Predictive In Vitro-In Vivo Extrapolation for Time Dependent Inhibition of CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP2D6 Using Pooled Human Hepatocytes, Human Liver Microsomes, and a Simple Mechanistic Static Model

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ABSTRACT

Inactivation of Cytochrome P450 (CYP450) enzymes can lead to significant increases in exposure of comedrugs. The majority of reported in vitro to in vivo extrapolation (IVIVE) data have historically focused on CYP3A, leaving the assessment of other CYP isoforms insubstantial. To this end, the utility of human hepatocytes (HHEP) and human liver microsomes (HLM) to predict clinically relevant drug-drug interactions was investigated with a focus on CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP2D6. Evaluation of IVIVE for CYP2B6 was limited to only weak inhibition. A search of the University of Washington Drug-Drug Interaction Database was conducted to identify a clinically relevant weak, moderate, and strong inhibitor for selective substrates of CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP2D6, resulting in 18 inhibitors for in vitro characterization against 119 clinical interaction studies. Pooled human hepatocytes and HLM were preincubated with increasing concentrations of inhibitors for designated timepoints. Time dependent inhibition was detected in HLM for four moderate/strong inhibitors, suggesting that some optimization of incubation conditions (i.e., lower protein concentrations) is needed to capture weak inhibition. Clinical risk assessment was conducted by incorporating the in vitro derived kinetic parameters maximal rate of enzyme inactivation (min\(^{-1}\)) \(k_{\text{inact}}\) and concentration of inhibitor resulting in 50% of the maximum enzyme inactivation \(K_i\) into static equations recommended by regulatory agencies. Significant overprediction was observed when applying the basic models recommended by regulatory agencies. Mechanistic static models, which consider the fraction of metabolism through the impacted enzyme, using the unbound hepatic inlet concentration lead to the best overall prediction accuracy with 92% and 85% of data from HHEPs and HLM, respectively, within twofold of the observed value.

SIGNIFICANCE STATEMENT

Coupling time-dependent inactivation parameters derived from pooled human hepatocytes and human liver microsomes (HLM) with a mechanistic static model provides an easy and quantitatively accurate means to determine clinical drug-drug interaction risk from in vitro data. Optimization is needed to evaluate time-dependent inhibition (TDI) for weak and moderate inhibitors using HLM. Recommendations are made with respect to input parameters for in vitro to in vivo extrapolation (IVIVE) of TDI with non-CYP3A enzymes using available data from HLM and human hepatocytes.

Introduction

Evaluating the potential for a drug candidate to inactivate Cytochrome P450 (CYP450) enzymes is important to predict the likelihood of clinically relevant drug-drug interactions (DDIs). Enzyme inactivation is a process whereby, during the catalytic cycle of an enzyme, a reactive intermediate is produced that binds to and irreversibly inhibits the active site of that enzyme. Competitive inhibition and inactivation are mechanistically distinct processes: in the case of competitive inhibition,
enzyme activity is restored when the inhibitor is removed, whereas removal of the inactivator does not restore the activity of the inactivated enzyme. Consequently, recovery of enzyme activity depends on the rate of enzyme resynthesis. Regulatory agencies have provided industry guidance on the conduct of in vitro studies to predict the potential of a drug to mediate DDI through enzyme inactivation [European Medicines Agency (EMA), Food and Drug Administration (FDA) and Pharmaceutical and Medical Devices Agency (PMDA)]. Currently, human liver microsomes (HLM) or recombinantly expressed enzymes are the in vitro systems most often used to evaluate CYP450 inactivation (Grimm et al., 2009); however, although HLM have been successfully used to predict clinically relevant DDIs for known CYP450 inactivators (Obach et al., 2007), there is a tendency to overpredict clinical DDIs (Chen et al., 2011). As with any in vitro system, the environment of the typical microsomal and recombinant enzyme assays differs significantly from the in vivo environment that they strive to model. Consequently, assumptions are made about a drug, for example, complete permeability across biologic membranes, minimal binding to microsomal proteins, and minimal contribution of non-CYP450 metabolism. By using a more physiologically complete system such as human hepatocytes, which have an intact plasma membrane, functional membrane transporters, a complete set of hepatic CYP450 and non-CYP450 enzymes, some assumptions associated with HLM and recombinant systems may no longer be necessary. Although human hepatocytes can be used to evaluate the potential for CYP450 inactivation, there are only a few peer-reviewed examples, prompting the need for additional research as a prerequisite for more routine use of this model. Nevertheless, human hepatocytes could provide mechanistic insight that supports conventional inactivation studies where non-CYP450 metabolites are generated or significant intracellular accumulation of a drug is suspected. In human hepatocytes, active transport of a drug into or out of cells can affect the concentration of a drug present at the CYP450 active site, ultimately affecting the inactivation parameters determined. Xu et al. and Chen et al. postulated that differences in inactivation parameters between human hepatocytes and HLM were due to active transport of drugs in hepatocytes (Xu et al., 2009; Chen et al., 2011). A decrease in active uptake of a compound with limited permeability will decrease the inactivation potential by decreasing the concentration of a drug at the site of inactivation. Conversely, a decrease in active efflux will increase the inactivation potential by increasing the amount of a drug available for metabolism (Lam et al., 2006). Indeed, erythromycin, diltiazem, and troleandomycin are known or suspected substrates of membrane transporters and result in the greatest discrepancy in inactivation parameters between HLM and hepatocytes (Seeff and Landwojtowicz, 2000; Kostrubsky et al., 2003; Kurnik et al., 2006). Additionally, CYP450 inactivators subject to extensive non-CYP450 metabolism, for example, glucuronidation in vivo, may be mistakenly determined to be clinically relevant inactivators when evaluated using HLM. For example, ezetimibe, a cholesterol-lowering drug, displayed potent in vitro inactivation of CYP3A4 using HLM but did not result in clinically meaningful inhibition, likely due to its extensive glucuronidation (Parkinson et al., 2010). Alternatively, a drug that is significantly metabolized via a non-CYP450 pathway and forms a metabolite that inactivates CYP450 enzymes cannot be detected in conventional microsomal CYP450 inactivation assays, as was observed with an aldehyde oxidase metabolite (Zetterberg et al., 2016). This was also the case with gemfibrozil, which led to significant CYP2C8 DDIs subsequently revealed to be mediated by its major metabolite gemfibrozil-1-O-β-glucuronide, the potential for DDIs could have been detected if the inactivation studies were initially performed using human hepatocytes (Ogilvie et al., 2006; Parkinson et al., 2010). Although IVIVE efforts for time-dependent inhibition (TDI) have focused primarily on CYP3A inactivation (Eng et al., 2020), it is unclear whether the recommendations made for CYP3A inactivators may translate to other major CYPs or whether hepatocytes could also be a predictive in vitro model to assess TDI of other CYP isoforms. The purpose of the work described here was to assess suspended hepatocytes and HLM as tools for predicting DDI caused by TDI of CYPs 1A2, 2C8, 2C9, 2C19, and 2D6.

