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

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Abbreviations: ABT, 1-aminobenzotriazole; AKR, aldo-keto reductase; AO, aldehyde oxidase; CBR, carbonyl reductase; CES, carboxylesterase; $CL_{int,}$ intrinsic clearance; CYP, cytochrome P450; DDI, drug-drug interaction; $f_{m,}$ fraction metabolized; ISEF, intersystem extrapolation factor; K_m, apparent substrate concentration at half-maximal velocity; MBI, mechanism-based inactivator; OATP, organic anion-transporting polypeptide; P450, cytochrome P450; PK, pharmacokinetics; RAF, relative activity factor; rhCYP, recombinant human cytochrome P450; RT, room temperature; SULT, sulfotransferase; TAO, troleandomycin; TI, therapeutic index; UGT, uridine diphosphate glucuronyltransferase; WEM, Williams E medium.

Abstract

A novel P450 reaction phenotyping method for low clearance compounds has been developed for eight cytochrome P450 enzymes (CYP1A2, 2B6, 2D6, 2C8, 2C9, 2C19, 3A and 3A4) and pan-CYP using the hepatocyte relay approach. Selective mechanism-based inhibitors were used to inactivate the individual P450 enzymes during pre-incubation, and inactivators were removed from the incubation prior to 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 pre-incubation times: furafylline (1 μM, 15 min) for CYP1A2, phencyclidine (20 μM, 15 min) for 2B6, paroxetine (1.8 μM, 15 min) for CYP2D6, gemfibrozil glucuronide (100 μM, 30 min) for 2C8, tienilic acid (15 μM, 30 min) for 2C9, esomeprazole (8 μM, 15 min) for 2C19, troleandomycin (25 μM, 15 min) for 3A4/5, CYP3cide (2 μM, 15 min) for 3A4, and 1-aminobenzotriazole (1 mM, 30 min) supplemented with tienilic acid (15 μM, 30 min) for pan-CYP. 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 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 in order 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 inter-subject PK 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 co-administration 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 mainly metabolized by a single isozyme, it possesses a greater risk in the clinic for DDI if co-administered with an inhibitor or an inducer of the isozyme. This can lead to 'black box' warnings on co-medications, 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 ultra-rapid metabolizers. This can cause a greater risk of adverse effects, toxicity, or lack of efficacy. Therefore, compounds with 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 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 ADME study with radiolabelled material in the later stage of drug development. These data are integrated with *in vitro* reaction phenotyping results to inform clinical DDI study design or PK studies in genotyped subjects. Ultimately, reaction phenotyping data are included in the product label to help inform physicians and pharmacists of co-medications 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 (rhCYPs) with the correction of RAF (relative activity factor) (Venkatakrishnan et al., 2000; Emoto et al., 2006) or ISEF (intersystem extrapolation factor) (Proctor et al., 2004; Chen et al., 2011), and (3) correlation analysis. However, for low clearance compounds, it is challenging to conduct reaction phenotyping studies using these methods due 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 clinic for these "metabolically stable" compounds. This observation was previously considered to be a "mystery" due to 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 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. However, this approach can be challenging and resource-consuming when multiple metabolites are formed, or when metabolic pathways or metabolite structures are not well characterized in early stages of drug discovery (preclinical). There was therefore a need to develop a relatively inexpensive method with quick turn-around time to address the reaction phenotyping challenges of low clearance compounds in fast-paced drug discovery projects. Recently, the hepatocyte relay method has been developed to address low clearance challenges in drug discovery (Di et al., 2012; Di et al., 2013). Here, a novel approach is addressed that applies the hepatocyte relay method to P450 reaction phenotyping using MBI in drug discovery. Similar to any standard hepatocyte assay, non-metabolic decline should first be evaluated. If the standard relay assay shows non-metabolic decline, it will not be possible to run the phenotyping relay for that compound.

Material and Methods

Material

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 (St. Louis, MO) unless specified. 48-well polystyrene plates were purchased from Corning (Corning, NY).

