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Novel Cytochrome P450 Reaction Phenotyping for Low-Clearance Compounds Using the Hepatocyte Relay Method

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ABSTRACT

A novel cytochrome P450 (P450) reaction phenotyping method for low-clearance compounds has been developed for eight P450 enzymes (CYP1A2, 2B6, 2D6, 2C8, 2C9, 2C19, 3A, and 3A4) and pan-cytochrome using the hepatocyte relay approach. Selective mechanism-based inhibitors were used to inactivate the individual P450 enzymes during preincubation, and inactivators were removed from the incubation before adding substrates to minimize reversible inhibition and maximize inhibitor specificity. The inhibitors were quite selective for specific P450 isoforms using the following inhibitor concentrations and preincubation times: furafylline (1 μM, 15 minutes) for CYP1A2, phencyclidine (20 μM, 15 minutes) for 2B6, paroxetine (1.8 μM, 15 minutes) for CYP2D6, gemfibrozil glucuronide (100 μM, 30 minutes) for 2C8, tienilic acid (15 μM, 30 minutes) for 2C9, esomeprazole (8 μM, 15 minutes) for 2C19, troleandomycin (25 μM, 15 minutes) for 3A4/5, CYP3cide (2 μM, 15 minutes) for 3A4, and 1-aminobenzotriazole (1 mM, 30 minutes) supplemented with tienilic acid (15 μM, 30 minutes) for pan-cytochrome. The inhibitors were successfully applied to the hepatocyte relay method in a 48-well format for P450 reaction phenotyping of low-clearance compounds. This novel method provides a new approach for determining the fraction metabolized of low-turnover compounds that are otherwise challenging with the traditional methods, such as chemical inhibitors with human liver microsomes and hepatocytes or human recombinant P450 enzymes.

Introduction

Determination of fraction metabolized (f_m) by drug-metabolizing enzymes (i.e., reaction phenotyping) is critical for drug candidates to: 1) understand the potential risk associated with being a victim of drug-drug interaction (DDI); 2) estimate the impact of genetic polymorphic enzymes on in vivo exposure; and 3) anticipate intersubject pharmacokinetic variability for compounds with a narrow therapeutic index. Polypharmacy has become a common practice to treat multiple conditions within a single patient. DDI caused by coadministration of multiple drugs has been cited as one of the major reasons for hospitalization, and even death (Lazarou et al., 1998). Cytochrome P450 enzymes metabolize ~75% of the marketed drugs, and members of several P450 subfamilies are responsible for metabolizing most of the xenobiotics (Williams et al., 2004). Inhibition and induction of P450 enzymes are the most common mechanisms of DDI. When a compound is metabolized metabolized by a single isozyme, it has a greater risk in the clinic for DDI if coadministered with an inhibitor or an inducer of the isozyme. This can lead to "black box" warnings on comedications, dose adjustment in the clinic, and even market withdrawals of the victim drugs (Wienkers and Heath, 2005). If the clearance pathway of a compound is predominately metabolized by a genetic polymorphic enzyme, higher systemic exposure could be observed in poor metabolizers and lower exposure in ultrarapid metabolizers, which can cause a greater risk of adverse effects, toxicity, or lack of efficacy. Therefore, compounds with a high f_m (e.g., > 0.8) of polymorphic enzymes (e.g., CYP2D6) do not usually progress through the drug discovery pipeline. Structural modification strategies are typically applied to introduce additional clearance pathways and reduce the f_m of the polymorphic enzymes. Drug candidates with a balance of different clearance pathways are desirable to minimize the potential of victim DDI and the impact of polymorphic enzymes.

In vivo reaction phenotyping data are usually obtained from a human absorption, distribution, metabolism, and excretion (ADME) study with radiolabeled material in the later stage of drug development. These data are integrated with in vitro reaction phenotyping results to inform a clinical DDI study design or pharmokinetic studies in genotyped subjects. Ultimately, reaction phenotyping data are included in the product label to help inform physicians and pharmacists of comedications and dose adjustments. In drug discovery, several in vitro approaches are available for P450 reaction phenotyping (Parkinson, 1996; Mei et al., 1999; Rodrigues, 1999; Zhang et al., 2007), including: 1) chemical inhibitors or antibodies with human liver microsomes or hepatocytes; 2) recombinant human P450 enzymes with the correction of relative activity factor (Venkatakrishnan et al., 2000; Emoto et al., 2006) or intersystem extrapolation factor (Proctor et al., 2004; Chen et al., 2011); and 3) correlation analysis. For low-clearance compounds, it is challenging to conduct reaction phenotyping studies using these methods owing to no or low turnover of test compounds in the in vitro systems (Di and Obach, 2015). Frequently, in vitro-in vivo discrepancies are observed for low-clearance compounds with no detectable in vitro metabolism, but metabolites are formed in vivo. Since in vitro systems fail to detect any significant metabolism, f_m cannot be determined using the traditional approach, and negligible DDI is predicted; however, DDIs are observed in the