### Materials and Methods

#### Chemicals and Reagents

Cryopreserved Hepatocyte Recovery Medium was purchased from Life Technology (cat # CM7000, Carlsbad, CA). Pooled 200 donor mixed gender human liver microsomes were purchased from XenoTech (cat# H2610, Kansas City, KS). Cinetidine, ciprofloxacin, clopidogrel, dronedarone, fluconazole, fluvoxamine,
miconazole, moclobemide, omeprazole, paroxetine, tamsulosin, ticlopidine trimethoprim, NADPH, and William's medium E were purchased from Millipore Sigma (St. Louis, MO). Mirabegron was obtained from MyBioSource (San Diego, CA), prim, NADPH, and William

Hepatocytes

Experiments were performed using cryopreserved human hepatocytes (Cat. # 454427, Corning Life Sciences, Woburn, MA) pooled from three donors (lot # 305, 346, and 347, except for ticlopidine, which used lot #305, 289, and 293). Donor demographics are displayed in Supplemental Table 1.

Methods

Identification of Clinically Relevant Inhibitors for IVIVE Analysis. Clinical data were collected according to Fig. 1, by searching the University of Washington Drug-Drug Interaction Database (UW-DIDB) for published studies with and without observed changes in area under the curve (AUC) or clearance for sensitive substrates of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6, indicated on the FDA website (https://www.fda.gov/drugs/ drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers), Supplemental Table 2. Clinical data were reviewed and analysis was extended to CYP2B6. Data compiled from literature are presented in Supplemental Table 7. Additionally, HLM incubations were conducted within Takeda, using standard experimental conditions for inhibitors with no published data.

Experimental. Hepatocyte incubations. Hepatocytes from three individual lots (reference Supplemental Table 1) were thawed and pooled in Cryopreserved Hepatocyte Recovery Medium, then centrifuged at 100 g for 10 minutes at room temperature. The supernatant was aspirated, and the pellet was washed with William’s medium E (WME) followed by centrifugation at 40 g for 3 minutes. Hepatocytes from three individual lots (reference Supplemental Table 1) were thawed and pooled in Cryopreserved Hepatocyte Recovery Medium, then centrifuged at 100 g for 10 minutes at room temperature. The supernatant was aspirated, and the pellet was washed with William’s medium E (WME) followed by centrifugation at 40 g for 3 minutes. Hepatocyte incubations.

To expand the analysis to understand whether the recommendations identified for improving the prediction of TDI for CYPs 1A2, 2C8, 2C9, 2C19, and 2D6 could be extended to data generated in HLM, an attempt was made to collate time-dependent inhibition parameters derived from either human hepatocytes or HLM and analysis was extended to CYP2B6. Data compiled from literature are presented in Supplemental Table 7. Additionally, HLM incubations were conducted within Takeda, using standard experimental conditions for inhibitors with no published data.

TABLE 1

| CYP Isoform | P450 Probe Substrate | Substrate Concentration (µM) | Incubation Time (min) | HHEP | HLM |
|-------------|----------------------|-----------------------------|-----------------------|------|-----|
| CYP1A2      | Phenacetin           | 100                         | 30                    | 8    |
| CYP2C8      | Amiodarone           | 100                         | 10                    | 12   |
| CYP2C9      | Diclofenac           | 100                         | 10                    | 8    |
| CYP2C19     | s-Mephenytoin        | 100                         | 30                    | 8    |
| CYP2D6      | Dextromethorphan     | 25                          | 10                    | 8    |

HLM final protein concentration (1 mg/mL)

TABLE 2

| CYP Isoform | Inhibitor     | Clinical Inhibition | Concentration Range in Preincubation (µM) | Preincubation Times (min) |
|-------------|---------------|---------------------|-------------------------------------------|---------------------------|
| CYP1A2      | Cimetidine    | Weak                | 16–2000                                   | 0, 15, 30, 45, 60         |
| CYP1A2      | Ciprofloxacin | Moderate            | 3.9–500                                   | 0, 15, 30, 45, 60         |
| CYP1A2      | Fluvoxamine   | Strong              | 0.0046–10                                 | 0, 5, 10, 15, 20          |
| CYP2C8      | Trimethoprim  | Weak                | 1.5–200                                   | 0, 15, 30, 45, 60         |
| CYP2C8      | Clopidogrel   | Moderate            | 0.3–600                                   | 0, 15, 30, 45, 60         |
| CYP2C8      | Gemfibrozil   | Strong              | 0.1–300                                   | 0, 15, 30, 45, 60         |
| CYP2C9      | Fluoxamine    | Weak                | 3.1–300                                   | 0, 5, 10, 20, 30          |
| CYP2C9      | Miconazole    | Moderate            | 1.6–200                                   | 0, 5, 10, 20, 30          |
| CYP2C9      | Tassilum      | Strong              | 0.003–10                                  | 0, 5, 10, 20, 30          |
| CYP2C19     | Omeprazole    | Weak                | 0.03–100                                  | 0, 15, 30, 45, 60         |
| CYP2C19     | Fluvoxamine   | Strong              | 0.01–300                                  | 0, 5, 10, 20, 30          |
| CYP2C19     | Fluconazole   | Strong              | 0.03–100                                  | 0, 5, 10, 15, 20          |
| CYP2C19     | Ticlopidine   | Strong              | 0.01–30                                  | 0, 15, 30, 45, 60         |
| CYP2C19     | Olsodrostat   | Moderate            | 0.1–300                                   | 0, 15, 30, 45, 60         |
| CYP2C19     | Moclobemide   | Moderate            | 0.1–300                                   | 0, 15, 30, 45, 60         |
| CYP2D6      | Dronedaron    | Weak                | 3.1–300                                   | 0, 15, 30, 45, 60         |
| CYP2D6      | Mirabegron    | Moderate            | 0.0046–10                                 | 0, 15, 30, 45, 60         |
| CYP2D6      | Paroxetine    | Strong              | 0.0091–20                                 | 0, 15, 30, 45, 60         |

Weak = AUCR (≥1.2- to <2.0-fold), Moderate = AUCR (≥2.0- to <5.0-fold), Strong = AUCR (≥5.0-fold).
were determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis using previously validated analytical methods (Perloff et al., 2009). Linearity of metabolite formation with time was confirmed and Michaelis constant (Km) values determined for all substrates prior to inhibition experiments. Substrate concentrations threefold the Michaelis constant (Km) were chosen for all isoforms. Inhibitors (Table 2) were identified to represent weak, moderate, and strong clinical outcome and incubation concentrations were selected based on available clinical data (reference Supplemental Table 9). Initial preincubation and strong clinical outcome and incubation concentrations were selected based on the hepatocyte model in each assay. Effects of known TDI inhibitors were quantitated at each inhibitor concentration (min−1) (kobs) determination.

