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Reaction Phenotyping of Low-Turnover Compounds in Long-term Hepatocyte Cultures Through Persistent Selective Inhibition of Cytochromes P450

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Abbreviations: ABT, 1- aminobenzotriazole; AZM, azamulin; BNZ, (+)-N-3-benzylnirvanol; CL, clearance; CL_{int} intrinsic clearance; CYP, cytochrome P450; CRP, CYP reaction phenotyping; DDI, drug-drug interactions; ESI, electrospray ionization; f_m, fraction of metabolism; FUR, furafylline; HLM, human liver microsomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MPCC, micropatterned co-cultures; NCE, new chemical entity; PXT, paroxetine; TA, tienilic acid.

Abstract

Recognizing the challenges of determining the relative contribution of different drug metabolizing enzymes to the metabolism of slowly metabolized compounds, a cytochrome P450 reaction phenotyping (CRP) method, using co-cultured human hepatocytes (HEPATOPAC®), has been established. In this study, the emphasis on the relative contribution of different cytochrome P450 (CYP) isoforms was assessed by persistently inhibiting CYP isoforms over 7 days with human HEPATOPAC®. CYP isoform selective inhibition was achieved with the chemical inhibitors furafylline (CYP1A2), tienilic acid (CYP2C9), (+)-N-3-benzylnirvanol (CYP2C19), paroxetine (CYP2D6), azamulin (CYP3A), and a combination of 1-aminobenzotriazole and tienilic acid (broad spectrum inhibition of CYPs). We executed this CRP method using HEPATOPAC by optimizing for the choice of CYP inhibitors, their selectivity, and the temporal effect of inhibitor concentrations on maintaining selectivity of inhibition. In general, the established CRP method using potent and selective chemical inhibitors allows to measure the relative contribution of CYPs and to calculate the fraction of metabolism (f_m) of low turnover compounds. Several low turnover compounds were used to validate this CRP method by determining their hepatic CLint and f_m, with comparison to literature values. We established the foundation of a robust CRP for low turnover compound test system which can be expanded to include inhibition of other drug metabolizing enzymes. This generic CRP assay, using human long-term hepatocyte cultures, will be an essential tool in drug development for new chemical entities in the quantitative assessment of the risk as a victim of drug-drug interactions.

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Significance Statement

An ongoing trend is to develop drug candidates which have limited metabolic clearance. The current studies report a generic approach to conducting reaction phenotyping studies with human HEPATOPAC, focusing on CYP metabolism of low turnover compounds. Potent and selective chemical inhibitors were used to assess the relative contribution of the major human CYPs. Validation was achieved by confirming hepatic CL_{int} and f_m for previously reported low turnover compounds. This approach is adaptable for assessment of all drug metabolizing enzymes.

Introduction

When determining the potential for drug-drug interactions (DDI) of a new chemical entity (NCE), it is important to elucidate both the perpetrator profile of drugs on transporters and metabolizing enzymes, i.e. drugs as inhibitors, inactivators and/or inducers of these proteins and also understand the mechanisms by which drugs are eliminated in humans (victim profile). The potential victim DDI risk in the clinic is predicted from in vitro assays early in drug discovery by identifying the routes of metabolism and transporter-mediated disposition of NCEs (Zientek and Youdim, 2014; Di, 2017). There is a focus on understanding the cytochrome P450 (CYP)-mediated drug interactions as many drugs are metabolized by CYPs in the liver (Zientek and Youdim, 2014; Cerny, 2016; Di, 2017; Ogilvie et al., 2019). Phenotyping is a term which was adopted to define the enzymes responsible for metabolism of a compound (e.g. Fujino et al., 1982). CYP reaction phenotyping (CRP) aims to determine the relative quantitative contribution of CYP

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isoforms to the metabolism (f_m) of an NCE in vitro. There have been fundamentally four approaches for CRP, namely metabolism by human recombinant CYPs (rCYPs), inhibition of metabolism with human liver microsomes (HLM) by isoform selective chemical inhibitors, inhibition by isoform selective inhibitory antibodies, and correlation analysis. Recommendations by PhRMA (Bjornsson et al., 2004) and regulators, for example US Food and Drug Administration (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions) proposed that two independent studies should be sufficient which are typically metabolism by rCYP and inhibition of metabolism using isoform-selective chemical inhibitors.

With drug discovery scientists striving to generate drug candidates that are suitable for once daily or less frequent dosing to increase patient adherence and ensure pharmacological coverage (Bleeker et al., 2012; Smith et al., 2018), these NCEs often exhibit low metabolic turnover in liver preparations. Consequently, evaluation of those compounds with the traditional CRP methods is challenging since HLM, recombinant enzymes, and human hepatocyte suspensions gradually lose activity of drug-metabolizing enzymes beyond a standard incubation period (Elaut et al., 2006, Stringer et al., 2008). The relay method (Di et al., 2014) and incubation with co-cultured hepatocytes (Chan et al., 2013; Lin et al., 2017) have been used to assess hepatic clearance for low turnover compounds.

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Long term viable cultures of hepatocytes have proved to be valuable in maintenance of enzyme activity for extended periods of time (Khetani and Bhatia, 2008; Chan et al., 2013, Bonn et al., 2016). Furthermore, accurate in vitro to in vivo extrapolation (IVIVE) using co-cultured human hepatocytes has been observed for low turnover compounds (Chan et al., 2013; Lin et al., 2017) which should provide an additional layer of confidence in applying these systems to assess f_m.

Our goal was to develop a generic system to conduct CRP studies for low turnover compounds with confidence. For proof-of-principle we focused on phenotyping of CYP mediated reactions since these are the enzymes for which most of the selective inhibitors and substrates have been identified. Initial discussions concentrated on identifying highly efficient CYP selective inactivators as the extent of inhibition would then be defined primarily by the turnover of the CYP protein. However, k_{deg} values have not been reported for all CYPs and reported values can have broad ranges (Yang et al., 2008). As such we elected to merely identify potent inhibitors and apply a daily routine for inhibition/inactivation. We also recognize that while inactivation is a potential mechanism for oxidative enzymes this mechanism cannot be applied to many other drug metabolizing enzymes (DME).