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ABBREVIATIONS: ABT, 1-aminobenzotriazole; ADME, absorption, distribution, metabolism, and excretion; AKR, aldo-keto reductase; AO, aldehyde oxidase; CBR, carbonyl reductase; CES, carboxylesterase; CL_{int}, intrinsic clearance; DDI, drug-drug interaction; f_m, fraction metabolized; K_m, apparent substrate concentration at half-maximal velocity; LC-MS/MS, liquid chromatography-tandem mass spectroscopy; MBI, mechanism-based inactivator; P450, cytochrome P450; SULT, sulfotransferase; UGT, uridine diphosphate glucuronyltransferase.

clinic for these "metabolically stable" compounds. This observation was previously considered a "mystery" because of the lack of available low-clearance tools (Lu et al., 2003). Reaction phenotyping of low-clearance compounds is particularly challenging since the lower limit of intrinsic clearance needs to be 10-fold lower than the regular intrinsic clearance (CL_{int}) limit to detect 90% inhibition. Therefore, even for moderately cleared compounds, low-clearance methods are needed for reaction phenotyping to be able to cover greater than 90% inhibition.

One approach of reaction phenotyping for low-clearance compounds is to monitor metabolite formation using metabolite standards since it is a more sensitive assay than parent depletion. This approach can be challenging and resource-consuming, however, when multiple metabolites are formed or when metabolic pathways or metabolite structures are not well characterized in early stages of drug discovery (preclinical). Therefore, a relatively inexpensive method with quick turnaround time is needed to address the reaction phenotyping challenges of low-clearance compounds in fast-paced drug discovery projects. The hepatocyte relay method was developed to address low-clearance challenges in drug discovery (Di et al., 2012, 2013). Here, a novel approach is addressed that applies the hepatocyte relay method to P450 reaction phenotyping using mechanism-based inactivator (MBI) in drug discovery. Similar to any standard hepatocyte assay, nonmetabolic decline should first be evaluated. If the standard relay assay shows nonmetabolic decline, it will not be possible to run the phenotyping relay for that compound.

Materials and Methods

Materials. Test compounds were obtained from Pfizer Global Material Management (Groton, CT) or purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). All reagents were obtained from Sigma-Aldrich unless specified; 48-well polystyrene plates were purchased from Corning (Corning, NY).

P450 Inhibitor Specificity Study. Cryopreserved human hepatocytes (lot no. DCM) consisting of 10 donors, both males and females, were custom-pooled and prepared by BioreclamationIVT (Baltimore, MD). Upon thawing, the hepatocytes were resuspended in Williams' E medium (GIBCO-BRL, custom formula supplemented with 50 mM HEPES and 26 mM Na_2CO_3; Gibco/Invitrogen Corp., Grand Island, NY). The cells were counted, and viability was determined using the trypan blue exclusion method. Inhibitors were dissolved in methanol, diluted with 1:1 acetonitrile:water to the appropriate concentrations, and 3 μl was added to 300 μl suspended hepatocytes at 0.5 million cells/ml. The inhibitors were preincubated with hepatocytes for either 15 or 30 minutes (enzyme-specific incubation time predetermined in selectivity experiments) to inactivate the enzymes. The inhibitors, concentrations, and preincubation times were furafylline (1 μM, 15 minutes) for CYP1A2, phencyclidine (20 μM, 15 minutes) for 2B6, paroxetine (1.8 μM, 15 minutes) for CYP2D6, gemfibrozil glucuronide (100 μM, 30 minutes) for 2C8, tienilic acid (15 μM, 30 minutes) for 2C9, esomeprazole (8 μM, 15 minutes) for 2C19, troleandomycin (25 μM, 15 minutes) for 3A, CYP3cide (2 μM, 15 minutes) for 3A4, and 1-aminobenzotriazole (1 mM, 30 minute) supplemented with tienilic acid (15 μM, 30 minutes) for pan-CYP. The final amount of organic solvent was 0.5% in hepatocytes during preincubation. After preincubation, the media containing the inhibitors were removed from the hepatocytes, and fresh media with cytochrome substrates were added to the cells with specific isozymes inactivated to a final cell density of 0.5 million cells/ml, substrate concentration of 1 μM, and 0.6% organic solvent. The cytochrome substrates for inhibitor specificity studies were midazolam (3A) to 1′-OH-midazolam, dextromethorphan (2D6) to dextrorphan, diclofenac (2C9) to 4′-OH-diclofenac, amodiaquine (2C8) to N-desethylamodiaquine, S-mephenytoin (2C19) to 4′-OH-S-mephenytoin, phenacetin (1A2) to acetaminophen, and bupropion (2B6) to OH-bupropion. Metabolite formation was monitored to detect the specific metabolic reaction by the isozymes (see MRM transitions in Table 1). The plates were incubated at 37°C and 150 rpm on an orbital shaker (VWR, Radnor, NJ) in an incubator with 95% O_2/ 5% CO_2 and 75% relative humidity. At various time points (0, 5, 10, 20, 30, 45, and 60 minutes), 20 μl of hepatocyte suspension was collected and added to 100 μl of cold acetonitrile containing internal standards (a cocktail of 250 ng/ml indomethacin and 10 ng/ml terfenadine). The solution was centrifuged (Eppendorf, Hauppauge, NY) at 3000 rpm for 10 minutes at room temperature, and the supernatant was transferred for liquid chromatography-tandem mass spectroscopy (LC-MS/MS) analysis. Each inhibitor was tested against all P450 substrates to examine the inhibitor specificity for each isozyme. Percent inhibition was calculated using the area under the curve of metabolite formation. Only the linear region of the metabolite LC-MS/MS peak area ratio-time course was included in the calculation (Prism 6 for Windows, Version 6.03, GraphPad Software, Inc., La Jolla, CA).