Cell viability was assessed by trypan blue exclusion for the highest concentration of inhibitor and vehicle control for the longest preincubation time points. Cell suspension aliquots (25 µL) were sampled and gently mixed with an equal volume of trypan blue (0.4%). Cell number and viability were determined.

Cell viability and observed CYP metabolic activity confirmed a properly functioning hepatocyte model in each assay. Effects of known TDI inhibitors were demonstrated at least once (furafylline (CYP1A2), gemfibrozil (CYP2C8), tienilic acid (CYP2C9), fluvoxamine (CYP2C19), and paroxetine (CYP2D6) but not included in each assay.

### TABLE 3
Parameter estimates from pooled human hepatocyte Incubations

| CYP Isoform | Inhibitor | Equation | k_inact (min−1) | K/Ki (µM) | k_inact/Ki (L·min−1·µmol−1) | 95% CI k_inact (min−1) | 95% CI K_i (µM) |
|-------------|-----------|----------|----------------|-----------|--------------------------------|----------------------|-----------------|
| CYP1A2      | Cimetidine| 6        | 0.011          | 152/142   | 0.00072                        | 0.0090              | 0.012           | 84              | 271 |
| CYP1A2      | Ciprofloxacin| 6    | 0.0066         | 7.57/04   | 0.00088                        | 0.0050              | 0.0082          | 2.9             | 23  |
| CYP1A2      | Fluvoxamine| 5      | 0.35           | 0.0480/0.0356| 7.3                             | Only 2 points used for k_inact determinations K_i = 1.95         |
| CYP2B6      | Ticlopidine*| 5     | 0.137          | 0.489/0.257| 0.280                          | 0.12                | 0.16            | 0.37            | 0.66 |
| CYP2C8      | Trimethoprim| 3     | 0.011          | 4.33/3.95  | 0.0025                          | 0.010               | 0.012           | 3.0             | 6.0  |
| CYP2C8      | Clopidogrel| 3      | 0.013          | 3.6/1.53   | 0.0036                          | 0.010               | 0.017           | 0.88            | 15   |
| CYP2C8      | Gemfibrozil| 3      | 0.088          | 1.5/0.91   | 0.061                           | 0.080               | 0.097           | 0.85            | 2.4  |
| CYP2C9      | Fluvoxamine| 6      | 0.11           | 32/24.0    | 0.0034                          | 0.082               | 0.16            | 16              | 71   |
| CYP2C9      | Miconazole| 3      | 0.21           | 15/0.271   | 0.014                           | 0.19                | 0.24            | 10              | 22   |
| CYP2C9      | Tassilum  | 6      | 0.10           | 2.31/0.5   | 0.044                           | 0.069               | 0.17            | 0.69            | 8.1  |
| CYP2C19     | Omeprazole| 3      | 0.0047         | 1.0/0.807  | 0.0048                          | 0.0037              | 0.0058          | 0.33            | 2.6  |
| CYP2C19     | Fluvoxamine| 6     | 0.20           | 5.3/3.94   | 0.037                           | 0.16                | 0.25            | 2.9             | 9.9  |
| CYP2C19     | Fluconazole| No TDI observed| No inhibition observed|
| CYP2C19     | Osidrostat| No TDI observed| No inhibition observed|
| CYP2C19     | Moclomobide| No TDI observed| No inhibition observed|
| CYP2C19     | Ticlopidine*| 3     | 0.045          | 0.52/0.273| 0.086                           | 0.038               | 0.052           | 0.25            | 1.0  |
| CYP2D6      | Dronedarone| 3      | 0.035          | 137/9.17   | 0.00026                         | 0.029               | 0.045           | 87.5            | 226  |
| CYP2D6      | Mirabegron| 3      | 0.021          | 13.1/1.12  | 0.016                           | 0.015               | 0.033           | 0.32            | 4.8  |
| CYP2D6      | Paroxetine| 5      | 0.031          | 0.61/0.333 | 0.051                           | 0.026               | 0.039           | 0.39            | 1.0  |

* Ticlopidine parameters were derived using a different pool of hepatocyte donors.

### TABLE 4
Summary of clinical inhibition data

| CYP Isoform | Inhibitor | # of Trials | # of Substrates |
|-------------|-----------|-------------|----------------|
| 1A2         | Cimetidine| 7           | 2              |
| CYP1A2      | Ciprofloxacin| 11     | 3              |
| CYP1A2      | Fluvoxamine| 9          | 6              |
| 2C8         | Trimethoprim| 6          | 3              |
| CYP2C8      | Clopidogrel| 6          | 3              |
| CYP2C8      | Gemfibrozil| 30         | 4              |
| 2C9         | Fluvoxamine| 1          | 1              |
| CYP2C9      | Miconazole| 11         | 1              |
| CYP2C9      | Tassilum  | 3           | 1              |
| 2C19        | Omeprazole| 4           | 2              |
| CYP2C19     | Osidrostat| 1           | 1              |
| CYP2C19     | Fluconazole| 6          | 3              |
| CYP2C19     | Fluvoxamine| 16         | 5              |
| 2D6         | Ticlopidine| 3           | 1              |
| CYP2D6      | Dronedarone| 3          | 1              |
| CYP2D6      | Mirabegron| 3          | 3              |
| CYP2D6      | Paroxetine| 10         | 6              |

**Human liver microsome incubations.** Pooled human liver microsomes supplied by Xenotech (H2610 lot#1710084) were used at a final primary incubation concentration of 1 mg/mL. The primary incubation was equilibrated in a 37°C incubator for 10 minutes followed by the initiation of the reaction by addition of NADPH (final concentration 2 mM). At 0, 5, 10, 20, and 30 minutes preincubation with model inactivator, 7.5 µL aliquots were transferred to a plate containing 142.5 µL saturating concentrations of probe substrate. Reactions were stopped with 300 µL of acetonitrile containing internal standard at 8 minutes for all CYP substrates except paclitaxel (CYP2C8), which was stopped at 12 minutes. Samples were analyzed as previously described (Nishihara et al., 2021).

Positive control inhibitors included furafylline (CYP1A2), gemfibrozil glucuronide (CYP2C8), tienilic acid (CYP2C9), ticlopidine (CYP2C19), and paroxetine (CYP2D6).

**LC-MS/MS analysis.** Probe substrate metabolites were quantified by LC-MS/MS analysis as described previously (Perloff et al., 2009; Nishihara et al., 2021).

