g. with midazolam as victim drug) this might not be sufficiently sustained to be suitable for co-incubation with low turnover compounds performed over several days. Ritonavir also showed strong CYP3A4 inhibition and might be used as an alternative to 3 μ M itraconazole for fm,CYP3A4 estimation in human long-term co-cultured hepatocytes, in alignment with a previous report (Greenblatt and Harmatz, 2015). Posaconazole provided a moderate inhibition of CYP3A4 possibly due to incomplete inhibition at the tested concentrations, leading to calculation of a lower fm,CYP3A4 of midazolam (0.59-0.73).

Hence, the use of 3 μ M itraconazole was selected to estimate fm,CYP3A4 in the current reaction phenotyping study.

Metabolism of the probe CYP substrates in the presence of chemical inhibitors. The well-known inhibitors furafylline (20 μ M; CYP1A2), PPP (25 and/or

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100 μ M; CYP2B6), ticlopidine (20 μ M; CYP2B6), montelukast (40 μ M; CYP2C8), sulfaphenazole (40 μ M; CYP2C9), NBPB (3 μ M; CYP2C19), quinidine (5 μ M; CYP2D6) and itraconazole (3 μ M; CYP3A4) inhibited their respective substrate depletion in human long-term co-cultured hepatocytes by up to 93%, 0%, 41%, 82%, 37%, 83%, 88% and 83%, respectively (Table 2; Figure 2).

PPP, a nominal CYP2B6 inhibitor, showed no inhibition of CYP2B6 at 25 and 100 μ M and another CYP2B6 inhibitor, ticlopidine, showed only 41% inhibition of total depletion of bupropion and inhibited the turnover of substrates selective for other enzymes by >30%, namely tacrine (CYP1A2, 84%), diclofenac (CYP2C9, 61%) and dextromethorphan (CYP2D6, 54%). Such off-target activity was also observed for other CYPs. 20 μ M furafylline (a CYP1A2 inhibitor) showed 48% inhibition of CYP2D6 and 100 μ M PPP showed 45% inhibition of CYP2D6. Furthermore, 40 μ M sulfaphenazole demonstrated only a weak inhibition (37%) of total depletion of diclofenac, a dual substrate of CYP2C9 and UGT2B7 (Table 2), which was lower compared to the inhibition degree of the selective metabolite formation (89%) due to CYP2C9 in HLM supplemented with NADPH (Njuguna et al., 2016). Weak inhibition (34% and 29% respectively) was seen on the depletion of tacrine (CYP1A2 substrate) and repaglinide (CYP2C8 substrate) with 3 μ M itraconazole (Table 2). This represents the partial contribution of CYP3A4 to the metabolism of these two substrates as has been reported previously (Cacabelos 2020; Kajosaari et al., 2005).

A non-specific CYP inhibitor (1 mM ABZ) supplemented with a CYP2C9 inhibitor tienilic acid (15 μ M), *i.e.* previously used as an application of the human hepatocyte relay method for fm estimation (Yang et al., 2016), inhibited depletion of the probe CYP substrates in long-term co-cultured hepatocytes by up to 92% (CYP1A2), 0% (CYP2B6), 77% (CYP2C8), 51% (CYP2C9), 100% (CYP2C19), 96%

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(CYP2D6) and 83% (CYP3A4). This was aligned with the corresponding results when using the selective CYP inhibitors (Table 2). Further investigations of fm,CYP2B6 estimation in the current assay format were limited by the lack of available selective CYP2B6 substrates.