While chemical inhibitors in HEPATOPAC have been used to ablate specific CYP isoforms (Lin et al., 2017), characterization of selectivity toward different CYP isoforms, as required for CRP analysis, has not been demonstrated. These long-term cultures need to ensure complete inhibition under conditions where the inhibitor may be

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metabolized and where enzymes are regenerated as part of a normal cell function. Simply applying supersaturated concentrations of inhibitors is not a viable option as most inhibitors are only selective within a specific range of concentrations (Ogilive et al., 2019). An additional challenge with these studies is the potential for compounds to induce DME during the extended incubation period.

This report describes the development of a specific CRP method using chemical inhibitors with human HEPATOPAC. Potent and selective chemical inhibitors were identified to assess the relative contribution of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A, i.e. to calculate the fraction of metabolism (f_m) of low turnover compounds. The established CRP method was validated with low turnover compounds by determining their hepatic CL_{int} and f_m, with comparison to literature values. In addition, the report will discuss the selection of CYP inhibitors and unique challenges that needed to be overcome through the investigation.

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Materials and Methods

Chemicals

Furafylline (FUR), phenacetin, acetaminophen, tizanidine, tienilic acid (TA), diclofenac, 4'-hydroxydiclofenac, tolbutamide, (+)-*N*-3-benzylnirvanol (BNZ), *S*-mephenytoin, 4-hydroxymephenytoin, voriconazole, paroxetine (PXT), dextromethorphan, dextrorphan, risperidone, azamulin (AZM), midazolam, 1'-hydroxymidazolam, disopyramide, 1-aminobenzotriazole (ABT), labetalol, and imipramine were purchased from Sigma-Aldrich (St. Louis, MO), Toronto Research Chemicals (North York, ON, Canada), or Cayman Chemical Company (Ann Arbor, MI). HEPATOPAC® maintenance medium and metabolic stability application medium were obtained from BioIVT (Medford, MA). Other reagents were of analytical grade or higher.

HEPATOPAC

HEPATOPAC Micro-patterned co-cultures (MPCC) plates were purchased from BioIVT (Westbury, NY) and were prepared from pooled cryopreserved primary human hepatocyte lots (AMH, ACR and KCB; 10-subject, mixed gender pool for each lot) or from a single-donor lot (VKB; male). Human hepatocytes were seeded with a density of approximately 20,000 primary hepatocytes per well in a 24-well plate format, with a cell density ratio of 3:1 for hepatocytes and 3T3 murine fibroblasts as specified by the vendor. Fibroblast controls were included for intrinsic clearance experiments. Upon arrival of the human HEPATOPAC plates, shipping media was replaced with human HEPATOPAC Maintenance Media. Plates were kept in an incubator equilibrated at 37°C under a 10% CO_2 atmosphere and 95% relative humidity for 48 hours prior to an

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experiment. HEPATOPAC plates were changed to serum-free media 2 hours prior to initiating CRP studies at 37° C with 5% CO_2 atmosphere and 95% relative humidity.

Application of chemical inhibitors

The CYP-selective chemical inhibitors and their incubation concentrations used were FUR at 1 µM (CYP1A2), TA at 0.015 µM (CYP2C9), BNZ at 0.5 µM (CYP2C19), PXT at 1.8µM (CYP2D6), AZM at 1 µM (CYP3A), and ABT at 1 mM supplemented with TA at 15 µM (broad spectrum inhibitor of CYPs), with conditions optimized in prior experiments. All inhibitors were prepared in 95% ethanol/5% DMSO with the exception of ABT, which was dissolved with sterile water. Each HEPATOPAC well was preincubated with an inhibitor(s) for 24 hours prior to the initiation of the experiment. The final amount of organic solvent was ≤0.1% in HEPATOPAC wells during preincubation. After the preincubation period, the media containing inhibitor(s) was removed and HEPATOPAC wells were incubated with media containing chemical inhibitor(s) supplemented with a low-turnover probe substrate. An additional aliquot of the chemical inhibitor(s) was added to the incubation daily. One exception to this daily aliquot was CYP2D6 inhibition with PXT. The addition of PXT was optimized and required to be added as a pre-dose and on days 4 and 7, only.

The concentration of the chemical inhibitors was measured for the 7- day incubation period based on daily aliquots applied. The measured concentration increased by 2 to 3-fold for the duration of 7 days. The final amount of organic solvent accumulated between 0.5 to 1% throughout the 7-day incubations for all inhibition conditions, based on daily aliquots of chemical inhibitor(s), and no solvent effects were observed upon microscopic evaluation of cell morphology.

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CYP enzyme activity and chemical inhibitor selectivity study

Enzyme activities were used to evaluate CYP chemical inhibitor selectivity, conducted by measuring oxidative metabolite formation of CYP-selective substrates. After a preincubation period with the chemical inhibitor(s), the media was replaced with fresh media containing inhibitor at pre-treatment concentration levels and a cocktail of CYP-selective substrates. The substrates used were phenacetin for CYP1A2 at 100 µM final incubation concentration, diclofenac for CYP2C9 at 25 µM, and midazolam for CYP3A at 15 µM. In a separate cocktail S-mephenytoin for CYP2C19 at 150 µM and dextromethorphan for CYP2D6 at 25 µM were combined. The plates were incubated at 37°C with 5% CO_2 atmosphere and 95% relative humidity for 100 minutes (optimized for 24-well HEPATOPAC plates). After 100 minutes, all the collected samples were quenched with one volume of acetonitrile containing 200 nM of internal standards (labetalol, diclofenac, and imipramine) and stored at -70°C until all samples were collected. After all samples were collected, the quench plate was centrifuged at 2900 x *g* for 10 minutes, 100 µL supernatant was removed and transferred to clean injection plates (as shown in experimental scheme Figure 1). The formation of the corresponding primary oxidative metabolites (acetaminophen, 4'-hydroxydiclofenac, 4-hydroxymephenytoin, dextrorphan, 1'-hydroxymidazolam) was measured by LC-MS/MS analysis to examine the enzyme activity and inhibitor specificity for each isoform on each of the 7 days. Representative CYP activity was calculated and denoted as an average ± standard error from n=4 to 8 experiments, each obtained from triplicates determinations. Percent activity was calculated for inhibitor specificity for each isoform. The percent activity remaining of the CYPs as a function of time was calculated from

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triplicate determinations for each CYP and was plotted as mean ± standard deviation HEPATOPAC incubations without chemical inhibitors were conducted in the same manner as described above with the exclusion of chemical inhibitor(s).