**Calculations.** For each assay, metabolite concentrations in the incubated samples were quantified using LC-MS/MS analysis by interpolating from the regression line of the standard curves. Standard curves were produced from least squares linear regression analysis of the ratio of metabolite peak area to internal standard peak area versus concentrations of metabolite.

For each concentration of test compound, the raw data from LC-MS/MS quantitation at each time point were normalized to the corresponding solvent control (no inhibitor) to determine % CYP activity remaining as shown in Eq. 1. The normalized data were transformed to natural log (In) % CYP activity remaining and plotted versus the primary incubation time. The slope was determined from the linear portion of the ln % CYP activity remaining versus primary incubation time curve by linear regression analysis. The negative value of the slope represents k_obs, the observed rate constant for inactivation at a specified concentration of inactivator.

\[
\text{CYP activity remaining at } [I] = \frac{C_I}{C_{solvent}} \times 100 \quad (1)
\]

Where \( C_I \) is the concentration of metabolite formed in the secondary incubation for each concentration of inhibitor at a primary incubation time point and \( C_{solvent} \) is the concentration of metabolite formed in the secondary incubation for the corresponding solvent control primary incubation time point.

An alternate method to determine the kobs was used where % CYP activity remaining at each concentration of inhibitor at each time point was normalized by the CYP activity in the 0 minute vehicle control (Eq. 2). The ln % CYP activity remaining was plotted versus primary incubation time. This method resulted in a kobs value for the solvent control, first order rate
constant for inactivation estimated from the slope of residual activity (In) versus preincubation time for the solvent control [min⁻¹] (may be constrained to equal the kobs for the vehicle control) (k_{solvent}), which is a measure of nonspecific loss of activity during incubation.

% CYP activity remaining at [I] = C_{i, min}/C_{i, 0 minute solvent} * 100 (2)

Where C_{i, min} is the concentration of metabolite formed in the secondary incubation for each time point and C_{i, 0 minute solvent} is the concentration of metabolite formed in the secondary incubation in the 0 minute vehicle control.

Non-linear regression models to derive inhibition kinetic parameters. For this study, clinically relevant inactivators of CYPs 1A2, 2C8, 2C9, 2C19, and 2D6 were used to assess suspended hepatocytes as a DDI prediction model. The characteristics of CYP inactivators and the determination of kinact and KI is extensively described in several papers (Orr et al., 2012; Nagar et al., 2014b; Leow and Chan, 2019) and will be minimally addressed here.

Since the inactivator is considered a substrate of the enzyme being inactivated, the Michaelis-Menten (MM) model was used to determine kinact and KI as shown in Eq. 3 and an adjusted version of the MM model shown in Eq. 6.

If the k_{obs} was determined using Eq. 1 for % CYP activity remaining, then the adjusted MM model described in Eq. 6, which includes an extra parameter, k_{solvent} for the nonspecific loss of activity in the solvent control. The parameters kinact and KI were then determined by plotting k_{obs} versus [I] and applying nonlinear regression analysis with GraphPad Prism (v. 8, GraphPad Software LLC).

For some experiments nonhyperbolic or atypical MM kinetics such as biphasic and substrate inhibition was observed and Eq. 4 (biphasic) or Eq. 5 (substrate inhibition) models were used. These types of atypical kinetics are considered an artifact of the in vitro system and are discussed in detail elsewhere (Nagar et al., 2014a). Data points to determine k_{obs} values were chosen using the linear portion of the curves. The best fit models for kinact and KI determination were chosen using Akaike Information Criterion value and evaluation of the 95% confidence intervals for the parameter estimates.

\[
\text{k}_{\text{obs}} = \frac{\text{kinact} \cdot [I]}{K_I + [I]} \quad (3, \text{Michaelis Menten})
\]

\[
\text{k}_{\text{obs}} = \frac{\text{kinact} \cdot [I]}{K_I + [I]} + \frac{k_{\text{ratio}} \cdot [I]}{K_I + [I]} \quad (4, \text{Biphasic Kinetics})
\]

\[
\text{k}_{\text{obs}} = \frac{\text{kinact} \cdot [I]}{K_I + [I]} \left(1 + \frac{K_I}{[I]} \right) \quad (5, \text{Substrate Inhibition})
\]

\[
\text{k}_{\text{obs}} = \frac{\text{ksolvent} - \text{kinact} \cdot [I]}{K_I + [I]} \quad (6, \text{ksolvent correction})
\]

Where:
- kinact is the maximal inactivation reaction rate constant;
- k_{obs} is the observed rate constant for inactivation;
- K_I is the concentration of inactivator at which the rate constant of inactivation is half maximal;
- k_{solvent} is the observed rate constant for non-specific loss of activity without inhibitor;
- k_{ratio} is the kinact/inhibition constant for reversible inhibition (K_I) ratio for the second inactivation site that does not reach saturation; and
- [I] is the concentration of inactivator in the primary incubation.

For some datasets, the k_{solvent} parameter was added as a constant by adding the absolute value of the slope for the solvent control to the model. This reduced the number of parameters and therefore the degrees of freedom to achieve a better fit.

Evaluation of in vitro to in vivo extrapolation. Basic models in the regulatory guidance documents were used for the initial analysis (Supplementary Table 5). Equation 7 is the equation recommended in the FDA and PMDA DDI guidelines and incorporates a 50-fold correction factor to the unbound Cmax,sub value. The R2 equation is presented in Eq. 8 and incorporates the enzyme specific rate of degradation (kdeg), (reference Table 5).

In addition, the evaluation of IVIVE without the correction factor (Eq. 9) was considered by using alternative correction factors such as 3, 5, 10, and 15.

\[
\text{k}_{\text{obs}} = \frac{\text{kinact} \times 50 \times C_{\text{max,sub}}}{K_{I, u} + 50 \times C_{\text{max,sub}}} \quad (7)
\]

\[
R_2 = \frac{\text{k}_{\text{obs}} + \text{kdeg}}{\text{k}_{\text{deg}}} \leq 1.25 \quad (8)
\]

### Table 5

| CYP Isoform | kinact (min⁻¹) | Reference |
|-------------|----------------|-----------|
| CYP1A2      | 0.00030        | (Faber and Fuhr, 2004) |
| CYP2B6      | 0.00036        | (Renwick et al., 2000) |
| CYP2C8      | 0.00053        | (Backman et al., 2009) |
| CYP2C9      | 0.00011        | (Renwick et al., 2000) |
| CYP2C19     | 0.00044        | (Liston et al., 2002; Venkatarkashna and Obach, 2005) |
| CYP2D6      | 0.00023        | |