In vitro inhibition by chemical inhibitors of non-CYP enzyme activities in the liver. The chemical inhibitors furafylline (20 μ M; CYP1A2), PPP (100 μ M; CYP2B6), sulfaphenazole (100 μ M; CYP2C9), NBPB (3 μ M; CYP2C19) and quinidine (5 μ M; CYP2D6) did not or only slightly inhibited formation of direct glucuronides of 17 β -estradiol (10 μ M) and zidovudine (10 μ M) in incubations with recombinant human UGT1A1 and UGT2B7, respectively (Table 3). However, co-incubation with 40 μ M montelukast inhibited these isoforms by 96% and 65%, respectively. Also, itraconazole at 3 μ M reduced the UGT1A1 marker activity by 62% with no impact on UGT2B7. At lower concentrations of 1 μ M and 0.1 μ M, respectively, montelukast and itraconazole did not significantly affect the UGT1A1 or 2B7 activities. This might reflect the incubation conditions as these molecules have extremely low fu(inc) values (montelukast: <0.003 and itraconazole: 0.01) in the incubation medium supplemented with 10% FCS (Supplementary Figure S1).

No inhibition effects of the inhibitors on recombinant human FMO3 activity (benzylamine oxidation) were exhibited. Metabolism of carbazepine (10 μ M) was not affected by co-incubation of the inhibitors using human liver cytosol, indicating no off-target activity of the chemical inhibitors on AO. In addition, no alteration of CLint for a SULT substrate troglitazone (10 μ M) was observed in human liver cytosol supplemented with PAPS in the absence and presence of the CYP inhibitors except for 40 μ M montelukast (CYP2C8 inhibitor). A lower concentration of montelukast (1 μ M) did not inhibit CYP2C8.

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In vitro-in vivo extrapolation of fm,CYP3A4 for reference drugs. Values of fm,CYP3A4 of 13 drugs (all at 1 μ M except for idasanutlin which was at 10 μ M), eliminated mainly by hepatic metabolism, were obtained by measuring the inhibitory effect of 3 μ M itraconazole on the parent drug depletion during four-days incubation in human long-term co-cultured hepatocytes (Table 4). The selected drugs were mainly eliminated by hepatic metabolism without significant involvement of other elimination pathways such as urinary and biliary excretion. Therefore, the use of the reference fm,CYP3A4 estimated using the data from the clinical victim DDI studies (Cleary and Gertz et al., 2017; Nemunaitis et al., 2018; Ridditid et al., 2005; Ohno et al., 2008) was justified. Metabolic CLint of the reference compounds without inhibition effects were categorized from low (idasanutlin: 1.30 μ L/min/mg) to high (nifedipine: >200 μ L/min/mg) according to the current measurement, and 7 drugs showed low to moderate intrinsic clearance in vitro (approximately < 10 μ L/min/mg). Irrespective of the diverse drug dispositions with a various fm,CYP3A4 (Ohno et al., 2008), currently measured fm,CYP3A4 values estimated in this study were within 0.5- to 2-fold difference compared to the reference data of a number of drugs except for gefitinib, prednisolone and simvastatin (Figure 4).

Assuming negligible first pass effects, the statically predicted systemic exposure change of the reference 13 drugs with co-medications of a strong CYP3A4 inhibitor was successfully categorized moderate/strong or moderate/weak victim DDI risk potential using the measured fm,CYP3A4 values, which were in line with those using the reference data (Table 4).

Discussion

Calculation of f_m , CYP from the ratio of metabolic CL_{int} in the presence and the absence of a chemical inhibitor in human hepatocytes or HLM is an established approach. However, the in vitro CL_{int} can be difficult to determine precisely for low clearance compounds. In this study, chemical inhibition has been applied to long-term co-cultured human hepatocytes and estimation of f_m , CYP was thoroughly investigated for a set of drugs including those with low clearance. The HepatoPac® co-culture system is routinely used over an incubation period of four days without media exchange (Docci et al., 2020; Umehara et al., 2020). In the interest of achieving a simple assay format, depletion of the parent drug was pursued in this study without evaluation of metabolite formation except for 4'-hydroxymephenytoin formation (CYP2C19 marker activity).