Determining the intrinsic clearance of a test compound

After a 24-hour pre-treatment with specific chemical inhibitor(s), incubation reactions were started by adding 0.5 mL HEPATOPAC metabolic stability medium containing the test compound at a final concentration of 0.3 µM and CYP-selective chemical inhibitor(s) at concentrations specified in the previous section, to each well of the HEPATOPAC plate. CYP-selective inhibitors were aliquoted into each well on a daily basis (except for CYP 2D6 inhibition, see previous section for details). The incubations were terminated at 0, 24, 48, 72, 96, 120, 144 or 168 h. Sample work-up and preparation of the LC-MS/MS sample were conducted in the same manner as described in the previous section. HEPATOPAC incubations without chemical inhibitors were conducted in the same manner as described above with the exclusion of chemical inhibitor(s). An additional plate containing stromal cells (mouse embryonic 3T3 fibroblasts) was incubated with the test compound and served as a control. All assays and measurements were performed in triplicate determinations. Parent disappearance versus time profiles of low turnover substrates were generated for untreated and treated with chemical inhibitor or incubated with stromal cells.

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LC-MS/MS analysis

Samples were analyzed by LC-MS/MS using a Waters Acquity UPLC (Milford, MA) coupled to an AB Sciex 4500 triple quadrupole mass spectrometer (Framingham, MA). Chromatographic separation was obtained using a Waters Xbridge Shield RP18 column (2.1 × 50 mm, 3.5 µm). Solvent A consisted of 0.1% formic acid in water, and solvent B of acetonitrile containing 0.1% formic acid, and were delivered at a constant flow rate of 0.5 mL/min. The solvent gradient initiated at 5% B for 0.5 min and then increased linearly to 80% B over 4 min. The gradient was further increased to 90% B in 0.1 min, held for 0.9 min, and then returned to 5% B in 0.1 min. The column was re-equilibrated at initial conditions for 0.9 min before injection of the next sample. Mass spectrometric analysis was performed with electrospray ionization (ESI) in the positive mode. For quantification, selected reaction monitoring experiments were performed to detect ion pairs at *m/z* 312/231 (4'-hydroxydiclofenac), 235/150 (4-hydroxymephenytoin), 258/157 (dextrorphan), 342/203 (1'-hydroxymidazolam). Internal Standards ion pairs at *m/z* 329/162 (labetalol), or 281/193 (imipramine) were used to monitor the instrument performance. The internal standard closest to the retention time of the analyte of interest was used. Internal standards labetalol and imipramine are interchangeable since both are close to analytes retention time (Supplemental data). Acetaminophen chromatography separation was obtained using a Phenomenex Synergi C18 column (2.1 x 50mm, 3.5 µm). Solvent A and B were identical to the method mentioned in the beginning of this section and delivered at a constant flow rate of 0.5 mL/min. The solvent gradient initiated at 2% B for 0.4 min and then increased linearly to 90% in 0.5 min, held for 0.6 min and then returned to 2% in 0.7 min. The column was re-

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equilibrated at initial conditions for 0.93 min before injection of the next sample. Mass spectrometric analysis was performed with electrospray ionization (ESI) in the positive mode. For quantification, selected reaction monitoring experiments were performed to detect ion pairs at *m/z* 152/110 (acetaminophen).

Modifications in chromatography conditions were made for several test compounds as needed. Chromatographic separation was achieved using Acquity UPLC BEH Shield RP18 column (2.1 x 50 mm, 1.7µm). Solvent A consisted of 0.1% formic acid in water, and solvent B of acetonitrile containing 0.1% formic acid, and was delivered at a constant flow rate of 0.75 mL/min. The solvent gradient initiated at 5% B for 0.2 min and then increased linearly to 95% in 1.8 min, held for 0.7 min and then returned to 5% in 0.7 min. The column was re-equilibrated at initial conditions for 0.6 min before injection of the next sample. Mass spectrometric analysis was carried out with electrospray ionization (ESI) in the positive mode. For quantification, selected reaction monitoring experiments were performed to detect ion pairs at *m/z* 271/91 (tolbutamide), 340/194 (disopyramide), 350/281 (voriconazole), 411/191 (risperidone), and 254/44 (tizanidine).

Data analysis and clearance calculations

For low turnover compounds, data showing time-dependent reduction in substrate concentration, as indicated by the peak area ratio, were fitted to the mono-exponential decay model (eq.1) using GraphPad Prism 8 (San Diego, CA):

$C_t = C_0 \times e^{-kt}$ Equation 1

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Where *k* represents the elimination rate constant determined based on the substrate concentration remaining at t=0 (C_o; 100%) and at time *t* (C_t).

Intrinsic clearance (CL_{int}) values were calculated as seen in equation 2:

$$CL_{int} = \frac{k \times SF \times HLW}{P \times fu_{hep}} \qquad \text{Equation 2}$$

Where SF is the hepatocellularity of 99 million cells/g liver (Barter et al., 2007), HLW is the human liver weight of 25 g liver/kg body weight (Howgate et al., 2006), P is the concentration of hepatocytes in the incubation mixture, and fu_{hep} is the fraction unbound in hepatocyte incubation calculated based on Log *D* values (Kilford et al., 2008). Log *D* values were predicted using ACD/Labs software (Advanced Chemistry Development, Toronto, ON, Canada).

The calculation $f_{m:}$ was obtained as described (eq. 3):

$$f_m = \frac{CL_{int,\ no\ inhibitor} - CL_{int,\ with\ inhibitor}}{CL_{int,\ no\ inhibitor} - CL_{int,\ stromal}} \qquad \text{Equation 3}$$

Where $CL_{int,\ no\ inhibitor}$ represents the intrinsic clearance value obtained in the absence of inhibitor, $CL_{int,\ with\ inhibitor}$ represents the intrinsic clearance value obtained in the presence of the inhibited data set, and $CL_{int,\ stromal}$ is the intrinsic clearance value obtained from stromal cells, with the assumption that the stromal cells behave in the same manner with or without inhibitor.