### Table 6

| Inhibitor     | Molecular Weight g/Mol | Log P or D | V_{app} | F_{u} | F_{p} | K_{m} min⁻¹ | R_{2} | References |
|---------------|------------------------|------------|---------|------|------|-------------|------|------------|
| Cimetidine    | 252.34                 | 0.48       | 0.81    | 1    | 0.92 | 0.012       | 0.97 | (Varma et al., 2010; Burt et al., 2016) |
| Ciproflaxacin | 331.346                | 0.60       | 0.75    | 0.98 | 0.01 | 0.75        |      |            |
| Fluvoxamine   | 318.337                | 0.23       | 1       | 0.5  | 0.01 | 1.5         |      | (Jogiraju et al., 2021) |
| Trimethoprim  | 290.321                | 0.50       | 0.8     | 0.0082 | 1     | (Kim et al., 2016) |
| Clopidogrel   | 321.826                | 0.52       | 0.5     | 0.08 | 0.57 | (Xu et al., 2020) |
| Clopidogrel glucuronide | 483.92 | 0.1  | NA | NA | NA | 0.57 | (Tormio et al., 2014) |
| Gemfibrozil   | 250.336                | 0.03       | 1       | 0.1  | 0.825 | (Varma et al., 2015) |
| Gemfibrozil glucuronide | 426.5   | 0.115  | NA | NA | NA | 0.825 | |
| Miconazole    | 416.134                | 0.08       | 0.1     | 0.01 | 0.03 | 1.5         |      | (O'Reilly et al., 1992; Miki et al., 2011) |
| Tassinulum    | 437.09                 | 0.01       | NA | NA | NA | NA | (Perkins et al., 2018) |
| Omeprazole    | 345.42                 | 0.05       | 1       | 1    | 0.1  | 1           | (Marsoussi et al., 2018) |
| Osilodrostat  | 227.241                | 0.636      | 1       | 0.0467 | 0.85 | (Armani et al., 2017) |
| Fluvonozole   | 306.725                | 0.89       | 0.98    | 0.0292 | 1     | (Marsoussi et al., 2018) |
| Ticlopidine   | 263.786                | 0.02       | 1       | 0.5  | 0.55 | Default values used |
| Dromedaron    | 556.764                | 0.01       | 1       | 0.898 | 0.0136 | 1     | (Djebl et al., 2015) |
| Mirabegron    | 396.513                | 0.27       | 1       | 0.68 | 0.00617 | 1.42 | (Konishi et al., 2019) |
| Paroxetine    | 329.369                | 0.05       | 0.93    | 1    | 0.017 | 1.26 | (Marsoussi et al., 2018) |
TABLE 7

Comparison of model fits

| Model | R² | R² (Reversible Inhibition only) | Model 1 (Same Data-Set) | Model 3 (Same Data-Set) |
|-------|----|--------------------------------|-------------------------|-------------------------|
| R, 3  | 0.98 | 0.67 (0.04–2.70) | 1.42 (0.25–7.78) | 0.90 (0.71–1.35) |
| Model 1 | 1.16 | 0.86 (0.04–1.14) | 0.76 (0.23–1.30) | 0.76 (0.23–1.30) |
| Model 2 | 1.16 | 0.76 (0.23–1.30) | 0.76 (0.23–1.30) | 0.76 (0.23–1.30) |
| Model 3 | 1.16 | 0.76 (0.23–1.30) | 0.76 (0.23–1.30) | 0.76 (0.23–1.30) |

GMFE (90% CI) 9.42 (8.75–10.1) 1.12 (0.89–1.34) 1.16 (0.89–1.34) 1.16 (0.89–1.34)

IVIVE of Non-CYP3A Inhibition

119

$$k_{obs} = \frac{k_{max} \times C_{max,u}}{K_{i,u} \times C_{max,u}}$$  \hspace{1cm} (9)

To refine the quantitative prediction, the mechanistic static model (MSM), which enables additive perpetrators, reported elsewhere (Fahmi et al., 2008; Isoherranen et al., 2012) was also used (Eq. 10).

$$\frac{AUCc}{AUC} = \frac{1}{F_g + (1 - F_g) \times \left( \sum_{k=1}^{n} \frac{f_{E_{k}}(E_{k})}{A_{k} \times C_{max,u} + 1 - \sum_{k=1}^{n} f_{E_{k}}(E_{k})} \right) \times \left( 1 - \sum_{k=1}^{n} f_{E_{k}}(E_{k}) \right)}$$  \hspace{1cm} (10)

Where:

- A = reversible inhibition, B = time-dependent inhibition, C = induction, g = gut, h = liver and k = enzyme.

Since the enzymes used in this analysis are minimally expressed in enterocytes (Paine et al., 2006; Thelen and Dressman, 2009; Xie et al., 2016), or their expression does not impact DDI outcome (CYP2C intestinal expression to DDI) the gut component was removed, resulting in Eq. 11, which includes Eq. 12, representing the reversible inhibition, and Eq. 13, the time-dependent inhibition in liver.

$$\frac{AUCc}{AUC} = \frac{1}{\sum_{k=1}^{n} \frac{f_{E_{k}}(E_{k})}{A_{k} \times C_{max,u} + 1 - \sum_{k=1}^{n} f_{E_{k}}(E_{k})}} + \frac{1}{B_{h,k} + 1} K_{i,u}$$  \hspace{1cm} (11)

$$B_{h,k} = 1 + \frac{k_{max} \times f_{E_{k}}(E_{k})}{K_{i,u}}$$  \hspace{1cm} (12)

Calculation of maximal hepatic inlet concentration was conducted using Eq. 14, and the unbound hepatic inlet concentration (I_{inlet,max,u}) was calculated with Eq. 15.

$$I_{inlet,max} = \frac{R_h \times C_{max,plasma} + Fa \times Fg \times Ka \times Dose}{Qh}$$  \hspace{1cm} (14)

$$I_{inlet,max,u} = \frac{(1 - H) \times f_{E_{k}}(E_{k}) \times I_{inlet,max}}{Rb}$$  \hspace{1cm} (15)

Where H is the hematocrit and assumed to be 0.45 and Rb is the blood-to-plasma ratio.

Multiple iterations of the above model with various [I] input values were considered including:

- Model 1: Using unbound hepatic inlet concentration as described in regulatory guidance;
- Model 2: Using unbound hepatic inlet concentration calculated with default values; and
- Model 3: Inputting unbound systemic C_{max,u} in place of I_{inlet,max,u}.

All models were evaluated considering the range of published fraction metabolized through the pathway (Fm) values (Supplemental Table 3), and Model 1 used published Ka, Fa and Fg values where available (Table 6), default values used for Model 2 were Ka (0.03 minute⁻¹), Fa:Fg = 1 and Rb = 0.55. The best universally fitting Fm was selected for the optimized data and is depicted in bold in Supplemental Table 3.