As CYP3A4 is one of the major drug metabolizing enzymes and the victim DDI liability is of great concern for drug discovery and development, selection of the chemical inhibitor and optimization of the dosing concentration over the duration of incubation were carefully evaluated. By applying the measured total CL_{int} values of the four CYP3A4 inhibitors (ketoconazole, ritonavir, itraconazole and posaconazole) and assuming a linear one-compartment model, the unbound concentrations after dosing of these four inhibitors at total concentrations of 0.3, 1 and 3 μ M to the assay samples were simulated. It was assumed that unbound concentrations of the inhibitor media supplemented with 10% FCS would represent concentration within the hepatocytes, neglecting any contributions of active transport. Unbound ketoconazole was rapidly depleted, and was predicted to fall below the unbound reversible inhibition constant $K_{i,u}$ (0.015 μ M; SimCYP Version 18) at 24-48 h (Figure 3(A)). The respectively simulated unbound concentrations of itraconazole at an initial

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concentration of 3 μM were >3-fold higher than a reported $K_{i,u}$ of 0.001 μM (Chen et al., 2019a) (Figure 3(B)). The metabolite hydroxyl-itraconazole might also additively contribute to the net inhibition effect of itraconazole on CYP3A4 (Chen et al., 2019a). Despite the identical total CL_{int} of posaconazole and itraconazole (6.50 $\mu\text{L}/\text{min}/\text{mg}$), only moderate inhibition effects of posaconazole on midazolam were observed (Table 1). This is likely due to the lower inhibitory potency of posaconazole: As illustrated in Figure 3(D), unbound posaconazole concentrations after single dosing on the day 1 at 0.1, 1 or 3 μM (total) were close to or much below reported $K_{i,u}$ values for CYP3A4 (0.065 – 1.7 μM ; Cristofolletti et al., 2017) and dropped below effective inhibitory concentrations e.g. 20 x $K_{i,u}$ during the first day of incubation.

The current chemical inhibition assay using co-cultured human hepatocytes was successfully applied to 13 marketed drugs and provided reasonable $f_{m,CYP3A4}$ values relative to the reference data except for gefitinib, simvastatin and prednisolone (Table 4). Gefitinib is a dual substrate of CYP3A4 and CYP1A1, a highly variable and extra-hepatically expressed enzyme (Xu and Li, 2019; Lang et al., 2019). Therefore, hepatocytes without relevant expression of CYP1A1 might over-estimate the CYP3A4 contribution to total metabolism. Thus, an $f_{m,CYP3A4}$ of 0.83, is higher than determined in vivo (0.39) where CYP1A1 involvement was taken into account. Simvastatin $f_{m,CYP3A4}$ in vitro (0.54) was greatly under-estimated relative to the reference (1.00). However, DDI-PBPK modeling with an $f_{m,CYP3A4}$ of 0.9-1.0 occasionally over-estimated the effect of CYP3A4 inhibitors such as erythromycin and ketoconazole on the systemic exposure of simvastatin (SimCYP Version 20; model verification document); otherwise, conversion of simvastatin (lactone) to its acid form, besides the active uptake in liver, might impact characterization of this drugs disposition in vitro and in vivo. Although prednisolone is

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known to be a CYP3A4 substrate, the reported in vivo f_m ,CYP3A4 value was only 0.18, notably lower than the value of 0.64 estimated in this study. A report of a 2-fold increase in prednisolone exposure when co-administered with hormone contraceptives (University of Washington Drug Interaction Database: <https://didb.druginteractionsolutions.org/>) is also suggestive of a higher in vivo f_m value, perhaps more aligned with our in vitro estimate. Hence, irrespective of clearance classification, the f_m ,CYP3A4 values could be now calculated with high resolution and complemented the standard enzymology assay using HLM and recombinant CYP enzymes at preclinical stage. Involvement of extra-hepatic metabolism, renal and fecal excretion to total clearance is also critical to assess the victim DDI liability.