P450 Reaction Phenotyping Using the Hepatocyte Relay Method. Details of the 24-well hepatocyte relay method have been described previously (Di et al., 2012, 2013), and the method has been modified to a 48-well format for reaction phenotyping studies. In the hepatocyte relay assay, buffer controls are included to monitor nonmetabolic decline resulting from buffer instability or nonspecific binding. Only compounds with adequate intrinsic clearance values and without nonmetabolic decline will be considered for hepatocyte relay-reaction phenotyping. Nonspecific binding has not been an issue in our experience (only 4% of the 350 compounds tested in our laboratories showed nonmetabolic decline, and much of that was due to buffer instability). At the beginning of each relay, 2 μl of inhibitors is added to 200 μl of hepatocytes (final cell density of 0.5 or 2 million cells/ml, 0.5% final organic solvent) at the appropriate concentrations and preincubation times as described herein to inactivate the enzymes. The selection of the different cell densities is based on previous determination of test compound intrinsic clearance, the targeted percent inhibition of the clearance pathways, and the lower measurable limit of intrinsic clearance of the assay. The plate containing hepatocytes is incubated at 37°C, 95% O_2/ 5% CO_2 and 75% relative humidity. The plate is not shaking during the enzyme-specific inactivation preincubation so that the cells settle to the bottom of the plate to more easily enable removal of the inhibitors after preincubation. After preincubation, media containing inhibitors are removed from the hepatocytes. Hepatocytes with specific isozymes inactivated are added to the substrates (1 μM final concentration with total organic solvents of 0.6%). Substrate concentration of 1 μM is, in general, appropriate for most test compounds. For compounds with low K_m, lower substrate concentrations can be used. The plates are incubated at 37°C and 150 rpm on an orbital shaker in an incubator with 95% O_2/5% CO_2 and 75% relative humidity. At time 0 and 4 hours, 10 μl of the hepatocyte suspension is removed from the incubation. After the 4-hour suspension is collected, the remaining hepatocyte suspension in the incubation plate is centrifuged (3000 rpm, 10 minutes, room temperature), and 10 μl of supernatant is removed to determine the concentration of the supernatant. All the collected samples are quenched with 100 μl of cold acetonitrile containing internal standards and centrifuged at 3000 rpm for 10 minutes at room temperature, and 50 μl of supernatant is transferred to a clean plate for LC-MS/MS analysis. Then 90 μl of the supernatant remaining from the incubation plate is transferred to a new 48-well plate and stored at −80°C until the next relay experiment. For the second relay experiment, freshly thawed hepatocytes are inactivated with MBIs as described here in the first relay. The media containing inhibitors are removed after preincubation, and the remaining hepatocytes are reconstituted with Williams' E medium buffer and resuspended. In the meantime, the plates containing the supernatant from the first relay are prewarmed at 37°C for 30 minutes. The hepatocytes with specific isozymes inactivated are added to prewarmed supernatant from the previous relay to give a final cell density of 0.5 (or 2.0) million cells/ml. The plates are incubated at 37°C and 150 rpm on an orbital shaker in an incubator with 95% O_2/5% CO_2 and 75% relative humidity. The relay suspension is sampled at 8, 12, 16, and 20 hours and processed as described already. Five relays are performed using MBI-inactivated hepatocytes to give a total accumulative incubation time of 20 hours. Controls without inhibitors (solvent control) are run in parallel on the same plate. Percent inhibition is calculated based on parent depletion.

