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Time-dependent enzyme inactivation: numerical analyses of in vitro data and prediction of drug-drug interactions

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Abstract

Cytochrome P450 (CYP) enzyme kinetics often do not conform to Michaelis-Menten assumptions, and time-dependent inactivation (TDI) of CYPs displays complexities such as multiple substrate binding, partial inactivation, quasi-irreversible inactivation, and sequential metabolism. Additionally, in vitro experimental issues such as lipid partitioning, enzyme concentrations, and inactivator depletion can further complicate the parameterization of in vitro TDI. The traditional replot method used to analyze in vitro TDI datasets is unable to handle complexities in CYP kinetics, and numerical approaches using ordinary differential equations of the kinetic schemes offer several advantages. Improvement in the parameterization of CYP in vitro kinetics has the potential to improve prediction of clinical drug-drug interactions (DDIs). This manuscript discusses various complexities in TDI kinetics of CYPs, and numerical approaches to model these complexities. The extrapolation of CYP in vitro TDI parameters to predict in vivo DDIs with static and dynamic modeling is discussed, along with a discussion on current gaps in knowledge and future directions to improve the prediction of DDI with in vitro data for CYP catalyzed drug metabolism.

Keywords

cytochrome P450; time-dependent inactivation; numerical methods; non-Michaelis-Menten kinetics

1. Introduction

The Centers for Disease Control and Prevention's 2016 National Health and Nutrition Examination Survey reported that in the years 2011–2014, 21.5% of the US population reported use of 3 or more prescription drugs within 30 days prior to being surveyed (<https://>

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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www.cdc.gov/nchs/data/abus/abus16.pdf#079). The report highlighted the frequency of comedications in the US population, underlying the importance of drug-drug interactions (DDIs) among co-prescribed drugs, and the resulting safety, adverse events, and associated healthcare costs due to DDIs. Evaluation of DDI potential is critical in drug discovery and development, and identification of a new drug as a victim or perpetrator of DDIs is important for prescribing the information and product labeling (Levy & Ragueneau-Majlessi, 2019; Rekic, et al., 2017; Yu, et al., 2018). Knowledge of mechanisms underlying potential DDIs is critical in designing dosing regimens to avoid clinical DDI with co-prescribed medication. DDIs can occur via pharmacokinetic or pharmacodynamic mechanisms (Niu, et al., 2019), with cytochrome P450 (CYP)-mediated metabolic interactions being the most common pharmacokinetic mechanism for potential DDI in drug discovery (Dresser, et al., 2000; Zhang & Wong, 2005).

DDIs mediated by CYPs usually occur due to enzyme inhibition or induction. For CYP inhibition, the perpetrator drug increases the plasma concentration-time area under the curve (AUC) of the victim, while for induction, the victim AUC is decreased. For CYP metabolism, most substrates have relatively high (micromolar) K_m values, and therefore competitive inhibition between substrates is uncommon in vivo (unbound concentrations at clinical doses are generally sub-micromolar). However, some drugs cause a time-dependent loss of enzyme activity, resulting in a significant decrease in enzyme levels and serious DDIs. The ability to predict DDIs due to time-dependent inactivation (TDI) is therefore important in drug discovery and development.

Details of CYP mechanisms and kinetics have been studied for many years. Until recently, many of the known complexities in CYP kinetics have not been considered in the analysis of CYP TDIs. Some complexities include multi-substrate binding interactions, quasi-irreversible inactivation, partial inactivation, and sequential metabolism. This review focuses on these complexities, and the application of numerical methods to analyze in vitro TDI data. We define numerical methods as methods to solve a series of ordinary differential equations. Standard replot approaches to the analysis of TDI data are based on Michaelis-Menten (MM) kinetics and the underlying assumptions of irreversible inactivation, steady-state kinetics, initial-rate conditions, and single substrate binding. Numerical approaches do not rely on these assumptions, and CYP complexities can therefore be modeled.

2. CYP kinetics and TDI mechanisms

I. CYP Kinetics

The CYP superfamily includes numerous functional isozymes in humans, but a relatively small number of enzymes are responsible for most of the CYP mediated oxidations. CYPs 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4 mediate over 90% of human drug oxidations (Zanger, et al., 2008). CYPs are understood to be promiscuous with respect to ligand selectivity, and many drug molecules are substrates for the same CYP enzyme (Ekroos & Sjögren, 2006). Crystal structures support the conclusion that the active sites of some CYPs are flexible, particularly those involved in the metabolism of xenobiotics (Li & Poulos, 2004; Sevrioukova & Poulos, 2017). These non-specific binding interactions between CYPs and ligands can result in some unusual binding and metabolism kinetics. This topic is outside the

scope of the present review, and the reader is referred to comprehensive publications on this research (Hsu & Johnson, 2019; Lampe, 2017; Reed & Backes, 2017).