The chemical inhibitors of CYP1A2 (furaflavone, 20 μ M), CYP2C8 (montelukast, 40 μ M), CYP2C19 (NBPB, 3 μ M), CYP2D6 (quinidine, 5 μ M) and CYP3A4 (itraconazole, 3 μ M) inhibited their respective depletion of the probe substrates by up to 93%, 82%, 83%, 88% and 83-88%, respectively (Table 1 and Table 2). Regarding the inhibition of CYP1A2, CYP2C19, CYP2D6 and CYP3A4, the nominal inhibitor concentrations at time 0 were equivalent or 2-fold higher compared to the standard chemical inhibition study using HLM (Cai et al., 2004; Njuguna et al., 2016; Walsky and Obach et al., 2007). On the contrary, a 20-fold higher concentration of montelukast (40 μ M) in the co-cultured hepatocyte incubation than HLM (2 μ M) was deployed to achieve sufficient marker activity inhibition. The different inhibitor concentrations in the two systems could be derived from protein abundance in the incubation. Provided an extremely low f_u (inc) of montelukast in the hepatocyte media samples (<0.003) with 10% FCS, a lowest unbound concentration after initial incubation with montelukast at 20 μ M was approximately estimated to be

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<0.12 μM , which could reach the $K_{i,u}$ in HLM (0.007-0.18 μM) indicating the sufficient inhibition of CYP2C8 (Supplementary Figure S1). However, this might not be accomplished if a low montelukast concentration at 2 μM as used in HLM was applied initially. Along the line, the lowest unbound concentrations of furafylline in the human hepatocyte co-cultures were >10-fold higher than the $K_{i,u}$ (Supplementary Figure S1). Quinidine unbound concentration on the day 4 (~0.111 μM) similar with or below the $K_{i,u}$ in HLM (0.019-0.931 μM) still provided the sufficient inhibition on the CYP2D6 marker activity (Table 2; Supplementary Figure S1).

37% Inhibition of sulfaphenazole (40 μM) on diclofenac metabolism as the CYP2C9 marker activity (Table 2) was completely aligned with the respective fraction clearance in vivo. Diclofenac was metabolized by hepatic CYP2C9, hepatic/renal UGT2B7 followed by renal excretion with corresponding fractions of 0.29, 0.64 and 0.07, respectively (SimCYP Version 16; model verification document). No off-target activity of sulfaphenazole at a high concentration 100 μM on UGT2B7 was also confirmed in this study (Table 3). McGinnity et al (2006) demonstrated the long-term knockdown of CYP2C9 by tienilic acid in plated hepatocytes and a good estimation of $f_{m,CYP2C9}$. Effective inhibition of tolbutamide turnover (a CYP2C9 probe substrate) was reported on co-incubation of tienilic acid at an extremely low concentration 0.015 μM using human co-cultured hepatocytes without supplementation of protein in the media (Smith et al., 2021). Hence, tienilic acid can be used as a mechanism-based CYP2C9 inhibitor in place of the competitive inhibitor sulfaphenazole. Estimation of $f_{m,CYPs}$ using time-dependent inhibitors will work and can benefit from it to characterizing a competition between enzyme inactivation and de novo synthesis of CYP enzymes (Fowler and Zhang, 2008).

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Due to reliability of fm,CYP3A4 estimation within 2-fold of the reference data for 10 out of the 13 CYP3A4 substrates tested in this study (Table 4, Figure 4), fmCYP3A4 $\geq$ 0.5 representing moderate to sensitive victim DDI risk (AUC ratio $\geq$ 2) in systemic circulation with co-medication of a strong CYP3A4 inhibitor can be flagged with confidence even for low clearance drug candidates (FDA, 2020). An identical concept can be applied to other CYP enzymes and this and other studies have shown the feasibility to extend the methodology. Validation of the approach for other CYP enzymes using multiple drug examples with in vivo fm data will be needed to build confidence in the approach for these enzymes as well as for CYP3A4.

In conclusion, the successful use of 3 μ M itraconazole to estimate fm,CYP3A4 by co-incubating with the test drug candidate in long-term of pooled human hepatocytes was demonstrated effective in vitro-in vivo extrapolation of fm,CYP3A4 values. The CYP enzyme reaction phenotyping method could be further applied for other CYP enzymes (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6) using the inhibitors: 20 μ M furafulline, 40 μ M montelukast, 40 μ M sulfaphenazole, 3 μ M NBPB and 5 μ M quinidine, respectively. Qualification of the chemical inhibition assay lies in selecting chemical inhibitors retaining sufficient unbound concentrations in the samples over an extended incubation time period.