P450 Inhibitor Specificity Study

Cryopreserved human hepatocytes (lot# DCM) consisting of 10 donors, both males and females, were custom-pooled and prepared by BioreclamationIVT (Baltimore, MD). Upon thawing, the hepatocytes were re-suspended in Williams E Medium (WEM GIBCO-BRL, custom formula supplemented with 50 mM HEPES and 26 mM Na_2CO_3). 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 pre-incubated with hepatocytes for either 15 or 30 minutes (enzyme specific incubation time pre-determined in selectivity experiments) to inactivate the enzymes. The inhibitors, concentrations, and pre-incubation times were furafylline (1 µM, 15 min) for CYP1A2, phencyclidine (20 µM, 15 min) for 2B6, paroxetine (1.8 µM, 15 min) for CYP2D6, gemfibrozil glucuronide (100 µM, 30 min) for 2C8, tienilic acid (15 µM, 30 min) for 2C9, esomeprazole (8 µM, 15 min) for 2C19, troleandomycin (25 µM, 15 min) for 3A, CYP3cide (2 µM, 15 min) for 3A4 and 1-aminobenzotriazole (1 mM, 30 min) supplemented with tienilic acid (15 µM,

30 min) for pan-CYP. The final amount of organic solvent was 0.5% in hepatocytes during pre-incubation. After pre-incubation, the media containing the inhibitors were removed from the hepatocytes, and fresh media with CYP 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 CYP 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 RT and the supernatant was transferred for 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 AUC 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 Hepatocyte Relay Method

The details of the 24-well hepatocyte relay method have been described previously (Di et al., 2012; Di et al., 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 non-metabolic decline due to buffer instability or non-specific binding. Only compounds with adequate intrinsic clearance values and without non-metabolic 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 labs showed non-metabolic decline, and much of that was due to buffer instability). At the beginning of each relay, 2 µL inhibitors are added to 200 µL hepatocytes (final cell density of 0.5 or 2 million cells/mL, 0.5% final organic solvent) at the appropriate concentrations and pre-incubation times as described above 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 pre-incubation so that the cells settle to the bottom of the plate to more easily enable removal of the inhibitors after the pre-incubation. Following pre-incubation, 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 hour, 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, RT) and 10 µL supernatant is removed to determine the concentration of the supernatant. All the collected samples are quenched with 100 µL cold acetonitrile containing internal standards and centrifuged at 3000 rpm for 10 minutes at RT and 50 µL of supernatant is transferred to a clean plate for LC-MS/MS analysis. 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 2nd relay experiment, freshly thawed hepatocytes are inactivated with MBIs as described above in the 1st relay. The media containing inhibitors are removed after pre-incubation and the remaining hepatocytes are reconstituted with WEM buffer and re-suspended. In the meantime, the plates containing the supernatant from the 1st relay are pre-warmed at 37°C for 30 min. The hepatocytes with specific isozymes inactivated are added to pre-warmed 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 above. Five relays are performed using MBI-inactivated hepatocytes to give a total accumulative incubation time of 20 hr. Controls without inhibitors (solvent control) are run in parallel on the same plate. Percent inhibition is calculated based on parent depletion.

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LC-MS/MS Conditions

The LC mobile phases were: (A) HPLC 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, 30x3 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 HPLC AD30 pumps (Columbia., MD) connected to an AB Sciex (Foster City, California) 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; Di et al., 2013). For inhibitor specificity studies, % inhibition was calculated based on the AUC 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, % inhibition was calculated based on intrinsic clearance of parent depletion using Eq. (2), since metabolite standards of test compounds

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are usually not available at early stages of drug discovery. Fraction metabolized (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 % inhibition from all isozymes. A parallel line test was used to test if the slopes of the Ln % remaining vs. time are not equal (Prism 6 for Windows, Version 6.03, GraphPad Software, Inc., La Jolla, CA). When a P-value is > 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.