Results

Chemical inhibition studies with CYP inhibitors FUR, TA, BNZ, PXT, AZM, and ABT/TA, were conducted to assess their selectivity against five CYP enzymes (CYP1A2, 2C9, 2C19, 2D6, and 3A) and broad spectrum inhibition of CYPs using 10-donor human

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hepatocyte pool with HEPATOPAC. The scheme for the chemical inhibition method for low turnover compounds is depicted in Figure 1. Initial studies were conducted to demonstrate that CYP activity was maintained for 7 days using CYP probe substrates incubated with Human HEPATOPAC (Figure 2, Table 1). We selected five CYP substrates because each has long been recognized as CYP member-specific reactions (Table 1). CYP activities for CYP2C9, 2C19, 2D6 and 3A maintained approximately ≥80% activity throughout the 7 days of culture. CYP1A2 activities in the absence of inhibitor decreased with time and will require further investigation to ensure that there enzyme activity is maintained over 7 days and complete inhibition observed with time. The potency and selectivity of CYP chemical inhibitors are displayed in Figure 3. The data shown that the five CYP chemical inhibitors: FUR, TA, BNZ, PRX and AZM had good potency and selectivity for their respective CYP-isoform. In the current study, FUR (1 µM) exhibited potent inhibitory effect toward the targeted isoform CYP1A2 (~70% inhibition) and good selectivity (<20% inhibition of other CYP activities) within the first day of HEPATOPAC incubation, similar to the results obtained from HLM and hepatocyte suspensions (Newton et al., 1995; Yang et al., 2016). Evaluation of native CYP1A2 activity revealed a time-dependent loss of enzyme activity (Figure 3) that mirrored the reduction in inhibitory potency of FUR. TA reduced ~80% of CYP2C9 activity with good selectivity over the 7-day incubation period (Figure 3). BNZ reduced ~80% of CYP2C19 activity with good selectivity over the 7-day incubation period (Suzuki et al., 2002) (Figure 3). An increase of CYP3A activity up to 2-fold by day 7 was detected following a daily administration of BNZ. PXT exhibited an average of ~80% inhibition of CYP2D6 activity over 7 days when the compound was applied three

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times over the course of the experiment at pre-treatment, Day 1, and Day 4. While the selectivity profile toward CYPs 1A2 and 2C9 appeared to be greater than 80%, considerable inhibition (~50%) of CYP2C19 activity by PXT was also observed (Figure 3), a phenomenon that was previously reported (Kobayashi et al., 1995). CYP3A activity appeared to be ~80% for Days 0 to 4 and CYP 3A activity declines to ~ 60% for Day 5 to 7. Utilization of AZM in human HEPATOPAC resulted in persistent reduction (≥80%) in CYP3A activity and good selectivity (< 20% inhibition of other CYP activities) over a 7-day incubation period (Figure 3). Using global inhibition of CYPs with the relatively non-selective inhibitor ABT and TA to inhibit CYP2C9 effectively, demonstrated that ~95% activity was lost for the five CYPs and sustained inhibition of CYP activity was possible with daily addition of these inhibitors (Figure 4).

The inhibitory effects of the selective inhibitors were also evaluated with several low-turnover compounds using human HEPATOPAC (Figure 5). The intrinsic clearance and the respective f_m values for the low turnover compounds in the presence and absence of the chemical inhibitors is shown in Table 2. Utilization of FUR for CRP was evaluated with tizanidine, a compound predominantly metabolized by CYP1A2 (Granfors et al., 2004). Treatment with FUR reduced clearance of tizanidine by 91% (Figure 5, Table 2) and is comparable to the value previously obtained with human liver microsomes (Gransfors et al., 2004). TA greatly reduced CYP2C9-mediated tolbutamide clearance in human HEPATOPAC with an estimated f_m value of 1.0 for CYP2C9 (Figure 5; Table 2), which is consistent with the reported values ranging from 0.8 to 1 (Veronese et al.,1991; Yang et al., 2016). Treatment with BNZ reduced voriconazole CL_{int} by 40%,

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which is also in line with previously published data showing CYP2C19 contributed to ~35% of the formation of the major *N*-oxide metabolite (Figure 5) (Yanni et al., 2010). A CPR study was conducted with risperidone, a compound predominantly metabolized by CYP2D6 (Berecz et al., 2004), and the f_m was estimated to be 0.83 (Figure 5; Table 2) which is consistent with previously reported in vitro f_m values. Applicability of AZM for CRP in human HEPATOPAC was validated with disopyramide, a compound metabolized predominantly by CYP3A (Echizen et al., 2002; Ma et al., 2017). CYP3A was found to be contributing to the total disopyramide clearance in the current assay (Figure 5; Table 2), consistent with results reported in the literature (Echizen et al., 2002).

Discussion

Determination of f_m via CRP studies is an important aspect in assessing DDI liabilities of NCEs (Zhang et al., 2007; Zientek and Youdim, 2015; Ogilive et al., 2019). Typical CRP approaches rely on a combination of inhibition of activity with human liver microsomes, using either specific chemical inhibitors or inhibitory antibodies, complemented with metabolism by individual recombinant CYP isoforms. With enzyme activities of these cell-free preparations deteriorating substantially in a matter of hours, the applicability of traditional CRP approaches to low-turnover compounds is limited. Hence, development of a CRP method in hepatocyte co-cultures provides an opportunity to evaluate DDI liabilities for low-turnover NCEs.

Recent development of long-term human hepatocyte co-cultures showed promising results in predictions of metabolic clearance (Chan et al., 2013; Kratochwil et al., 2017; Gibson et al., 2021). For human HEPATOPAC, the accuracy of prediction (defined as

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within 3-fold of the observed in vivo clearance) ranged from 82 to 92% across laboratories, and the tendency for under-prediction or inter-individual variability was lower than those obtained from hepatocyte suspensions or plated hepatocytes (Gibson et al., 2021). In the current study, the ability to selectively inhibit CYP activities with chemical inhibitors in human HEPATOPAC has been demonstrated for an incubation period of up to 7 days. Overall, chemical inhibitors utilized in the current method demonstrated reasonably good selectivity profiles in human HEPATOPAC, as supported by consistent results obtained from confirmatory f_m determinations (Figure 4; Table 2). It is anticipated that the current method is also applicable to other human hepatocyte co-culture models.