LC-MS/MS Conditions. The LC mobile phases were: (A) high-performance liquid chromatography–grade water containing 0.1% formic acid, and (B) acetonitrile containing 0.1% formic acid. The following solvent gradient or equivalent was used: 95%(A)/5%(B) for 0.3 minutes, 95%(A)/5%(B)–5%(A)/95%(B) from 0.3 to 1.0 minutes, 5%(A)/95%(B) from 1.0 to 1.7 minutes, 5%(A)/95%(B)–95%(A)/5%(B) from 1.7 to 2.0 minutes. A flow rate of 0.5 ml/min was used to elute the compounds from the column (Kinetex C18, 30 × 3 mm, 2.6 μm; Phenomenex, Torrance, CA). A sample aliquot of 3 μl was injected for analysis using a CTC PAL autosampler (Leap Technology, Carrboro, NC). Shimadzu

TABLE 1

Summary of preincubation time, P450 inhibitors, substrates, and metabolites for specificity study

P450 Isozymes	Preincubation Time	Inactivators and Concentrations	Substrates	Metabolites	Mass Transitions of Metabolites
	min				
1A2	15	1 μM Furafylline	Phenacetin	Acetaminophen	152 > 110
2B6	15	20 μM Phencyclidine	Bupropion	OH-bupropion	256 > 238
2D6	15	1.8 μM Paroxetine	Dextromethorphan	Dextrorphan	258 > 199
2C8	30	100 μM Gemfibrozil glucuronide	Amodiaquine	*N*-desethylamodiaquine	328 > 283
2C9	30	15 μM Tienilic acid	Diclofenac	4′-OH diclofenac	312 > 231
2C19	15	8 μM Esomeprazole	Mephenytoin	4′-OH-mephenytoin	235 > 150
3A	15	25 μM Troleandomycin	Midazolam	1′-OH-midazolam	324 > 203
3A4	15	2 μM CYP3cide	Midazolam	1′-OH-midazolam	342 > 203
Pan-CYP	30	1 mM ABT, 15 μM tienilic acid	All substrates listed above	All metabolites listed above	NA

high-performance liquid chromatography AD30 pumps (Columbia, MD) connected to an AB Sciex (Foster City, CA) 5500 triple quadrupole mass spectrometer equipped with a TurboIonSpray source using MRM mode was also used. Analyst 1.5.2 software (Applied Biosystems, Foster City, CA) was applied to data collection, processing, and analysis. Terfenadine was used as an internal standard for LC-MS/MS quantification in positive ion MRM mode, and indomethacin was used in negative mode. Area ratio of analyte/internal standard was used to calculate CL_{int} and f_m.

Data Analysis. The intrinsic clearance calculation from hepatocyte relay method has been described in detail previously (Di et al., 2012, 2013). For inhibitor specificity studies, percent inhibition was calculated based on the area under the curve of metabolite formation using eq. 1 from the linear region of the metabolite LC-MS/MS peak area ratio-time course curve. For hepatocyte relay-reaction phenotyping, percent inhibition was calculated based on intrinsic clearance of parent depletion using eq. 2 since metabolite standards of test compounds are usually not available at early stages of drug discovery. The f_m in the hepatocyte relay-reaction phenotyping assay was calculated using eq. 3 by normalization of total percent inhibition, which was the sum of the percent inhibition from all isozymes. A parallel line test was used to test whether the slopes of the Ln % remaining versus time were not equal (Prism 6 for Windows, Version 6.03, GraphPad Software, Inc.). When a P value > 0.05, the slopes were assumed to be equal. The intrinsic clearance with inhibitor was considered to be the same as clearance without inhibitor. No significant contribution of the enzyme to the clearance pathway was concluded.