These assay capabilities are extremely useful for drug discovery and development of a drug candidate in terms of victim DDI assessment together with running the respective PBPK modeling. The new fm estimation methodology using long-term hepatocyte cultures and selective inhibition of individual CYP metabolism pathways offers a substantial opportunity to estimate fm of moderate clearance drug candidates at an earlier stage in drug development, before detailed knowledge of metabolism products has been gained and before metabolite standards and

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radiolabeled drug substance are available. The method is also significantly resource-sparing since few only a few experiments need to be run using a standardized experimental protocol and only requiring an analytical method for the drug candidate itself.

Authorship Contributions

Participated in research designs: Klammers, Ekiciler, Umehara

Conducted experiments: Klammers, Goetschi, Ekiciler, Walter

Performed data analysis: Klammers, Goetschi, Walter, Umehara

Wrote or contributed to the writing of the manuscript: Klammers, Parrott, Fowler, Umehara

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Footnotes

a) This work was supported by F. Hoffmann-La Roche and no external funding was received.

b) FK, AG, AE, IW, NP, SF and KU are employees of F. Hoffmann-La Roche. No author has an actual or perceived conflict of interest with the contents of this article.

Figure legends

Figure 1. Depletion of CYP3A4 inhibitors over time in human long-term co-cultured hepatocytes.

Ketoconazole (A), itraconazole (B), ritonavir (C) and posaconazole (D) at 1 μ M were administered at time 0 on the day 1, and then incubated for four days in human hepatocytes co-cultured with mouse stromal cells (closed squares), and in mouse stromal cells (negative controls; closed circles). The compound concentrations on y-axis (μ M) in semi-logarithmic scale were plotted against the incubation time on x-axis (day). The depletion profiles of the compounds over time (mean, n=2) were approximated according to linear regression analysis, resulting in calculation of the CL_{int} values. Contribution of stromal cells to the total metabolic clearance was subtracted from the degradation observed in the co-cultured hepatocytes.

Figure 2. Probe CYP substrate or specific metabolite concentrations over time in the absence and presence of the selective CYP inhibitors in human long-term co-cultured hepatocytes.

The effects of selective CYP inhibitors furafylline (CYP1A2, 20 μ M), ticlopidine (20 μ M; CYP2B6), montelukast (40 μ M; CYP2C8), sulfaphenazole (40 μ M; CYP2C9), NBPB (3 μ M; CYP2C19), quinidine (5 μ M; CYP2D6) and itraconazole (3 μ M; CYP3A4), on probe CYP marker activities over four-days incubation time in human co-cultured hepatocytes was illustrated: (A) tacrine (CYP1A2), (B) bupropion (CYP2B6), (C) repaglinide (CYP2C8), (D) diclofenac (CYP2C9), (E) dextromethorphan (CYP2D6) and (F) midazolam (CYP3A4) and (G) 4'-hydroxymephenytoin formation at 40 μ M S-mephenytoin (CYP2C19), respectively.

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The probe substrate concentration was 1 μM ; otherwise noted as above. The probe CYP substrates and inhibitors were applied at time 0 on the day 1, and no further multiple administration of drug compounds or media changes were made in this assay. The concentrations of the parent drug or selective metabolite on y-axis (μM) in semi-logarithmic scale were plotted against the incubation time on x-axis (day) except for (G). The profiles over time (mean, $n=2$) were approximated according to linear regression analysis, resulting in calculation of the CL_{int} values. Contribution of stromal cells (closed blue circles: with inhibition; open blue circles: without inhibition) was subtracted from the metabolic activity observed in the hepatocyte co-cultures (closed red squares: with inhibition; open red squares: without inhibition), respectively. Limit of qualification values of the analytes are as follows: 0.42 nM for tacrine, 0.42 nM for bupropion, 2.54 nM for repaglinide, 2.66 nM for diclofenac, 6.82 nM for 4'-hydroxymephenytoin, 2.60 nM for dextromethorphan and 0.40 nM for midazolam.