One key aspect of establishing a CRP method is the demonstration of selective inhibition. For a cell-based hepatocyte system where a whole host of DME are functional, the prospect of a narrowing selectivity window over time as a result of non-selective inhibitory effects exhibited by metabolite(s) of the inhibitor is of particular concern. To circumvent this challenge, time-dependent inhibitors were readily used in hepatocyte-based CRP (Yang et al., 2016; Chanteux et al., 2020). This approach is also applied to the current method with human HEPATOPAC, achieving sustained selectivity for up to 7 days of incubation. It is also important to note that potent reversible inhibitors exhibiting an appreciative selectivity profile are useful in hepatocyte-based CRP (Yang et al., 2016). In human HEPATOPAC, BNZ displayed selective inhibition toward CYP2C19 (Figure 3A). Of note, the usage of omeprazole, a mechanism-based inhibitor of CYP2C19 (Shirasaka et al., 2013), did not replicate the selective inhibitory effect achieved at 8 µM in human hepatocyte suspensions (Yang et

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al., 2016) with only ~50% reduction in CYP2C19 activity obtained in human HEPATOPAC (data not shown). For inhibitors used in the current CYP method, further characterization of the selectivity profile toward other DME will be important when using hepatocytes such as HEPATOPAC. While inactivators of other important CYP isoforms have been recognized, e.g. clopidogrel for CYP2B6 and gemfibrozil glucuronide for CYP2C8 (Mohutsky and Hall, 2021), these were not evaluated in the current study. Since mechanism-based inhibitors require metabolic activation, this mechanism will not occur with many other DME and as such it was important that we were also able to demonstrate extended inhibition using a competitive inhibitor (i.e. BNZ).

FUR (1 µM) exhibited potent inhibitory effect toward the targeted isoform CYP1A2 (~70% inhibition) and good selectivity (<20% inhibition on other CYP activities) within the first day of HEPATOPAC incubation, similar to the results obtained from human liver microsomes and hepatocyte suspensions (Newton et al., 1995; Yang et al., 2016). Surprisingly, a gradual decline of inhibitory effect on CYP1A2 enzyme activity in HEPATOPAC was observed, with minimal/no inhibition detected beyond three days of incubation (Figure 2). Evaluation of native CYP1A2 activity revealed a time-dependent loss of enzyme activity (Figure 3) that mirrored the reduction in inhibitory potency of FUR. In a previous study, CYP1A2 activity in human HEPATOPAC was monitored on Days 7, 9, 15, 19, 25, and 30 (Lin et al., 2017) and the observed CYP1A2 activities were reasonably variable (~4-fold difference between the minimum and maximum values with no apparent trend) across time points. It is possible that the trend of declining CYP1A2 activity was not captured (Lin et al., 2017). To-date, the underlying

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mechanism for the observed reduction in CYP1A2 activity in human HEPATOPAC is not known.

Constant CYP activities over the incubation period is a prerequisite to evaluate the selectivity and potency of chemical inhibitors and enables scientists to determine the fraction by which a specific CYP isoform is involved in the metabolic clearance of compounds. Throughout the course of a 7-day incubation period, an increase in distinct enzyme activities was observed with TA, AZM, and BNZ but not with the other reported chemical inhibitors. AZM and TA increased CYP3A4 mRNA by 22.2-fold and 8.2-fold in cultured hepatocytes, respectively, and are in vitro inducers (supplemental material). Incubating HEPATOPAC with TA for 7 days showed a potent and selective inhibition of CYP2C9 and a 5-fold increase in CYP3A4 activity. A lower TA concentration of 0.015 µM decoupled the inhibition and induction effect and resulted in a potent and selective CYP2C9 inhibition while maintaining constant CYP3A activity.

Despite showing a concentration-dependent increase in CYP3A4 mRNA, AZM demonstrated potent inhibition of CYP3A enzyme activity in HEPATOPAC. At the same time, AZM increased the CYP2D6 activity by less than 2-fold when incubated in HEPATOPAC for 7 days at 1 µM. This was an unusual observation as CYP2D6 is believed to be not inducible in vitro (Ingelman-Sundberg, 2005). A plausible explanation for this observation could be protein-protein interactions which have been described in the literature to modulate CYP activity including CYP2D6 (Yamazaki et al., 1994; Subramanian et al., 2009; Subramanian et al., 2010; Ramsden et al., 2014; Reed and Backes, 2016). Incubations with BNZ lead to a 2-fold increase in CYP3A activity (Figure 3) suggesting that this close analogue of phenobarbital, a known inducer of

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CYP3A4 expression in vitro (Rhodes et al 2011), may also be an inducer in vitro. Further evidence was observed as the CL_{int} for disopyramide, prototypic substrate of CYP3A, was increased by 2-fold after treatment with BNZ (supplemental data).

A CRP method useful for low turnover compounds has been established using HEPATOPAC, and the applicability has been verified with confirmatory f_m determination using low clearance compounds. As a proof-of-principle study, this method offers a generic approach for CRP studies to be conducted in other hepatocyte co-culture models. This assay generates quantitative CRP data which can be used to identify victim DDI risks of low-turnover NCEs prior to first in human studies and helps for planning of clinical DDI studies.

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Authorship Contribution

Participated in research design: Smith, Menzel, Ma, Lyman

Conducted experiments: Smith, Lyman

Performed data analysis: Ma, Smith

Wrote or contribute to writing: Smith, Ma, Tweedie, Menzel, Lyman

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Footnotes

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Figure legends

Figure 1: Schematic overview of Reaction phenotyping using MPCC

Figure 2: Representative CYP activity over the 7-day human hepatocyte pool with HEPATOPAC incubation period. Each data point represents average ± standard error from n=4-8 experiments, each obtained from triplicates determinations, for CYP1A2 (★), CYP2C9 (■), CYP2C19 (▲), CYP2D6 (♦) or CYP3A (□ .)

Figure 3: P450 inhibitor (FUR, TA, BNZ, PXT, AZM) selectivity in HEPATOPAC 10-donor human hepatocyte pool with HEPATOPAC measuring CYP activity remaining over 7 days. Percent Activity of the CYPs as function of time was shown as mean ± standard deviation from triplicate determinations were plotted for CYP1A2 (★), CYP2C9 (■), CYP2C19 (▲), CYP2D6 (♦) or CYP3A (□).

Figure 4: Pan-cytochrome inhibitor ABT/TA in HEPATOPAC 10-donor human hepatocyte pool measuring CYP activity remaining over 7 days. Percent Activity of the CYPs as function of time was shown as mean ± standard deviation from triplicate determinations were plotted for CYP1A2 (★), CYP2C9 (■), CYP2C19 (▲), CYP2D6 (♦) or CYP3A (□).

Figure 5: Inhibition profiles of CYP reaction phenotyping using novel HEPATOPAC method for low-turnover compounds. All assays and measurements were performed in triplicate determinations. Parent disappearance versus time profiles of low turnover substrates were generated for untreated (○) and treated with chemical inhibitor (●) or incubated with mouse embryonic 3T3 fibroblasts (Stromal cells)(▲).