$$\%Inhibition = \frac{AUC_{no\ inh} - AUC_{inh}}{AUC_{no\ inh}} \times 100\% \quad (1)$$

$$\%Inhibition = \frac{CL_{int,\ no\ inh} - CL_{int,\ inh}}{CL_{int,\ no\ inh}} \times 100\% \quad (2)$$

$$F_m = \frac{\%Inhibition}{Sum\ of\ Total\ \%Inhibition} \quad (3)$$

Results

Nine mechanism-based inactivators were evaluated for their specificity against eight cytochrome P450 enzymes (CYP1A2, 2B6, 2D6, 2C8, 2C9, 2C19, 3A, and 3A4) and pan-cytochrome using pooled cryopreserved human hepatocytes with multiple donors. The inactivator concentration, preincubation time, substrates, and metabolites are summarized in Table 1. The scheme of the novel hepatocyte relay-reaction phenotyping method is shown in Fig. 1. The inactivators were removed from the hepatocytes after preincubation (15 or 30 minutes) to improve the specificity of the inhibitors (shown in Fig. 2). The data showed that all the inactivators had good specificity with the exception of paroxetine and phencyclidine. Under the assay conditions, paroxetine showed 66% inhibition of CYP2B6. 1-Aminobenzotriazole (ABT, 1 mM) showed potent inhibition of all P450 isozymes with the exception of CYP2C9 (Fig. 2). Only 23% inhibition of CYP2C9 was observed after 30-minute preincubation with 1 mM ABT. Therefore, a cocktail of 1 mM ABT and 15 μM tienilic acid was used as pan-cytochrome inhibitor to completely inhibit the P450 activities. Several low-clearance compounds were used to validate the relay P450 reaction phenotyping assay. The intrinsic clearance and f_m values are shown in Table 2. The percentages of remaining-time courses of the compounds with and without inactivation are illustrated in Fig. 3. The inactivators have marked inhibition of the substrates. All the f_m data are consistent with the clearance pathway and the reported data of the compounds.

Discussion

This is the first time that P450 reaction phenotyping of low-clearance compounds using the hepatocyte relay method with inactivator removal after preincubation is addressed. This novel approach provides a new way to measure the f_m for compounds with low intrinsic clearance that would otherwise be difficult to obtain in drug discovery. The unique experimental design, without the coexistence of the inactivators and the substrates during incubation, enhances the specificity of the inhibitors compared with the traditional approach of coincubation, as this eliminates any potential reversible inhibition of phase 1/2 enzymes and transporters. The selective inactivators, and the specific assay conditions of this method can also be applied to reaction phenotyping for high- and moderate-clearance compounds to achieve high specificity in hepatocytes without the relay format.

Although hepatocytes are not commonly used for reaction phenotyping in drug discovery, they offer several advantages: they are more physiologically relevant systems and contain the full complement of hepatic drug-metabolizing enzymes of both phase 1 and phase 2 transporters (Soars et al., 2007), and a cell-membrane barrier, allowing for simultaneous identification of the various competing clearance pathways. In addition to P450 enzymes, hepatocyte systems enable determination of clearance pathways involving non-P450 enzymes,

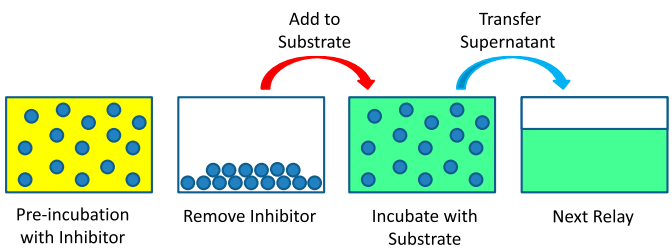

Fig. 1. Reaction phenotyping using hepatocyte relay method.