Figure 3. Simulated unbound concentration profiles of CYP3A4 inhibitors over time in human long-term co-cultured hepatocyte incubates.

By applying the measured total CL_{int} values of the four CYP3A4 inhibitors in co-cultured hepatocytes [see Results; (A) ketoconazole (24.1 $\mu\text{L}/\text{min}/\text{mg}$), (B) itraconazole (6.50 $\mu\text{L}/\text{min}/\text{mg}$), (C) ritonavir (3.50 $\mu\text{L}/\text{min}/\text{mg}$) and (D) posaconazole (6.50 $\mu\text{L}/\text{min}/\text{mg}$)], the unbound concentrations after single dosing of the four inhibitors at total concentrations of 0.3, 1 and 3 μM to the incubates were simulated for four days. The simulated unbound concentration profiles were visually compared with the corresponding $\text{K}_{\text{i,u}}$ or $\text{K}_{\text{I,u}}$ in HLM shown as black dotted lines in each

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panel. Reported $K_{i,u}$ values of ketoconazole, ritonavir, itraconazole and posaconazole were 0.015 μM (SimCYP Version 18), 0.172 μM (Umehara et al., 2018), 0.001 μM (Chen et al., 2019a) and 0.065 μM (Cristofolletti et al., 2017), respectively. Ritonavir was the only compound out of the four inhibitors showing time-dependent inhibition of CYP3A4 with a $K_{i,u}$ of 0.016 μM (Umehara et al., 2018).

* A reported lower value. A higher end of $K_{i,u}$ (1.7 μM) would result in no inhibition potential of posaconazole on CYP3A4 in the in vitro assay setting.

Figure 4. Comparison of $f_{m,CYP3A4}$ values in human long-term co-cultured hepatocytes with the reference data among a number of CYP3A4 substrates.

(A) $f_{m,CYP3A4}$ estimated in vitro in human co-cultured hepatocytes by incubating 13 CYP3A4 substrates with low to high clearance classification in the absence and presence of 3 μM itraconazole compared to the clinical reference data as summarized in Table 4. The solid lines represents 0.5- and 2-fold ranges of the line of unity (broken line).

(B) Differences between measured and reference values shown as a ratio plotted against the respective in vitro CL_{int} in the absence of inhibition effects.

Tables

Table 1. Metabolic clearance of midazolam with and without co-incubation of CYP3A4 inhibitors in human long-term co-cultured hepatocytes

	CYP3A4 inhibitor concentration (μ M)	Midazolam CL _{int} (μ L/min/mg)	Midazolam fm,CYP3A4
Control (midazolam alone)	NA	35.8 (CI95: 1.0)	NA
+Itraconazole	0.3	10.2 (1.1)	0.72
	1	3.5 (0.8)	0.90
	3	4.4 (0.2)	0.88
+Ketoconazole	0.3	14.3 (3.7)	0.60
	1	6.8 (2.1)	0.81
	3	6.0 (1.0)	0.83
+Ritonavir	0.3	3.8 (5.0)	0.89
	1	5.3 (2.6)	0.85
	3	Not tested	NA
+Posaconazole	0.3	14.5 (1.3)	0.59
	1	9.6 (0.7)	0.73
	3	13.2 (3.5)	0.63

NA: not applicable

Midazolam CL_{int} at 1 μ M in the absence or presence of CYP3A4 inhibitors at the designated initial concentrations in human co-cultured hepatocytes was measured as the midazolam depletion rate over a 4-day incubation period. Midazolam and the inhibitors were applied at time 0 on day 1 and there were no further applications. The fm,CYP3A4 values of midazolam under each inhibition assay condition were calculated as (1 – a ratio of midazolam CL_{int} with and without CYP3A4 inhibition). CL_{int} values the mean of duplicates. CI95 represents the respective CL_{int} with 95% interval for the fitting estimate of CL_{int} based on the midazolam depletion profiles over time according to linear regression analysis.