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Table 1: Selective substrates and corresponding metabolites for CYP isoforms

Substrate (Concentration)	P450 isoform	Metabolite
Phenacetin (100 µM)	1A2	Acetaminophen
Diclofenac (25 µM)	2C9	4'-Hydroxdiclofenac
S-Mephenytoin (150 µM)	2C19	4'-Hydroxymephenytoin
Dextromethorphan (25 µM)	2D6	Dextrorphan
Midazolam (15 µM)	3A	1'-Hydroxymidazolam

Table 2: Fraction of metabolism determination in human HEPATOPAC

Low turnover compound	In vitro Inhibitor (Concentration)	Observed in vitro CL_{int} (mL/min/kg)		f_m observed	f_m reported (CYP isoform)	Human Hepatocyte Lot (Number of donor(s))
		without inhibitor	with inhibitor			
Tizanidine	Furafylline (1.0 µM)	24	2.0	0.91	0.85 (CYP 1A2)[a]	VKB (single donor)
Tolbutamide	Tienilic acid (0.015 µM)	1.8	ND	1.0	0.80 to 1.0 (CYP2C9)[b]	KCB (10 donors)
Voriconazole	(+)-*N*-3-benzyl-nirvanol (0.5 µM)	11	6.7	0.40	0.35 (CYP2C19)[c]	KCB (10 donors)
Risperidone	Paroxetine (1.8 µM)	24	4.0	0.83	Mostly (CYP2D6)[d]	AMH (10 donors)
Disopyramide	Azamulin (1.0 µM)	12	0.10	0.99	Mostly (CYP3A)[e]	AMH (10 donors)