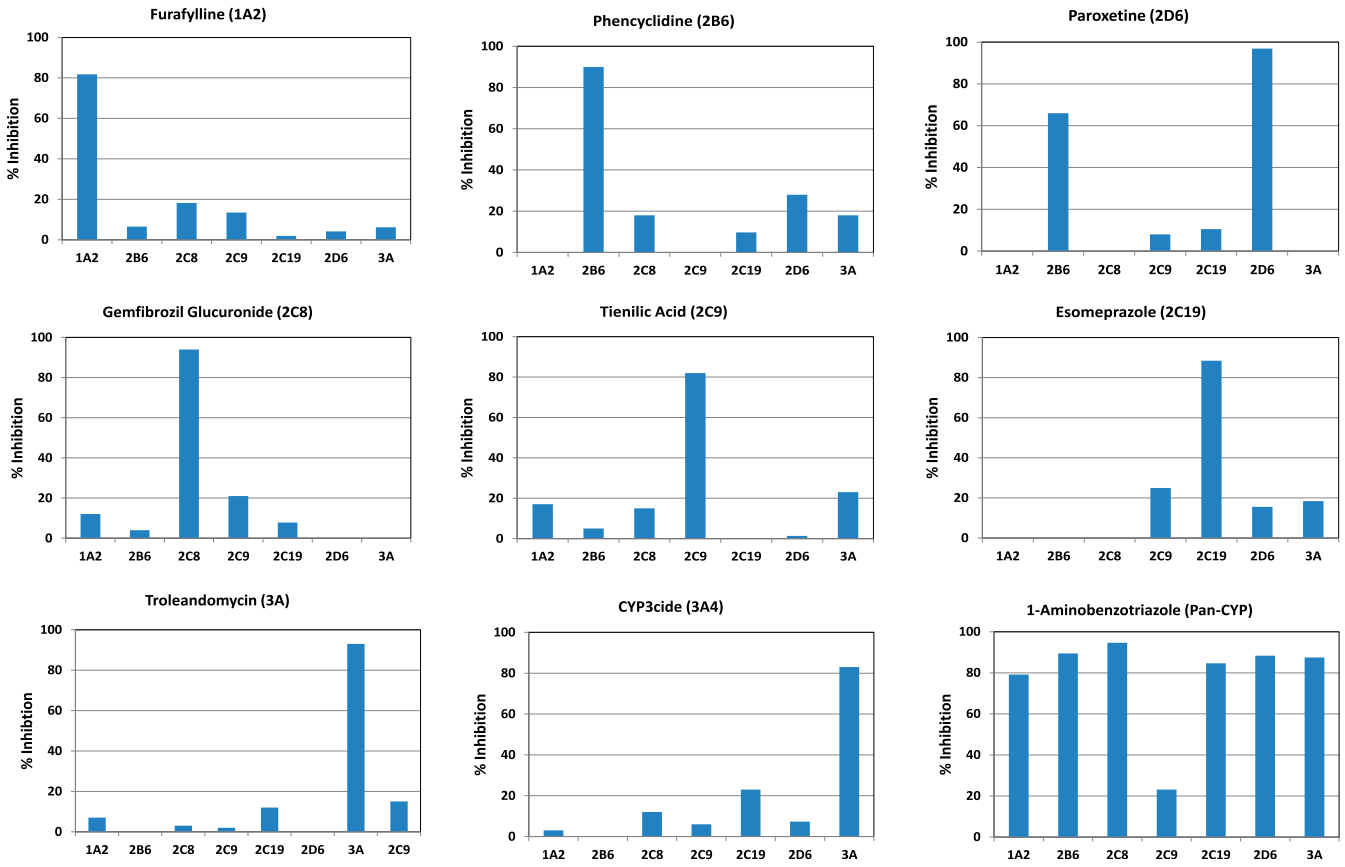

Fig. 2. P450 Inactivator specificity in human hepatocytes.

such as uridine diphosphate glucuronyltransferase (UGT), sulfotransferase (SULT), carboxylesterase (CES), aldehyde oxidase (AO), carbonyl reductase (CBR), and aldo-keto reductase (AKR), which is essential to more accurately assign f_m. The success of a reaction phenotyping assay using hepatocytes is highly dependent on the specificity of the chemical inhibitors. Nonspecific inhibitors can lead to confounding f_m results and, consequently, a less than optimal clinical study design. Therefore, it is critical to identify selective inhibitors and assay conditions for reaction phenotyping using hepatocytes to generate reliable high-quality data. Specificity of P450 inactivators has been evaluated under coincubation conditions of inhibitors and substrates using hepatocytes (Kazmi et al., 2014). Our studies showed that by removing inhibitors from the media after preincubation, the inactivator specificity improved significantly by minimizing reversible inhibition of various phase 1/2 enzymes and transporters (e.g., cytochromes, AO, UGT, CES, and organic anion-transporting polypeptide; data not shown). For example, troleandomycin (25 μM) showed some inhibition of CYP2C19 when coincubated with substrates. The specificity of the inhibitors generally improved when the inactivators and the substrates do not coexist in the incubation. This is particularly important for the selectivity MBIs against the non-P450 enzymes (e.g., AO, UGT, SULT, CES, CBR, AKR, and organic anion-transporting polypeptide), for which the inhibition has not been thoroughly evaluated. The likelihood of mechanism-based inactivation of these enzymes by the P450 inactivators is minimal.

Since the hepatocytes are alive during incubation, the deactivated enzymes will be regenerated during incubation once the inactivators are removed from the system. The estimated recoveries of the enzyme activity 4 hours postinactivation are 2%–10% for the eight P450 isozymes (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5) based on the enzyme turnover rate constants of 0.0067–0.0301 hour^{-1} from SIMCYP's healthy volunteer population data file. The recovery of the enzyme activity after preincubation is minimal and thus unlikely to have any significant impact upon the rate of substrate depletion.