i. MM kinetics—The Michaelis-Menten (MM) equation (equation 1) assumes that i) there is insignificant substrate depletion (typically, less than 10% (Seibert & Tracy, 2014)) as well as insignificant reverse reaction (the initial rate assumption), and ii) the rate of change of the concentration of the ES complex is zero (the steady-state assumption). Equation 1 can be derived from the following scheme: $E+S \leftrightarrow ES \rightarrow P$

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad \text{Equation 1:}$$

where, v is the initial velocity of the reaction, $[S]$ is the substrate concentration, V_{max} is the maximal velocity (turnover number k_{cat} multiplied by the total enzyme concentration E_t), and K_m or the MM constant is the substrate concentration yielding half-maximal velocity. The derivation of Eq. 1 along with detailed discussions on the underlying assumptions and interpretation of K_m are covered elsewhere (Gibaldi & Perrier, 1982; Michaelis & Menten, 1913; Segel, 1975). MM kinetics are not often observed for CYP-mediated TDI (Korzekwa, et al., 2014; Yadav, et al., 2018).

ii. non-MM (EII)—Non-hyperbolic saturation kinetics and atypical inhibition kinetics can occur when more than one substrate binds simultaneously to the active site (or to different regions on the protein). For multi-binding kinetics for a single substrate, four types of saturation kinetics are possible: hyperbolic, biphasic, sigmoidal, and substrate inhibition (Korzekwa, 2014). When two different substrates bind simultaneously, profiles that can be observed include complete inhibition, partial inhibition, activation, or a combination of activation followed by inhibition. These multi-substrate interactions are often seen during competitive inhibition experiments.

Kinetic analysis of non-MM schemes results in multiple binding parameters (e.g. K_{m1} , K_{m2}) and multiple velocity terms (e.g. k_{cat1} , k_{cat2}). Non-MM kinetics are often observed in CYP kinetics, and as discussed below, in CYP TDI. The interpretation of the resulting TDI kinetic parameters is not straightforward and can impact clinical DDI predictions, as discussed in the following sections.

II. CYP inactivation

Irreversible inhibition occurs through several molecular mechanisms ultimately resulting in an inactive protein (Fontana, et al., 2005; Kalgutkar, et al., 2007; Kamel & Harriman, 2013; Orr, et al., 2012; Zhou, et al., 2004). Compounds that cause irreversible inhibition can be classified as mechanism-based inactivators (MBIs), affinity labeling agents, slow-tight binders and transition state analogs (Silverman, 1995). MBIs must be transformed by the enzyme into a species that can inactivate the enzyme by forming either a covalent or a non-covalent bond. Since mechanism-based inactivation results in time dependence, it is also called time-dependent inactivation (TDI). Although slow-tight binders, affinity labeling agents and transition state analogs also cause irreversible inhibition, they are not MBIs since

such compounds do not have to be transformed into another species to inactivate the enzyme.

When a reactive intermediate is generated in the process of metabolism, the reactive intermediate can react with the nucleophiles present on the CYP enzyme, rendering the enzyme inactive. This reactive intermediate can either diffuse out of the active site or remain in the active site and inactivate the enzyme (Kent, et al., 2001). If the intermediate diffuses out of the active site, a decrease in inactivation is observed in the presence of scavengers like glutathione, superoxide dismutase and catalase (Johnson, 2008).

Silverman (Silverman, 1995) proposed the following properties of the inactivator that are required to classify a molecule as a TDI:

1. Time dependence of inactivation
2. Saturation of inactivator concentration
3. Substrate protection
4. Irreversibility
5. Inactivator stoichiometry
6. Involvement of the catalytic step
7. Inactivation prior to the release of active species