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

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

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

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-CYP using pooled cryopreserved human hepatocytes with multiple donors. The inactivator concentration, pre-incubation time, substrates, and metabolites are summarized in Table 1. The scheme of the novel hepatocyte relay reaction phenotyping method is shown the Figure 1. The inactivators were removed from the hepatocytes after pre-incubation (15 or 30 min) to improve the specificity of the inhibitors (shown in Figure 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. ABT (1 mM) showed potent inhibition of all P450 isozymes with the exception of CYP2C9 (Figure 2). Only 23% inhibition of CYP2C9 was observed after 30 minute pre-incubation with 1 mM ABT. Therefore, a cocktail of 1 mM ABT and 15 μM tienilic acid was used as pan-CYP 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 % remaining-time courses of the compounds with and without inactivation are illustrated in Figure 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 pre-incubation is addressed. This novel approach provides a new way to measure f_m for compounds with low intrinsic clearance that would otherwise be difficult to obtain in drug discovery. The unique experimental design, without the co-existence of the inactivators and the substrates during incubation, enhances the specificity of the inhibitors compared to the traditional approach of co-incubation, as this eliminates any potential reversible inhibition of phase I/II 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 utilized for reaction phenotyping in drug discovery, they offer several advantages as they are more physiologically relevant systems and contain the full complement of hepatic drug metabolizing enzymes of both Phase I and Phase II, transporters (Soars et al., 2007), and a cell membrane barrier. This allows 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, such as UGT, SULT, CES, AO, CBR and AKR, which is essential to more accurately assign f_m. The success of a reaction phenotyping assay using hepatocytes is highly dependent upon the specificity of the chemical inhibitors. Non-specific 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 in order to generate reliable high quality data. Specificity of P450 inactivators has been evaluated under co-incubation conditions of inhibitors and substrates using hepatocytes (Kazmi et al., 2014). Our studies showed that by removing inhibitors from the media after pre-incubation, the inactivator specificity improved significantly by minimizing reversible inhibition of various Phase I/II enzymes and transporters [e.g., CYPs, AO, UGT, CES and OATPs. Data are not shown.]. For example, troleandomycin (25 μM) showed some inhibition of CYP2C19 when it is co-incubated 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 OATPs), 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 four hours post-inactivation 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 h^{-1} from SIMCYP's® healthy volunteer population data file. The recovery of the enzyme activity after pre-incubation is minimal, and thus unlikely to have any significant impact upon the rate of substrate depletion.

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 pre-incubation to knock out all the P450s, to determine CYP contributions vs. non-CYP pathways (e.g., AO, UGT, SULT, CES, CBR and AKR). Paroxetine (1.8 μM) was found to not be completely selective for CYP2D6, and it also had significant inhibition of CYP2B6 under the assay conditions. Since substrates of CYP2B6 are not very common for most drug discovery compounds, due to the very 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 literature data. 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 being the major contributor (Volotinen et al., 2007; Volotinen et al., 2010),

consistent with the measured f_m value of 0.74. Tolbutamide f_m by CYP2C9 is estimated to be 0.8 to 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 minor contribution to tolbutamide clearance (Wester et al., 2000). This is comparable with our measured CYP2C9 f_m value of 0.9. Diazepam is mostly cleared through hepatic elimination by CYP3A and CYP2C19 in a one to one ratio (Andersson et al., 1994; Zvyaga et al., 2012), consistent with our study with 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 also suggest 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. Radio-labeled materials are expensive to make and are often 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, non-metabolic decline should be evaluated to assess the integrity of the assay. Compounds with significant non-metabolic 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 very useful to help project teams identify potential risks of victim DDI. Drug candidates with narrow therapeutic index (TI) and high victim DDI could potentially be terminated due to high risk associated with variable exposure under a less controlled dosing environment. The hepatocyte relay reaction phenotyping method provides initial f_m information of drug candidates. This 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

References

Andersson T, Miners JO, Veronese ME, and Birkett DJ (1994) Diazepam metabolism by human liver microsomes is mediated by both S-mephenytoin hydroxylase and CYP3A isoforms. *British Journal of Clinical Pharmacology* **38:**131-137.

Chen Y, Liu L, Nguyen K, and Fretland AJ (2011) Utility of intersystem extrapolation factors in early reaction phenotyping and the quantitative extrapolation of human liver microsomal intrinsic clearance using recombinant cytochromes P450. *Drug Metab Dispos* **39:**373-382.

Di L, Atkinson K, Orozco CC, Funk C, Zhang H, McDonald TS, Tan B, Lin J, Chang C, and Obach RS (2013) In vitro-in vivo correlation for low-clearance compounds using hepatocyte relay method. *Drug Metab Dispos* **41:**2018-2023.

Di L and Obach RS (2015) Addressing the Challenges of Low Clearance in Drug Research. *AAPS Journal* **17:**352-357.

Di L, Trapa P, Obach RS, Atkinson K, Bi Y-A, Wolford AC, Tan B, McDonald TS, Lai Y, and Tremaine LM (2012) A novel relay method for determining low-clearance values. *Drug Metab Dispos* **40:**1860-1865.

Dong D, Wu B, Chow D, and Hu M (2012) Substrate selectivity of drug-metabolizing cytochrome P450s predicted from crystal structures and in silico modeling. *Drug Metab Rev* **44:**192-208.