ND = No depletion was observed

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[a] Gransfors et al., 2004

[b] Miller et al., 1990

[c] Yanni et al., 2010

[d] Berecz et al., 2004

[e] Echizen et al., 2002

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Figure 1

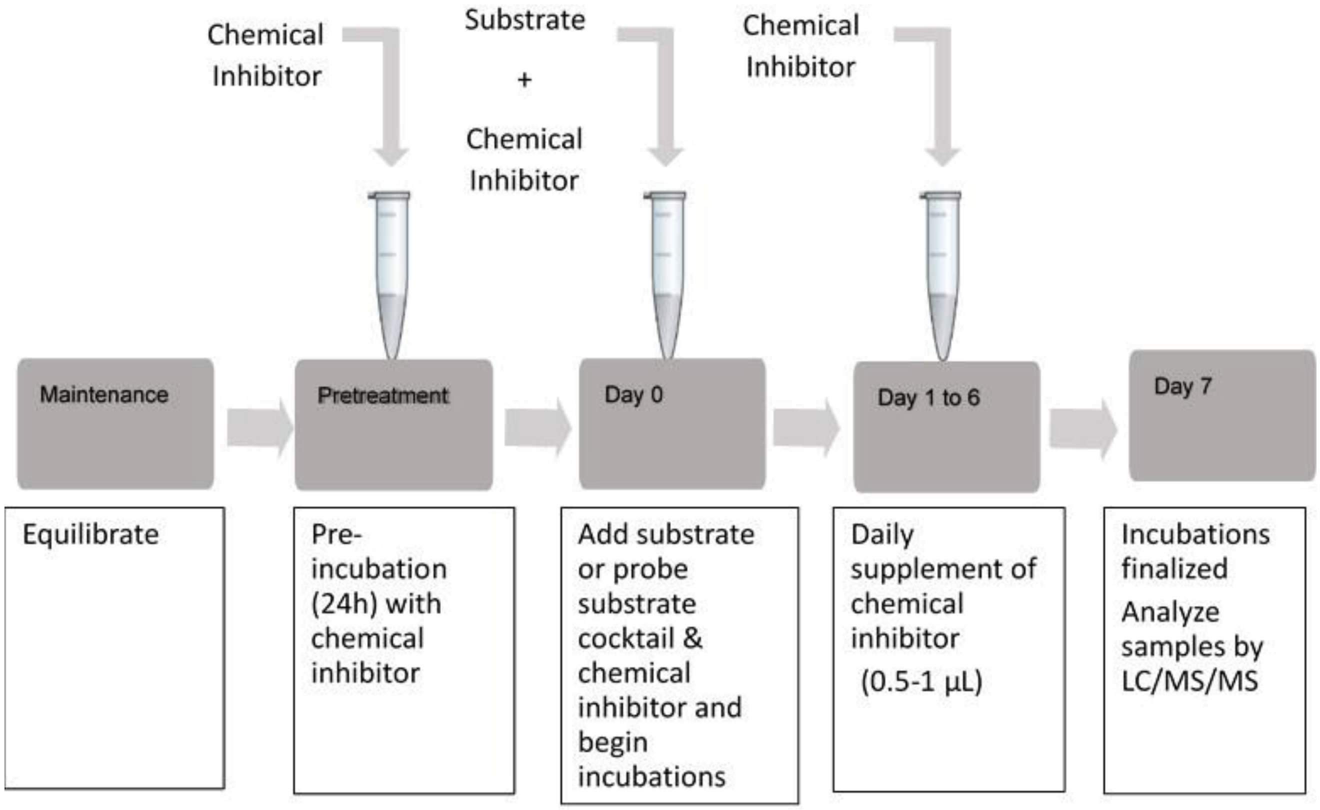

Figure 2

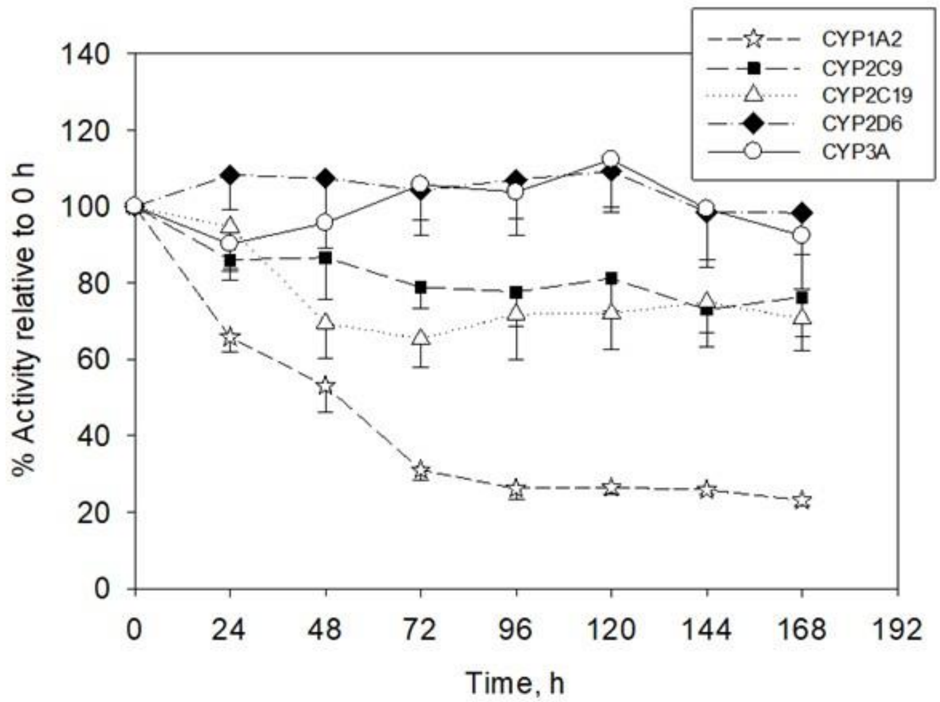
CYP1A2
CYP2C9
CYP2C19
CYP2D6
CYP3A
140
120
100
80
60
40
20
0
% Activity relative to 0 h
0
24
48
72
96
120
144
168
192
Time, h

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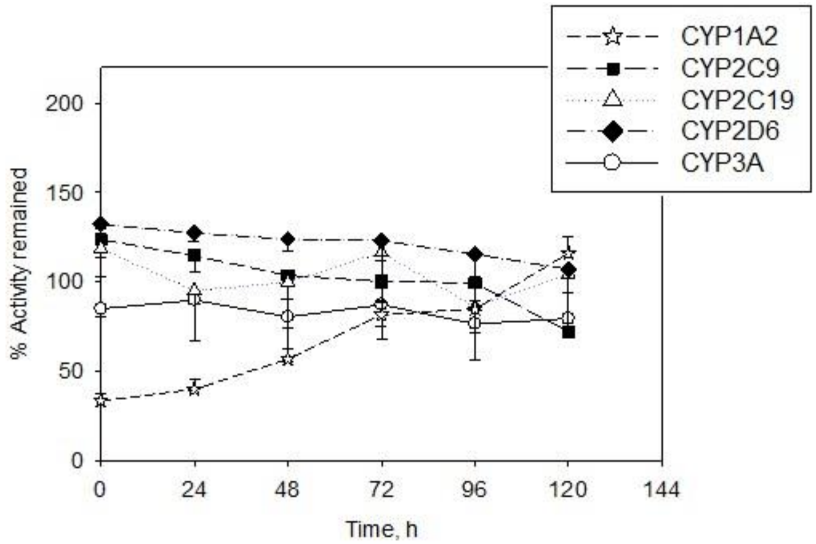
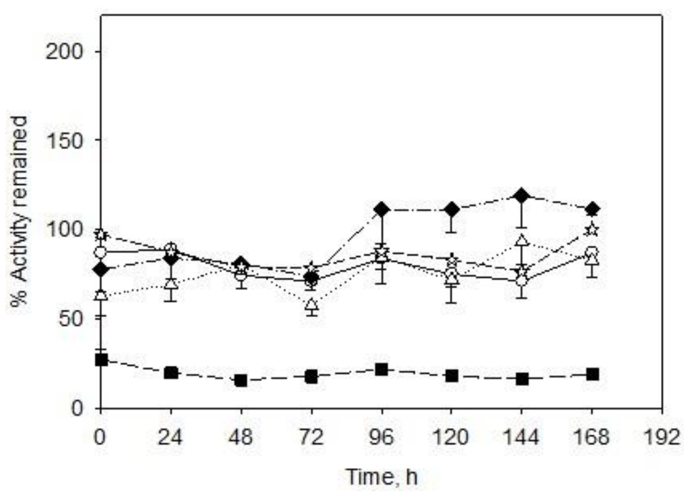
Figure 3

FUR (CYP1A2)



TA (CYP2C9)



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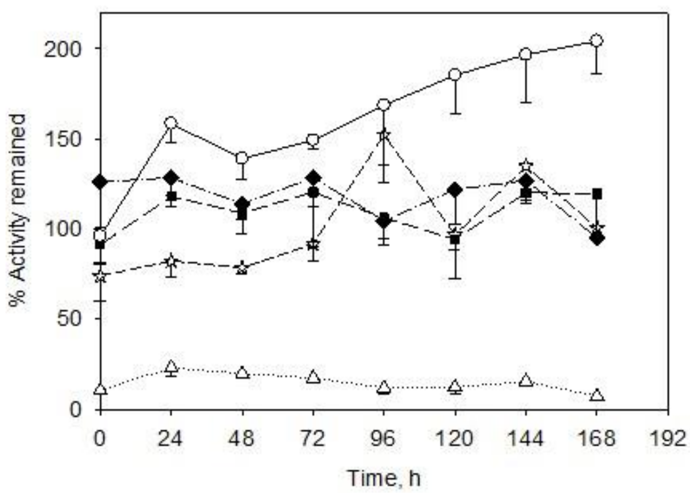
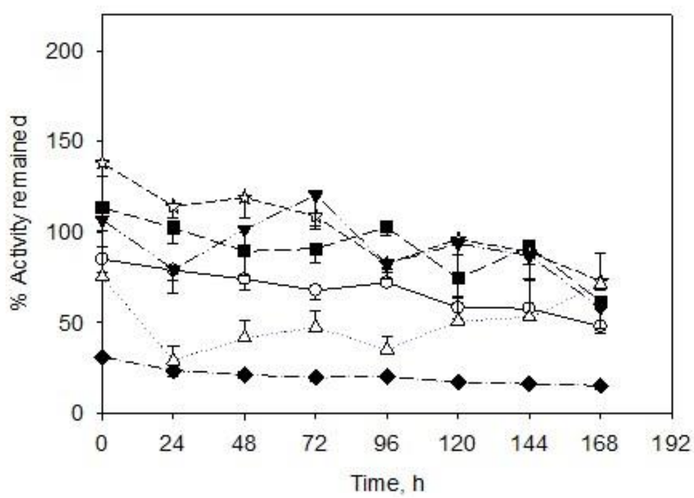
BNZ (CYP2C19)



PXT (CYP2D6)



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AZM (CYP3A)