However, not all inactivators satisfy all criteria. For example, CYP3A4 (Kenworthy, et al., 1999) and CYP2C9 (Kumar, et al., 2006) are shown to have multiple binding sites, hence substrate protection might not be observed. In another example, it was observed that CYP3A4 catalyzes the formation of iminium ion intermediate of DSP-1053, which leads to inactivation of CYP1A2 (Nishimuta, et al., 2018). Finally, in the case of KW-2449, it was observed that its iminium ion metabolite, catalyzed by monoamine oxidase-B, inhibited aldehyde oxidase by covalent binding (Hosogi, et al., 2018). Irreversibility is another criterion that is not satisfied by the metabolite intermediate complex (MIC) forming compounds (Blobaum, et al., 2004). Dialysis, gel filtration, microsomal washing or potassium ferricyanide treatment has been shown to reverse CYP inhibition (Grimm, et al., 2009). Terminal alkenes are also known to inactivate CYPs by the epoxide intermediate (Fontana, et al., 2005). It was shown that cytochrome P450 2E1 T303A (mutant CYP2E1) was inactivated by tert-butyl acetylene in a mechanism-based manner through the formation of two tert-butyl acetylene adducts to the P450 heme. However, upon overnight standing, activity and native heme spectra were restored (Blobaum, et al., 2004). A more general classification of CYP MBIs was noted by Hollenberg to include: (a) compounds that bind quasi-irreversibly to the iron atom of the prosthetic heme; (b) agents that covalently modify the porphyrin framework of the heme; (c) compounds that lead to the destruction of the prosthetic heme group with consequent irreversible modification of the enzyme active site by the ensuing heme fragments; and (d) compounds that form covalent adducts to amino acid residues in the apoprotein (Correia & Hollenberg, 2015).

TDI drugs have different mechanisms of CYP inactivation depending on the functional group present on the drug (Fontana, et al., 2005; Hollenberg, 2002; Kamel & Harriman, 2013; Orr, et al., 2012; Ortiz De Montellano, 2019). Some of the notable functional groups that inactivate CYPs are methylenedioxy aromatic groups, acetylenes, alkyl and aryl olefins, thiophenes, amines, furans, and phenols (Kalgutkar, et al., 2007; Kalgutkar & Soglia, 2005; Zhou, et al., 2004). However, not all compounds possessing these groups are TDIs. TDIs can be classified into different groups depending on the mechanism of inactivation as follows (Figure 1) (Correia & Hollenberg, 2015; Mohutsky & Hall, 2014; Zhou, et al., 2004; Zhou, et al., 2005):

1. Metabolite intermediate complex (MIC) formation with heme
2. Covalent modification of apoprotein
3. Heme destruction/modification

There are a number of clinical examples of drugs that show nonlinear accumulation and increased half-life in humans upon multiple dosing because of autoinactivation. These include diltiazem (DTZ; CYP3A4/5 inactivator) (Tsao, et al., 1990), verapamil (CYP3A4/5 inactivator), paroxetine (CYP2D6 inactivator), and ticlopidine (CYP2C9 inactivator). This inactivation of CYPs can lead to DDI (Backman, et al., 1994; Watanabe, et al., 2007; Zhou, et al., 2007).

i. MIC formation—MIC formation is the formation of a quasi-irreversible bond with the CYP heme, resulting in loss of enzyme activity. Many classes of drugs that contain amine functionalities form MICs, including calcium channel blockers such as verapamil and diltiazem, antidepressants like nortriptyline (Bensoussan, et al., 1995; Jones, et al., 1999; Pershing & Franklin, 1982), antibiotics including troleandomycin and erythromycin (Ludden, 1985; Periti, et al., 1992), and protease inhibitors. The amine group is hydroxylated and further oxidized to form a nitroso group that coordinates with the heme. The heme is then reduced to a more stable ferrous form (Bensoussan, et al., 1995; Mansuy, et al., 1977) with an absorbance maximum of 448 to 456 nm (Franklin, 1977). The MIC is stable and can be isolated (Ekroos & Sjögren, 2006; Murray & Reidy, 1990).

Drugs with a methylenedioxyphenyl group (e.g. podophyllotoxin, tadalafil, paroxetine) (Barnaba, et al., 2016; Bertelsen, et al., 2003; Ring, et al., 2005) form MICs through the formation of a carbene intermediate (Murray, 2000). MICs formed from methylenedioxyphenyl compounds have dual characteristic absorption peaks at 425–427 and 455 nm (“type III” spectrum) (Orr, et al., 2012; Philpot & Hodgson, 1972; Wilkinson, et al., 1984). The podophyllotoxin (PPT)-CYP3A4 interaction was shown to proceed through the formation of the reversible Fe^{3+} :carbene intermediate, which is reduced to the energetically more stable Fe^{2+} :carbene complex (Barnaba, et al., 2016; Taxak, et al., 2013). Moreover, the electron transfer ($\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$), which results in the irreversible inactivation of the enzyme, is the rate-limiting step (Barnaba, et al., 2016). This suggested an accumulation of Fe^{2+} :carbene during CYP3A4 inactivation. In contrast to this, more recently it was reported that the transition from the Fe^{3+} :carbene intermediate to the Fe^{2+} :carbene complex of 3, 4-methylenedioxymethamphetamine-CYP2D6 is rapid. Further, in contrast to PPT-derived Fe^{2+} :carbene complex of CYP3A4, the 3, 4-methylenedioxymethamphetamine-dependent

formation of the Fe^{2+} :carbene complex of CYP2D6 was found to be reversible, and no accumulation of the Fe^{3+} :carbene intermediate was observed (Rodgers, et al., 2018). These data suggest that the reversibility of the Fe^{2+} :carbene complex is an enzyme and/or substrate-specific.