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.

Granfors MT, Backman JT, Laitila J, and Neuvonen PJ (2004) Tizanidine is mainly metabolized by cytochrome P450 1A2 in vitro. *British Journal of Clinical Pharmacology* **57:**349-353.

Hall SD, Hamman MA, Rettie AE, Wienkers LC, Trager WF, Vandenbranden M, and Wrighton S (1994) Relationships between the levels of cytochrome P4502C9 and its prototypic catalytic activities in human liver microsomes. *Drug Metab Dispos* **22:**975-978.

Karlsson FH, Bouchene S, Hilgendorf C, Dolgos H, and Peters SA (2013) Utility of in vitro systems and preclinical data for the prediction of human intestinal first-pass metabolism during drug discovery and preclinical development. *Drug Metab Dispos* **41:**2033-2046.

Kazmi F, Yerino P, Ogilvie BW, Usuki E, Chladek J, and Buckley DB (2014) Assessment under initial rate conditions of the selectivity and time course of cytochrome P450 inactivation in pooled human liver microsomes and hepatocytes: Optimization of inhibitor conditions used for reaction phenotyping studies, in: *19th North American ISSX Meeting*, San Francisco, CA.

Komatsu K, Ito K, Nakajima Y, Kanamitsu S-I, Imaoka S, Funae Y, Green CE, Tyson CA, Shimada N, and Sugiyama Y (2000) Prediction of in vivo drug-drug interactions between tolbutamide and various sulfonamides in humans based on in vitro experiments. *Drug Metab Dispos* **28:**475-481.

Lazarou J, Pomeranz BH, and Corey PN (1998) Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA : the journal of the American Medical Association* **279:**1200-1205.

Linder CD, Renaud NA, and Hutzler JM (2009) Is 1-aminobenzotriazole an appropriate in vitro tool as a nonspecific cytochrome P450 inactivator? *Drug Metab Dispos* **37:**10-13.

Lu AYH, Wang RW, and Lin JH (2003) Cytochrome P450 in vitro reaction phenotyping: A re-evaluation of approaches used for P450 isoform identification. *Drug Metab Dispos* **31:**345-350.

Mei Q, Tang C, Assang C, Lin Y, Slaughter D, Rodrigues AD, Baillie TA, Rushmore TH, and Shou M (1999) Role of a potent inhibitory monoclonal antibody to cytochrome P-450 3A4 in assessment of human drug metabolism. *J Pharmacol Exp Ther* **291:**749-759.

Miller AK, Adir J, and Vestal RE (1990) Excretion of tolbutamide metabolites in young and old subjects. *European Journal of Clinical Pharmacology* **38:**523-524.

Parkinson A (1996) An overview of current cytochrome P450 technology for assessing the safety and efficacy of new materials. *Toxicologic pathology* **24:**48-57.

Proctor NJ, Tucker GT, and Rostami-Hodjegan A (2004) Predicting drug clearance from recombinantly expressed CYPs: intersystem extrapolation factors. *Xenobiotica* **34:**151-178.

Rodrigues AD (1999) Integrated cytochrome P450 reaction phenotyping: attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochemical Pharmacology* **57:**465-480.

Soars MG, Grime K, Sproston JL, Webborn PJH, and Riley RJ (2007) Use of hepatocytes to assess the contribution of hepatic uptake to clearance in vivo. *Drug Metab Dispos* **35:**859-865.

Srivastava PK, Yun CH, Beaune PH, Ged C, and Guengerich FP (1991) Separation of human liver microsomal tolbutamide hydroxylase and (S)-mephenytoin 4'-hydroxylase cytochrome P-450 enzymes. *Molecular Pharmacology* **40:**69-79.

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.

Veronese ME, Mackenzie PI, Doecke CJ, McManus ME, Miners JO, and Birkett DJ (1991) Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P4502C9. *Biochemical and Biophysical Research Communications* **175:**1112-1118.

Volotinen M, Korjamo T, Tolonen A, Turpeinen M, Pelkonen O, Hakkola J, and Maenpaa J (2010) Effects of selective serotonin reuptake inhibitors on timolol metabolism in human liver microsomes and cryo-preserved hepatocytes. *Basic & Clinical Pharmacology & Toxicology* **106:**302-309.