Table 2. Effects of chemical inhibitors on CYP substrate turnover in human long-term co-cultured hepatocytes

	Chemical inhibitor (initial concentration at time 0)	Substrate selectivity of the CYP inhibitors in human co-cultured hepatocytes (shown as fraction inhibited)						
		CYP1A2 (tacrine)	CYP2B6 (bupropion)	CYP2C8 (repaglinide)	CYP2C9 (diclofenac)	CYP2C19 (S-mephenytoin)	CYP2D6 (dextromethorphan)	CYP3A4 (midazolam)
CYP1A2	furafylline (20 μ M)	0.93	0.00	0.05	0.00	0.00	0.48	0.00
CYP2B6	PPP (25 μ M /100 μ M)	0.19/0.00	0.00/0.00	0.00/-	0.04/0.32	0.00/0.25	0.01/0.45	0.01/0.16
	ticlopidine (20 μ M)	0.84	0.41	0.00	0.61	0.17	0.54	0.00
CYP2C8	montelukast (40 μ M)	0.11	0.00	0.82	0.00	0.00	0.00	0.20
CYP2C9	sulfaphenazole (40 μ M)	0.04	0.04	0.11	0.37	0.00	0.00	0.00
CYP2C19	NBPB (3 μ M)	0.05	0.25	0.00	0.28	0.83	0.00	0.00
CYP2D6	quinidine (5 μ M)	0.07	0.00	0.10	0.18	0.00	0.88	0.00
CYP3A4	itraconazole (3 μ M)	0.34	0.00	0.29	0.18	0.00	0.10	0.83
CYPs	ABZ (1 mM) with tienilic acid (15 μ M)	0.92	0.00	0.77	0.51	1.00	0.96	0.83

PPP: 2-phenyl-2-(1-piperidiny) propane

NBPB: (-) N-3-benzyl-phenobarbital

ABZ: aminobenzotriazole

In vitro intrinsic metabolic clearance (CL_{int}) values were determined by measuring linear depletion rates of the parent drugs at 1 μ M after incubating with human co-cultured hepatocytes for 4 days, except for CYP2C19 (4'-hydroxymephenytoin formation rates at 40 μ M S-mephenytoin). Fractions inhibited were calculated as [1 – (ratio of CL_{int} in the presence vs. absence of the chemical inhibitor)]. The data represent mean values of duplicate experiments.

Table 3. Effects of chemical inhibitors on non-CYP enzyme marker activities

Chemical inhibitor		Off-target inhibition of selected non-CYP enzymes in individual enzyme preparations or liver cytosols (shown as fraction inhibited)				
		UGT1A1 (17 β -estradiol)	UGT2B7 (zidovudine)	FMO3 (benzylamine)	AO (carbazepine)	SULT (troglitazone)
CYP1A2	furafylline (20 μ M)	0.00	0.00	0.06	0.01	0.00
CYP2B6	PPP (100 μ M)	0.00	0.14	0.00	0.03	0.04
CYP2C8	montelukast (1/40 μ M) ¹⁾	0.32/0.96	0.14/0.65	0.00	0.10	0.21/0.75
CYP2C9	sulfaphenazole (100 μ M)	0.40	0.00	0.05	0.00	0.00
CYP2C19	NBPB (3 μ M)	0.18	0.15	0.04	0.05	0.00
CYP2D6	quinidine (5 μ M)	0.25	0.03	0.02	0.00	0.00
CYP3A4	itraconazole (0.1/3 μ M) ¹⁾	0.00/0.62	0.00	0.11	0.00	0.02

PPP: 2-phenyl-2-(1-piperidinyl) propane

NBPB: (-) N-3-benzyl-phenobarbital

1) Inhibition effects of montelukast and itraconazole at lower concentrations (1 μ M and 0.1 μ M, respectively) besides high concentrations (40 μ M and 3 μ M, respectively) on UGT1A1 and UGT2B7 marker activities were incubated; otherwise only the effects of the inhibitors at high concentrations were investigated.