In silico estimation of unbound Kᵢ and Kᵢ values. The Kilford equation (Kilford et al., 2008), Eq. 16, was used to estimate the unbound inhibition parameters. In the case of the experimental conditions employed in these studies, the hepatocyte concentration was 50,000 cells (1 × 10⁶ cells/mL), and there was no additional protein present in the media. An intracellular volume of 6.48 pl was used; the incubation volume was 50 µL, resulting in a Vᵢ of 0.00648 (Note Vᵢ = V_{cell} x V_{inc}). Log P or D values reported in the literature for the inhibitors were used (Table 6).
\[ f_{\text{h.e.p.}} = \frac{1}{1 + 125 \times V_R \times 10^{0.0001 \times \log D_{\text{ss}} \text{mg}^b \text{ or } D_{1.25} \times 10^{0.0001 \times \log D_{\text{ss}} \text{mg}^b \text{ or } D_{1.5} \times 10^{0.0001 \times \log D_{\text{ss}} \text{mg}^b}}} \]  

(16)

Correction of in vitro derived IC50 values based on saturating substrate concentrations used in the time-dependent inhibition assay was conducted according to Eq. 17 (Cheng and Prusoff, 1973) and assuming competitive inhibition.

\[ K_i = \frac{IC_{50}}{1 + \frac{\text{Substrate}}{K_m}} \]  

(17)

Statistical analysis of the goodness of fit for IVIVE models evaluated. The accuracy of the prediction of the individual models was evaluated by deriving the geometric mean fold error (GMFE) according to Eq. 18 and the root mean square error (RMSE) according to Eq. 19. GMFE closest to 1 represents the best fit, whereas RMSE approaching 0 does.

\[ \text{GMFE} = 10^{\text{geom} \left( \frac{\text{predicted DDI} - \text{observed DDI}}{\text{observed DDI}} \right) } \]  

(18)

\[ \text{RMSE} = \sqrt{\frac{\sum (\text{predicted DDI} - \text{observed DDI})^2}{\text{number of predictions}}} \]  

(19)

Results

Selection of Clinically Relevant Inhibitors for In Vitro Data Generation

The search of the UW-DIDB identified weak, moderate, and strong inhibitors toward CYPs 1A2, 2C8, 2C9, 2C19, and 2D6 (Table 2). Although an attempt was made to identify clinically relevant inhibitors of CYP2B6, studies were limited to no effect or weak inhibition, thus in vitro evaluation was not further pursued and IVIVE was conducted using reported and/or historical values (Supplementary Table 4). Clinical data used for the IVIVE evaluation of selected inhibitors of CYPs 1A2, 2C8, 2C9, 2C19, and 2D6 is presented in Supplementary Table 9.

TDI Results

Time-dependent inhibition was observed for 16 of the 19 evaluated inhibitors, and kinetic parameters could be confidently determined for them using hepatocytes (Table 3). Mild TDI was observed for cimetidine in HLM; however, inactivation parameters could not be confidently estimated. TDI was not observed in HLM, under the experimental conditions, for any other clinically weak inhibitors, but was observed for one moderate and three strong inhibitors. The positive control inhibitors used in the HLM assay demonstrated expected and robust response with kinetic parameters in line with those reported previously. In human hepatocyte incubations, the \( k_{\text{max}}/K_i \) ratios trended with the classification from weak to strong clinical inhibition such that the lower the ratio the weaker the observed clinical effect.

Time-dependent inhibition of CYP1A2 in hepatocytes was observed for cimetidine, ciprofloxacin, and fluvoxamine, using phenacetin as the probe substrate. Fluvoxamine showed potent and rapid inhibition of enzyme activity after a 5-minute preincubation but did not show a further decrease in activity with increasing preincubation time. As a result,
only 2 data points were available to estimate $k_{obs}$ values (Supplemental Fig. 1) resulting in a potential underestimation of inhibitory potency. TDI of CYP1A2 was observed in HLM for only the strong inhibitors, fluvoxamine and furafylline, and not for the weak and moderate inhibitors. The kinetic parameters for fluvoxamine were $K_i$ of 1.81 μM and a $K_{inact}$ of 0.0747 minute$^{-1}$ and for furafylline were $K_i$ of 22.5 μM and a $K_{inact}$ of 0.372 minute$^{-1}$.

Time-dependent inhibition of CYP2C8 in hepatocytes was observed for trimethoprim, clopidogrel, and gemfibrozil, using amodiaquine as the probe substrate. The inhibition parameters determined from clopidogrel and gemfibrozil were likely due to the glucuronide metabolites as described elsewhere (Ogilvie et al., 2006; Tornio et al., 2014). TDI of CYP2C8 was not observed with the test set in HLM although the positive control, gemfibrozil glucuronide, yielded a total $K_i$ of 29.8 μM and a $K_{inact}$ of 0.04 minute$^{-1}$.

Time-dependent inhibition of CYP2C9 in hepatocytes was observed for fluvoxamine, miconazole, and tasisulam, using diclofenac as the probe substrate. For fluvoxamine, substantial cytotoxicity was observed at concentrations of 100 μM and above (trypan blue viability of 33% at 200 μM with a 30-minute preincubation). As it is unclear what, if any, impact the decreased viability might have on CYP enzyme activity, the 200 and 300 μM data points were excluded from analysis. TDI of CYP2C9 was not observed with the test set in HLM although the positive control, ticlopidine, yielded a total $K_i$ of 4.35 μM and a $K_{inact}$ of 0.108 minute$^{-1}$.

Time-dependent inhibition of CYP2C19 was evaluated in human hepatocytes for omeprazole, fluvoxamine, fluconazole, osilodrostat, moclobemide, and ticlopidine. TDI was observed and $K_i$ and $k_{inact}$ values were determined for omeprazole and fluvoxamine. Fluconazole and osilodrostat did not demonstrate any inhibition of CYP2C19 activity in hepatocytes despite resulting in a clinically moderate inhibition of omeprazole clearance [area under the concentration curve ratio (AUCR) = 2.07]. TDI was not observed in HLM for omeprazole, osilodrostat, or fluvoxamine under the incubation conditions used. There was TDI observed for ticlopidine with a total $K_i$ of 85.7 μM and a $K_{inact}$ of 0.111 minute$^{-1}$.

The inhibition potential for ticlopidine was investigated in this pooled lot of human hepatocytes but did not demonstrate TDI. Of note, historical studies using an alternate set of three donors of hepatocytes have demonstrated 30% time-dependent inhibition of CYP2C19 by ticlopidine and kinetic parameters from those studies were used for the clinical risk assessment. The reason for the difference between donors is unclear. In pooling donors, any impact of polymorphic enzymes should be reduced (Ramsden et al., 2009); however, genotyping data for the donors used in these studies was not available.

Time-dependent inhibition of CYP2D6 was observed for dronedarone in hepatocytes only and for mirabegron and paroxetine in both HLM and human hepatocytes.