TABLE 2

Summary of intrinsic clearance and f_m of validation compounds

Compounds	Intrinsic Clearance	f_m (Hepatocyte Relay Reaction Phenotyping)	f_m (Literature Data)
	ml/mg/kg		
Erythromycin	9.0	CYP3A 0.84	Mostly CYP3A (Karlsson et al., 2013)
Tizanidine	8.8	CYP1A2 0.83	CYP1A2 0.85 (Granfors et al., 2004)
Timolol	14	CYP2D6 0.74	CYP2D6 (major), CYP2C19 (minor) (Volotinen et al., 2007; Volotinen et al., 2010)
Tolbutamide	7.4	CYP2C9 0.90	CYP2C9 0.8 to 1 (Miller et al., 1990; Srivastava et al., 1991; Veronese et al., 1991; Hall et al., 1994; Komatsu et al., 2000; Wester et al., 2000)
Diazepam	15	CYP3A 0.56 CYP2C19 0.42	CYP3A 0.50 CYP2C19 0.50 (Andersson et al., 1994; Zvyaga et al., 2012),

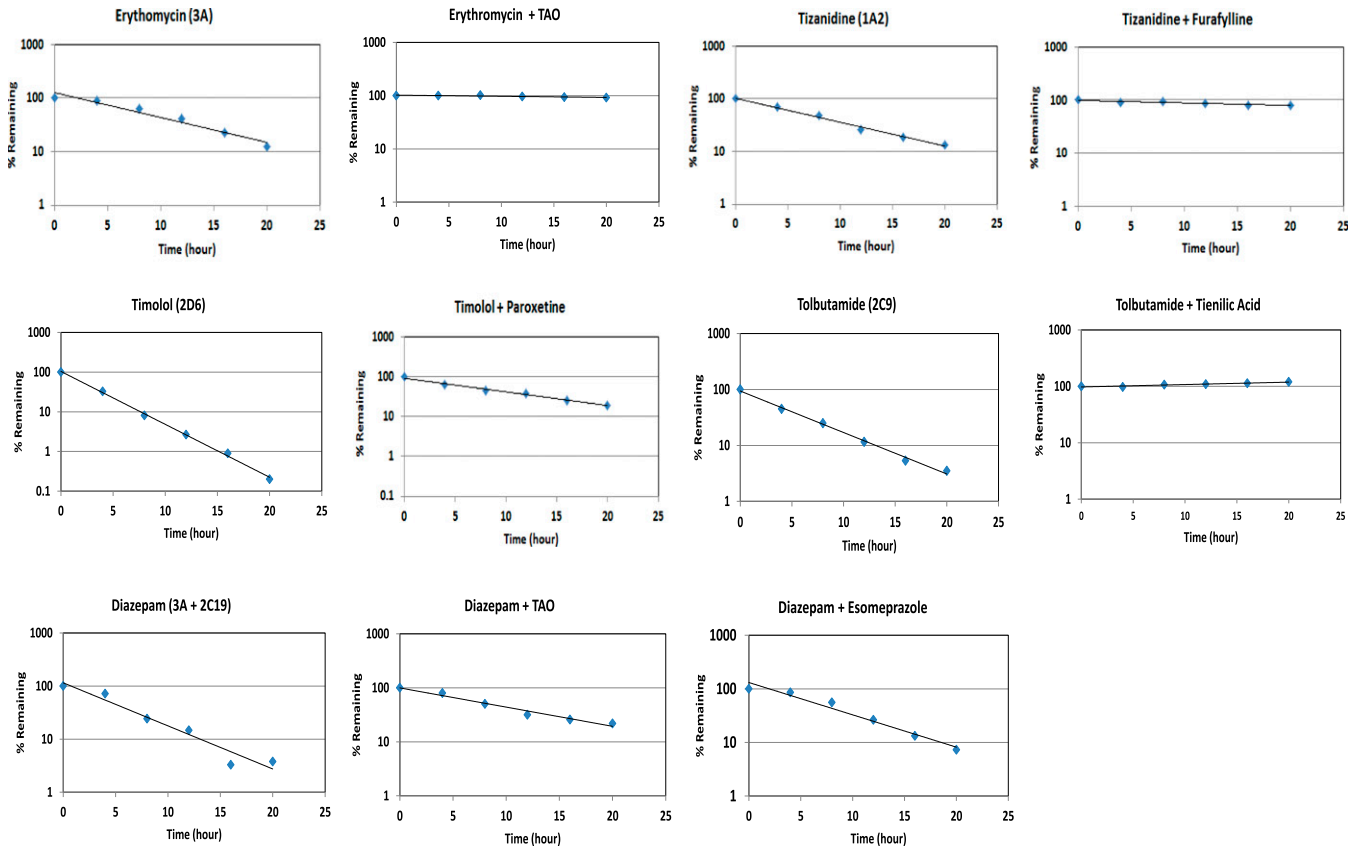

Fig. 3. Inhibition profiles of reaction phenotyping using hepatocyte relay method for low-clearance compounds (0.5 million cells/ml for erythromycin, tizanidine, and diazepam; two million cells/ml for timolol and tolbutamide).