Volotinen M, Turpeinen M, Tolonen A, Uusitalo J, Maenpaa J, and Pelkonen O (2007) Timolol metabolism in human liver microsomes is mediated principally by CYP2D6. *Drug Metab Dispos* **35:**1135-1141.

Wester MR, Lasker JM, Johnson EF, and Raucy JL (2000) CYP2C19 participates in tolbutamide hydroxylation by human liver microsomes. *Drug Metab Dispos* **28:**354-359.

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

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR, and Ball SE (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: A pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. *Drug Metab Dispos* **32:**1201-1208.

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.

Zvyaga T, Chang S-Y, Chen C, Yang Z, Vuppugalla R, Hurley J, Thorndike D, Wagner A, Chimalakonda A, and Rodrigues AD (2012) Evaluation of six proton pump inhibitors as inhibitors of various human cytochromes P450: focus on cytochrome P450 2C19. *Drug Metab Dispos* **40:**1698-1711.

Figure Legends

Figure 1. Reaction Phenotyping Using Hepatocyte Relay Method

Figure 2. P450 Inactivator Specificity in Human Hepatocytes

Figure 3. Inhibition Profiles of Reaction Phenotyping Using Hepatocyte Relay Method for Low Clearance Compounds (0.5 million cells/mL for erythromycin, tizanidine and diazepam; 2 million cells/mL for timolol and tolbutamide)

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Table 1. Summary of Pre-Incubation Time, P450 Inhibitors, Substrates and Metabolites for Specificity Study

P450 Isozymes	Pre-Incubation Time (min)	Inactivators and Concentrations	Substrates	Metabolites	Mass Transitions of Metabolites
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 (TAO)	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 Above	All Metabolites Above	NA

Table 2. Summary of Intrinsic Clearance and f_m of Validation Compounds

Compounds	**Intrinsic Clearance (CL_{int}, mL/min/kg)**	**f_m (Hepatocyte Relay Reaction Phenotyping)**	**f_m (Literature Data)**
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),