Resulting graphs depicting the ln % remaining CYP activity versus incubation time and $k_{obs}$ versus inhibitor concentration are provided in Supplemental Fig. 1.
CYP2C9 is the next most abundantly expressed CYP representing 14% of the detected intestinal CYP content, followed by CYP2C19 (2%) and CYP2D6, whereas neither CYP1A2 or CYP2C8 were detected (Paine et al., 2006; Xie et al., 2016). To evaluate the importance of CYP2C9 intestinal expression toward observed DDIs, the clinical inhibition and induction data were reviewed for inhibitors and inducers evaluated against the substrates when dosed IV (hepatic) and orally (hepatic + intestinal) (Supplemental Table 6). As evidenced by the similar magnitudes of change observed when substrates were dosed IV or orally, after administration of the inhibitor or inducer, the impact of intestinal CYP2C9 toward the observed DDI is limited. In addition, the Fg reported for common CYP2C9 substrates including warfarin, tolbutamide, celecoxib, and phenytoin are >0.9, whereby the max percent AUC increase from inhibition at the intestinal level is calculated to be 11%. Since the expression of CYP2C19 and CYP2D6 in the gut is much less than CYP2C9, an assumption is made that the impact of intestinal activity on the magnitude of DDI is also likely to be limited.

How Does the Data Generated in This Study Compare with Literature Values Reported in HLM? Although the scope of the enclosed work did not originally include comparative evaluation of TDI for non-CYP3A enzymes using recombinant CYPs or HLM, conducting these studies in human hepatocytes isn’t trivial and evaluation of the predictivity of parameters reported for HLM was performed. To facilitate this analysis, all available in vitro parameters for the selected inhibitors were collated from literature (Supplemental Table 7). In some cases, the inhibitor resulted in time-dependent inhibition of multiple CYPs (cimetidine, dronedarone, fluvoxamine, omeprazole, paroxetine, and ticlopidine). Thus, it is important to understand the selectivity of the clinical probe substrate and whether the potential inhibition of other CYPs involved in its metabolism needs to be considered in the DDI risk assessment. The available literature data for the inhibitors selected in the analysis conducted herein was limited. Published values were available for gemfibrozil, gemfibrozil glucuronide, omeprazole, osilodrostat, paroxetine, and ticlopidine (for both CYP2B6 and CYP2C19). The inhibition parameters were generated in-house for fluvoxamine, gemfibrozil glucuronide, ticlopidine, mirabegron, and paroxetine. The values were corrected to unbound values using the in silico approach reported by Hallifax and Houston, 2006. In addition, an attempt was made to derive the TDI parameters for the selected test set using the standard protocols established within Takeda. Kinetic parameters could only be derived for a limited number of the inhibitors using HLM under the incubation conditions used. A recent publication highlighted the critical role of passive permeability to differences between clearance and inhibition parameters derived from HHEP and HLM (Keefer et al., 2020). To understand whether the time dependency observed in hepatocytes may be an artifact of low/slow permeability followed by direct inhibition, reported information on the biopharmaceutics classification system and direct inhibition parameters was collected (Supplemental Table 8). These data were used to evaluate the potential for reversible inhibition to recover the observed clinical DDI using the MSM. Using only the reversible inhibition parameters resulted in 44 false negative trials and a large underprediction (58/119 over twofold below the observed magnitude) (Table 7). These results suggest that delayed permeability, followed by direct inhibition, cannot explain the lack of TDI observed in HLM for weak and moderate inhibitors. An additional approach would be to experimentally derive the Kpuu values in hepatocytes, rather than relying on in silico values. There are a number of proposed methods to derive this value although no consensus has been
Clinical Risk Assessment from In Vitro Inhibition Parameters.

The clinical risk assessment was conducted following the recommendations set forth in the regulatory documents (FDA, EMA, and PMDA). The first step was to use the basic models that consider the inhibition kinetic parameters and the maximal unbound plasma concentration at steady state ($C_{\text{max,ss,u}}$) but do not incorporate substrate specific parameters (Eq. 7–9). The nominal $K_i$ values were corrected to unbound $K_i$ values using the predicted nonspecific binding to hepatocytes or HLM (Eq. 10). The unbound $K_i$ values were used in the subsequent equations to assess the clinical risk. The degradation rates presented in Table 5 were input into Eq. 8, dependent on the CYP being inhibited. While the inhibitor $C_{\text{max,ss,u}}$ values for the enclosed dataset are known, it should be appreciated that this value is often based on a prediction using preclinical data prior to when first-in-human or multiple dose clinical studies have been conducted. The impact of the inhibitor concentration input value should therefore be considered during the clinical risk assessment for new chemical entities. The $R_2$ value generated with the basic model was compared with the observed AUCR (Supplementary Table 9). The resulting $R_2$ values using Eq. 8 significantly overpredicted the observed magnitude of DDI (Fig. 2A, Table 7) when $K_{puu}$ were calculated with 50x unbound $C_{\text{max}}$ (Eq. 9). When the 50-fold correction factor was removed according to Eq. 7, dronedarone, a weak to moderate inhibitor of CYP2D6, resulted in a false negative at all three clinical dose levels studied (Fig. 2B). The 800 mg dronedarone became a true positive when applying a correction factor of 3 to the $R_2$ equation; however, both the 400 and 600 mg dose level predictions were considered false negatives (Fig. 2C, Table 7). The next step was to evaluate various iterations of the MSM (Eq. 10). The MSM model incorporates both inhibitor and substrate specific parameters. The $F_m$ value(s) for each substrate was collected from the literature (Supplemental Table 3). In cases where multiple $F_m$ values were reported, individual and mean values were evaluated in the prediction. In terms of inhibitor specific parameters, the literature was searched for $K_{a,f}$, $F_{a,f}$, and $R_b$ to support estimation of the hepatic inlet concentration (Table 6) using Eqs. 14 and 15. When the reported values were used to estimate the unbound hepatic inlet concentration and the optimal $F_m$ values for the substrates were used, there were no false negatives and there was good quantitative prediction observed (Fig. 3, Table 7). In this case, 109 of the 119 (92%) clinical studies were predicted within twofold of the observed AUCR and 64 were predicted within bioequivalence or between 0.8- to 1.25-fold of the observed. The magnitude of seven clinical studies was overpredicted (>$2\text{fold predicted/observed}$) and three were underpredicted (<0.5 predicted/observed). Trimethoprim with repaglinide was overpredicted by 2.4-fold. There were three trials with gemfibrozil, which were overpredicted using repaglinide as the probe substrate for CYP2C8 ranging from 2.4- to 4.8-fold; of note, 20 other similarly designed trials fell within twofold of the observed with 15 of them within bioequivalence. A similar observation was made for ticlopidine with omeprazole where one trial was overpredicted by 2.6-fold and the other two trials were predicted within bioequivalence. If the average of the clinical results is used rather than discreet AUC values, these are no longer overpredicted. Similarly, the inhibitors that were underpredicted (fluconazole and fluvoxamine) were well-predicted in all of the other clinical studies, 4/6 and 8/9, respectively. The analysis of these trends is presented in Supplemental Fig. 2. Therefore, it is likely that the variability in outcome observed between clinical interaction studies should be considered in the risk assessment. The importance of substrate selectivity in the magnitude of DDI can be highlighted by the magnitude of inhibition observed for fluvoxamine against CYP1A2 substrates, where the predicted AUCR ranges from 2.38-fold with theophylline to
Since it is appreciated that the inhibitor specific parameters are often not known during early DDI risk assessment, default values of 1 for $F_{D,\text{g}}$, 0.03 minute$^{-1}$ for $K_a$, and 0.55 for $R_b$ were also evaluated to derive the unbound hepatic inlet concentration. This also resulted in zero false negatives and 108 of 119 (90.8%) trials within twofold and 58 (49%) within bioequivalence. In the case of missed predictions, most (8/11) were overpredicted (Fig. 4). Lastly, the MSM was evaluated using the $C_{\text{max,ss, u}}$ rather than the unbound hepatic inlet concentration.