ABT at 1 mM concentration has been shown to be a potent inactivator against most of the P450s, with the exception of CYP2C9. The data are consistent with the results reported previously that 1 mM ABT only partially inhibited CYP2C9 activity (Linder et al., 2009). Therefore, for pan-CYP inhibition, a cocktail of ABT (1 mM) and tienilic acid (15 μM) was used, with 30-minute preincubation to knock out all the P450s, to determine cytochrome contributions versus non-cytochrome pathways (e.g., AO, UGT, SULT, CES, CBR, and AKR). Paroxetine (1.8 μM) was found to be not completely selective for CYP2D6, and it also had significant inhibition of CYP2B6 under the assay conditions. Since substrates of CYP2B6 are not common for most drug-discovery compounds, because of the small binding pocket of this enzyme (Dong et al., 2012), it is not often an issue for most compounds; however, if both enzymes are involved in the clearance pathway, other approaches can be applied, such as hepatocytes from donors genotyped as CYP2D6 or CYP2B6 PM. This does call for identification of more selective CYP2D6 inactivators in the future.

The measured f_m values of the commercial drugs using this novel method are consistent with the data in the literature. Erythromycin is reported to be mostly metabolized by CYP3A (Karlsson et al., 2013), consistent with the high CYP3A f_m value observed in this study (Table 2). Tizanidine is a CYP1A2 substrate with reported inhibition of 85% by furafylline (Granfors et al., 2004), which is in line with our measured value of 83%. It has been reported that CYP2D6 and CYP2C19 are involved in the metabolism of timolol, with CYP2D6 the major contributor (Volotinen et al., 2007, 2010), consistent with the measured f_m value of 0.74. Tolbutamide's f_m by CYP2C9 is estimated to be 0.8–1 using various in vitro and in vivo approaches (Miller et al., 1990; Srivastava et al., 1991; Veronese et al., 1991; Hall et al., 1994; Komatsu et al., 2000; Wester et al., 2000), and CYP2C19 has a minor contribution to tolbutamide clearance (Wester et al., 2000), comparable to our measured CYP2C9 f_m value of 0.9. Diazepam is cleared mostly through hepatic elimination by CYP3A and CYP2C19 in a 1:1 ratio (Andersson et al., 1994; Zvyaga et al., 2012), consistent with our study with a CYP3A f_m of 0.56 and CYP2C19 f_m of 0.42. A number of internal compounds with human ^{14}C-ADME or DDI data have also suggested that data from this new method are consistent with the human in vivo f_m values.

Determination of f_m values for low-clearance compounds has been challenging in drug discovery because, at early stages, metabolite standards are not typically available and clearance pathways are not completely understood. Radiolabeled materials are expensive to make and often are not available for early drug-discovery programs. Even with high enzyme concentrations, many low-clearance compounds do not show any significant turnover of the parent compound, which makes reaction phenotyping exceptionally challenging. This novel hepatocyte relay-reaction phenotyping method enables early determination of f_m for low-clearance compounds based on parent depletion. Similar to the standard hepatocyte assay, nonmetabolic decline should be evaluated to assess the integrity of the assay. Compounds with significant nonmetabolic decline are not suitable for the CL_{int} determination or reaction phenotyping studies. The method can be applied to any drug-discovery compounds without the prerequisite of metabolite standards or radiolabeled material. Early f_m determination of drug candidates is quite useful to help project teams identify potential risks of victim DDI. Drug candidates with narrow therapeutic index and high victim DDI could potentially be terminated because of the high risk associated with variable exposure under a less controlled dosing environment. The hepatocyte relay-reaction phenotyping method provides initial f_m information on drug candidates, which can be

followed up by measuring metabolite formation using enzyme kinetics (i.e., V_{max} and K_m determination) as the metabolite standards become available and clearance pathways well understood. Human ^{14}C-ADME studies are typically conducted at later stages of drug development to provide more definitive reaction phenotyping information and clearance pathways of clinical candidates. The novel hepatocyte relay-reaction phenotyping method, in conjunction with the more definitive methods, provides a holistic approach to f_m determination of low-clearance compounds during the entire drug discovery and development processes.

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Authorship Contributions

Participated in research design: Yang, Atkinson, Di.
Conducted experiments: Yang, Atkinson.
Performed data analysis: Yang, Atkinson, Di.
Wrote or contributed to the writing of the manuscript: Yang, Atkinson, Di.

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