Figure 1

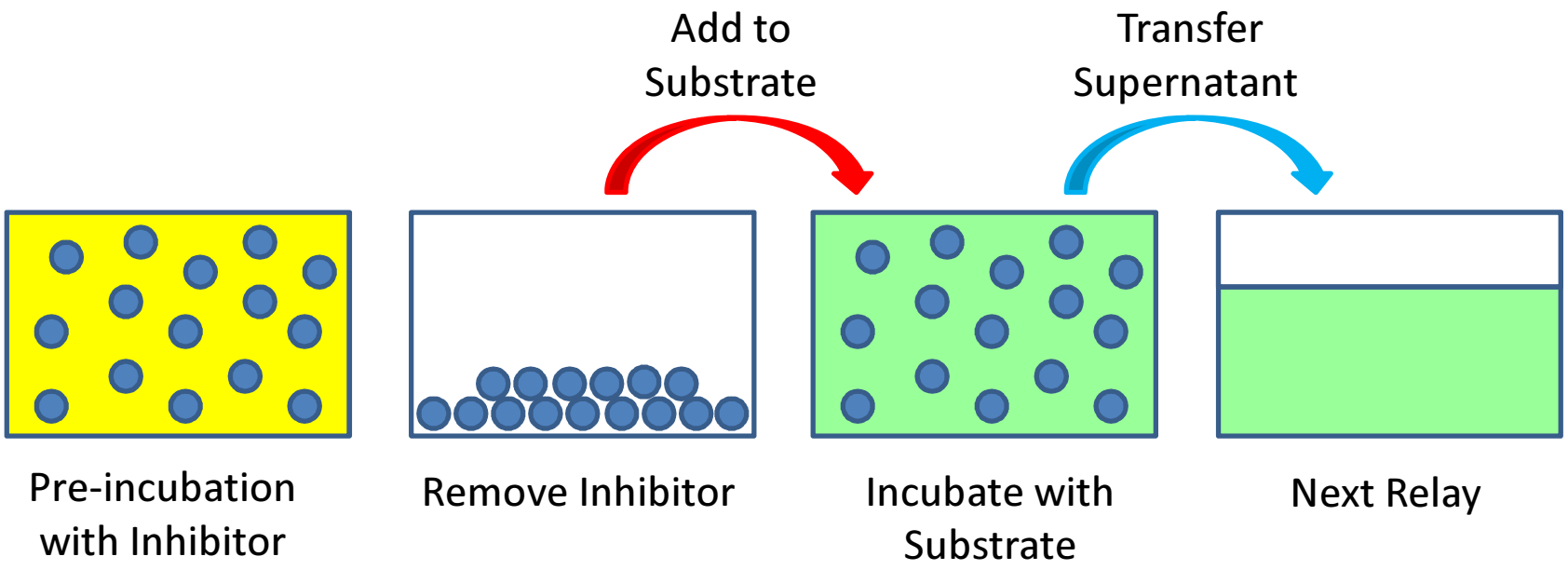

Figure 2

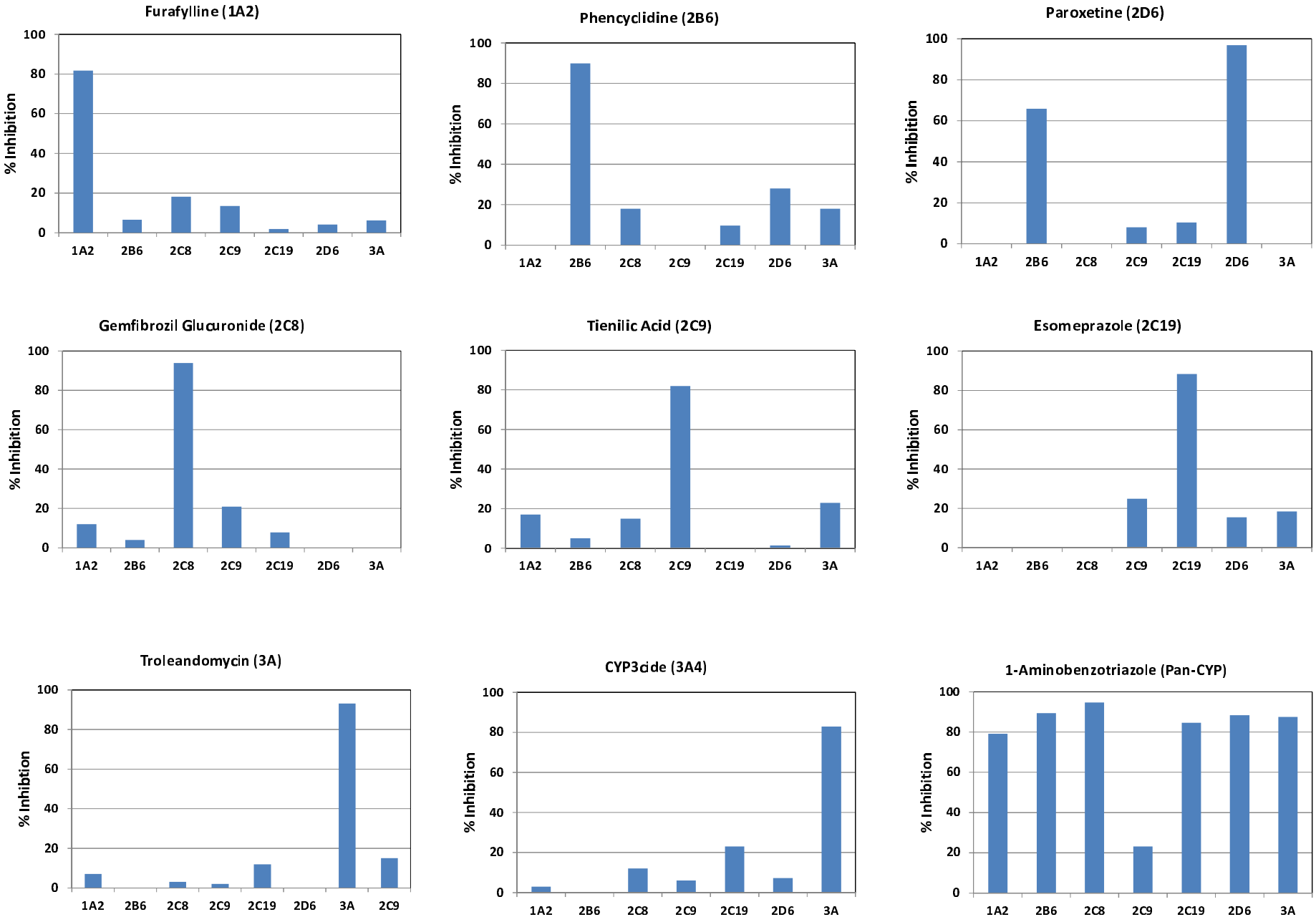

Figure 3