In vitro intrinsic metabolic clearance (CL_{int}) values were determined by measuring linear depletion rates of the parent drugs (carbazepine and benzylamine at 10 μ M, and troglitazone at 1 μ M) or the subsequent metabolite formation (glucuronidated metabolite of 17 β -estradiol at 1 μ M and zidovudine at 10 μ M) in i) recombinant UGTs supplemented with UDPGA, ii) recombinant FMO3 with NADPH, iii) human liver cytosols for AO or iv) human liver cytosols with PAPS for SULTs (n=1 or mean values of

duplicate experiments). Fractions inhibited were calculated as $[1 - (\text{ratio of CLint in the presence vs. absence of the chemical inhibitor})]$.

Table 4. Estimated fm,CYP3A4 values in human long-term co-cultured hepatocytes with the reference data among a number of CYP3A4 substrates

Compounds	CLint w/o CYP3A4 inhibition (μL/min/mg)	CLint with CYP3A4 inhibition (μL/min/mg)	fm,CYP3A4 [measured]	Victim DDI risk using measured fm,CYP3A4	fm,CYP3A4 [reference]	Victim DDI risk using reference fm,CYP3A4
Alectinib	12.5 (CI95: 0.60)	5.3 (CI95: 0.20)	0.57	moderate	0.40 ¹⁾	weak
Alprazolam	3.14 (0.14)	0.50 (0.15)	0.84	strong	0.75	moderate
Atorvastatin	14.3 (0.26)	1.91 (0.17)	0.87	strong	0.68	moderate
Buspirone	60.8 (0.00)	8.35 (0.65)	0.86	strong	0.99	strong
Gefitinib	20.1 (1.70)	3.36 (0.32)	0.83	strong	0.39	weak
Idasanutlin	1.30 (0.3)	1.00 (0.40)	0.23	weak	0.25 ²⁾	weak
Mefloquine	6.26 (0.32)	3.69 (0.13)	0.41	weak	0.47 ³⁾	weak
Nifedipine	>200 (0.00)	6.93 (0.33)	0.99	strong	0.78	moderate
Prednisolone	37.0 (8.11)	13.2 (0.65)	0.64	moderate	0.18	no
Simvastatin	49.1 (4.17)	23.2 (5.03)	0.54	moderate	1.00	strong
Triazolam	26.9 (2.08)	1.65 (0.30)	0.94	strong	0.93	strong
Zolpidem	9.64 (0.39)	3.57 (0.32)	0.63	moderate	0.40	weak
Zopiclone	10.3 (0.82)	3.29 (0.27)	0.68	moderate	0.44	weak

N.C.: not calculated

In vitro intrinsic metabolic clearance (CLint) values of 13 drugs were determined by measuring linear depletion rates of the parent drugs at 1 μM except for idasanutlin (10 μM) after incubating with human co-cultured hepatocytes for 4 days in the absence and presence of the CYP3A4 inhibitor 3 μM itraconazole. CI95 represents the respective CLint with 95% interval for the fitting estimate of CLint based on the parent drug depletion profiles over time according to linear regression analysis. Based on these CLint values of the fmCYP3A4 was estimated. The categories of victim DDI risks were given according to FDA (2020): no (AUCR < 1.25), weak (1.25 ≤ AUCR < 2), moderate (2 ≤ AUCR < 5), and strong (5 ≤ AUCR) using a static model predicted systemic exposure increase in

the presence of strong CYP3A4 inhibition effects, representing the situation in a preclinical research and development setting where further information related to oral bioavailability and non-metabolic clearance pathways are not yet available. Compounds classified into low – moderate CL_{int} category without CYP3A4 inhibition (approximately < 3 – 10 µL/min/mg; Umehara et al., 2020) were presented in bold. The data represent mean values of duplicate experiments. The reference fm, CYP3A4 data are taken from (Ohno et al., 2008) based on clinical DDI data unless otherwise noted.

- 1) Cleary and Gertz et al., 2017
- 2) Nemunaitis, et al., 2018
- 3) Thai population (Ridtitid et al., 2005)

Figure 1

Figure 2

(A) CYP1A2

(B) CYP2B6

(C) CYP2C8

(D) CYP2C9

(E) CYP2D6

(F) CYP3A4

(G) CYP2C19

Figure 3

Figure 4

(A)

(B)