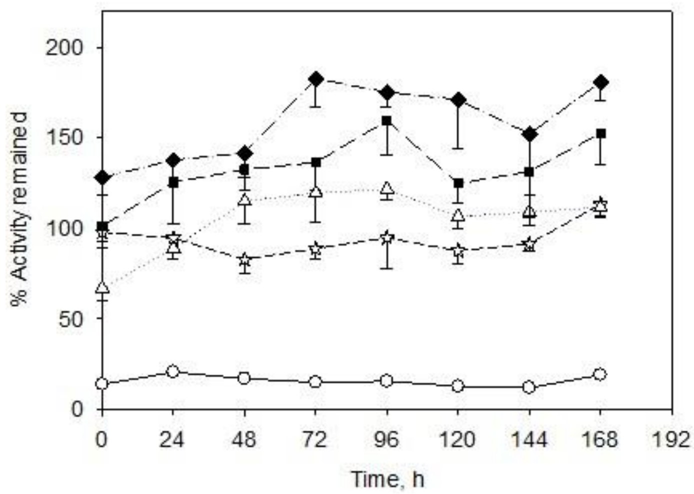

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Figure 4

Aminobenzotrazole-1 + TA

(Pan-CYP)

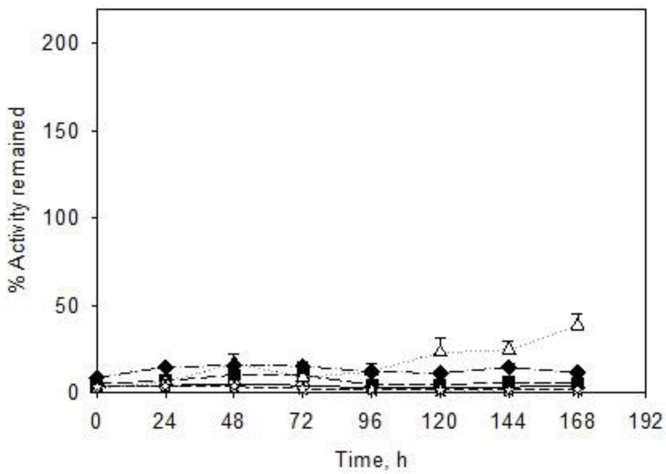

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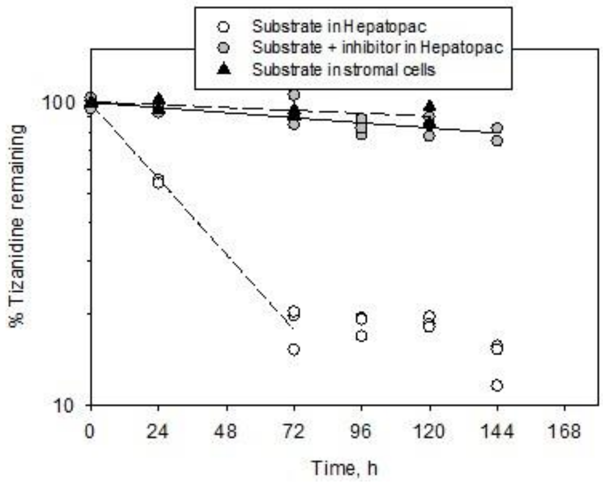
Figure 5

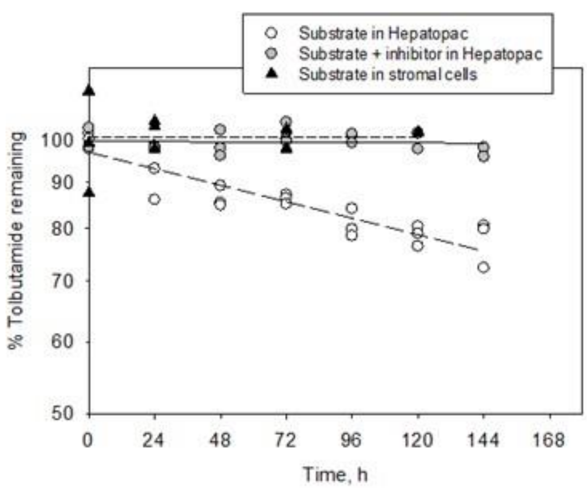
Tizanidine in the presence and absence of FUR

(CYP1A2)



Tolbutamide in the presence and absence of TA

(CYP2C9)



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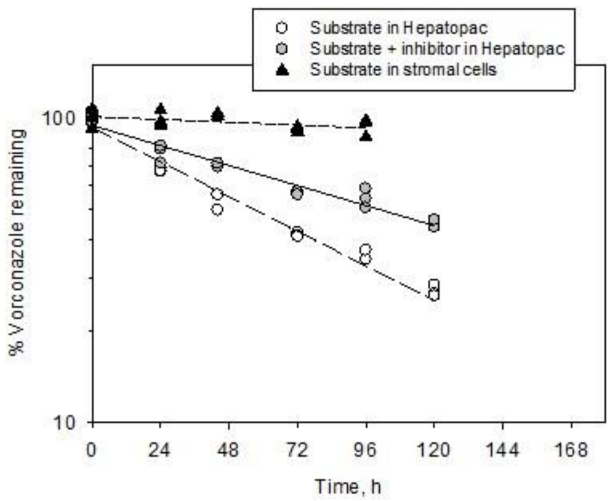
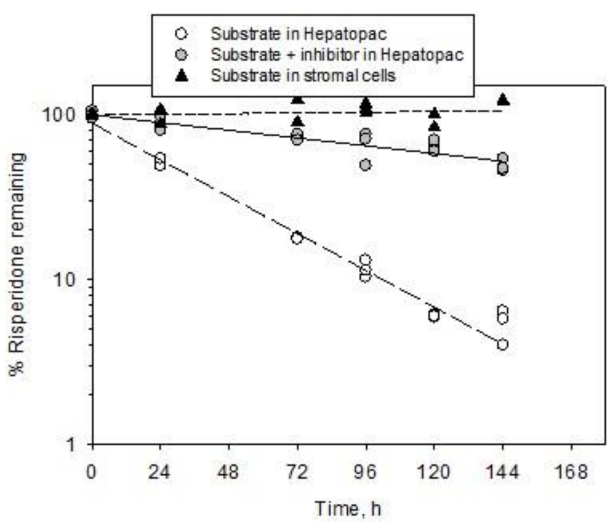
Voriconazole in the presence and absence of BNZ

(CYP 2C19)



Risperidone in the presence and absence of PXT

(CYP2D6)



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Disopyramide in the presence and absence of AZA

(CYP3A)