When using the $C_{\text{max,ss, u}}$ as the input, there were 3 and 13, for HHEPs and HLM, respectively, as false negatives (FN) when using the unbound hepatic inlet concentration; whereas there were 3 and 13, for HHEPs and HLM, respectively, when using the $C_{\text{max,ss, u}}$. In the case where $C_{\text{max,ss, u}}$ was used as the input parameter, there were 13 false negatives using the HLM data including trials with ticlopidine (3/3), mirabegron (1/3), and paroxetine (9/10) and 3 false negatives using the hepatocyte data (3/4, omeprazole trials), (Table 7). Taken together, these data suggest that parameters generated from either HLM or HHEPs coupled with the MSM using $I_{\text{inlet, max, u}}$ as the input results in quantitative prediction of magnitude of DDI with no false negatives. Since good quantitative predictions were possible using the MSM, which is much easier and more accessible to researchers, Physiologically-Based Pharmacokinetic (PBPK) modeling was not conducted. It is possible that some of the overpredictions might be reduced with PBPK modeling.

**Discussion**

Clinically relevant TDI has been reported for multiple CYP enzymes, although clinical risk assessments and IVIVE efforts have historically focused on CYP3A as the primary enzyme responsible for the majority of DDI (Obach et al., 2007; Mao et al., 2011; Kenny et al., 2012; Vieira et al., 2014; Tseng et al., 2021). Given the importance of identifying DDI liabilities during drug development, regulatory agencies have proposed guidance on evaluating the DDI potential for NCEs (EMA, FDA, and PMDA). Although clinically relevant TDI has been reported for non-CYP3A enzymes including CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6, to our knowledge a systematic review has not been conducted, and therefore it is unclear whether the proposed recommendations, based on CYP3A data, are appropriate for characterizing the clinical inhibition risk of these non-CYP3A isoforms.

It is appreciated that traditional in vitro studies tend to overpredict the risk for DDI and can be based on a number of assumptions. Similar observations have been reported for competitive inhibition, whereby the inhibitory parameters associated with compounds likely to accumulate within cells due to active uptake were markedly different in experiments conducted using HLM and human hepatocytes (Brown et al., 2010). To this end, experiments were designed using pooled human hepatocytes to derive TDI kinetic parameters with a focus on non-CYP3A enzymes. To investigate the utility of suspended hepatocytes as a tool to better predict TDI, the literature was mined for clinically relevant weak, moderate, and strong inhibitors of CYPs 1A2, 2C8, 2C9, 2C19, and 2D6 using the
UW-DIDB. Inhibitors were selected with clinical inhibition observed under steady state conditions. The literature was further searched to evaluate whether in vitro induction parameters were available for the inhibitor test set, and where these parameters were not available, the assumption was made that the inhibitor was not an inducer. Furthermore, whether the inhibitors were substrates or inhibitors of major drug transporters was also considered. In vitro induction conditions were established based on validation work conducted by Corning Life Sciences. The in vitro test concentrations used in the evaluation were determined considering the clinical concentrations, solubility, existing data, and toxicity potential with the goal to enable estimation of the in vitro kinetic parameters. The time points were selected to ensure adequate sensitivity for deriving the inhibition rate constants $k_{obs}$. The in vitro data were fit to various kinetic models to derive the $K_i$ and $K_{inac}$ values, and the model selected was dependent on the shape of the $k_{obs}$ versus concentration profile.

Of the 18 evaluated inhibitors, 16 demonstrated TDI in human hepatocytes, and kinetic parameters could be confidently derived for them. TDI toward CYP2C19 was not observed in hepatocytes for fluconazole or osilodrostat although reversible inhibition parameters could be derived for use in clinical risk assessment with basic models. Fluconazole is known to be a potent reversible inhibitor of CYP2C9, CYP2C19, and CYP3A. As TDI necessitates formation of a reactive metabolite, the lack of TDI by fluconazole is consistent with the knowledge that fluconazole is poorly metabolized and primarily eliminated unchanged via renal excretion (Bellmann and Smuszkiewicz, 2017). The lack of TDI observed for osilodrostat in hepatocytes was in contrast to data generated in HLM, where inactivation parameters could be determined considering the clinical concentrations, solubility, existing data, and toxicity potential with the goal to enable estimation of the in vitro kinetic parameters. The time points were selected to ensure adequate sensitivity for deriving the inhibition rate constants $k_{obs}$. The in vitro data were fit to various kinetic models to derive the $K_i$ and $K_{inac}$ values, and the model selected was dependent on the shape of the $k_{obs}$ versus concentration profile.

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parameters in the MSM, there were a number of FN and underpredictions observed when using the $C_{\text{Max,CLD}}$ and therefore evaluation of $C_{\text{Max,CLD}}$ was not pursued. A notable difference between the assessment for CYP3A and non-CYP3A DDI is the importance of gut CYP3A in the overall magnitude of the observed DDI. Presumably the concentration used to project hepatic DDI would be consistent across CYP enzymes, suggesting that further optimization of the gut input is warranted for predicting CYP3A inhibition DDI.

Results from this study show that incorporating kinetic parameters for TDI into the previously proposed MSM enables quantitative prediction of TDI for CYPs 1A2, 2B6, 2C8, 2C9, 2C19, and 2D6. Additionally, analysis of the available HLM data also demonstrates reasonable quantitative prediction using the MSM, confirming that in vitro parameters derived from HLM are likewise valuable for TDI risk assessment from non-CYP3A enzymes, although the analysis would benefit from additional data points.

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Wrote or contributed to the writing of the manuscript: Ramsden, Perloff, Whitter-Johnstone, Zhang.

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