ii. Covalent modification of apoprotein—Reactive intermediates formed during CYP-catalyzed oxidation can react either with the heme or the apoprotein. Apoprotein modification results from the formation of a covalent bond with nucleophilic amino acids such as lysine, serine, cysteine, and threonine (Hollenberg, et al., 2008; Kalgutkar, et al., 2007). Apoprotein modification can lead to alteration of CYP activity, ranging from complete loss of activity to partial loss of activity (Crowley & Hollenberg, 1995; Hollenberg, et al., 2008). It is often observed that both heme and apoprotein modifications occur simultaneously, as evident from CYP3A inactivation by gestodene (Guengerich, 1990) and erlotinib (Zhao, et al., 2018), CYP2B6 inactivation by bergamottin (Lin, et al., 2005), mifepristone (Lin, et al., 2009), and clopidogrel (Zhang, et al., 2011), CYP2B1 inactivation by secobarbital (He, et al., 1996), and CYP2J2 inactivation by 17α -ethynyl estradiol (Lin, et al., 2018; Zhao, et al., 2018). Carbon monoxide (CO) binding can be used to differentiate between the two inactivation mechanisms. Apoprotein modified proteins retain the capability to bind CO whereas heme modification will result in loss of CO binding (Foti, et al., 2011; Knodell, et al., 1987).

Some examples of functional groups responsible for apoprotein modification are furan (e.g. rofecoxib and L-745,394 (Karjalainen, et al., 2006; Lightning, et al., 2000)), thiophenes (e.g. ticlopidine, clopidogrel (Ha-Duong, et al., 2001; Nishiya, et al., 2009; Zhang, et al., 2011)), tienilic acid (Dansette, et al., 1991; Koenigs, et al., 1999; López-García, et al., 2005)), phenol (e.g. metabolite of AMG487 (Henne, et al., 2012)), alkene (e.g. secobarbital (He, et al., 1996)), and alkynes (e.g. 17α -ethynyl estradiol (Hollenberg, et al., 2008; Lin, et al., 2002; Lin, et al., 2018), mifepristone (He, et al., 1999; Lin, et al., 2009)). The acetylenes are initially oxidized either to a ketene intermediate (for terminal acetylenes) (Sridar, et al., 2012; Zhao, et al., 2018) or an oxirene intermediate (for internal acetylenes). Both these intermediates can either alkylate heme or the apoprotein depending upon the overall structure of the inactivator (Ortiz De Montellano, 2019; Roberts, et al., 1998; Roberts, et al., 1993; Roberts, et al., 1994). More recently, sulfenylation of the heme thiolate has been observed for CYP4A11 and may be involved in the modification of catalytic activity (Albertolle, et al., 2017).

iii. Heme destruction—In this mechanism, the reactive metabolite modifies the heme such that enzyme activity is lost (Peterson, et al., 2000). Although the pyrrole nitrogens of the heme porphyrin ring are not very nucleophilic, generation of reactive species in the proximity of the heme prosthetic group can lead to alkylation of the porphyrin ring. Changes in the porphyrin ring can be studied by UV/visible spectroscopy and CO binding studies (Foti, et al., 2011; Grab, et al., 1988). The native heme is typically monitored by UV at 400 nm by LC-UV. The difference in the HPLC elution profiles compared to control (absence of nicotinamide adenine dinucleotide phosphate) indicates heme adducts and/or heme loss. The isolation of the heme adduct depends on stability. This mechanism is seen for drugs

containing a terminal acetylene group (e.g. ethinylestradiol, gestodene) (Guengerich, 1990) and internal acetylenes (e.g. mifepristone) (Foroozesh, et al., 1997; He, et al., 1999). Mibefradil (Foti, et al., 2011; Prueksaritanont, et al., 1999) and gemfibrozil glucuronide inactivate CYP3A and CYP2C8 respectively by the destruction of heme (Baer, et al., 2009). There have also been reports of the covalent binding of heme fragments to the apoprotein (Correia & Hollenberg, 2015).

III. In vitro experimental approaches and assay design

i. Enzyme systems—The most common enzyme sources used for CYP TDI assays include liver microsomes, hepatocytes, and recombinant enzymes. Human liver microsomes (HLM) are the first choice for CYP inhibition assays for drugs primarily metabolized by CYPs (Grimm, et al., 2009). The advantages of HLM include availability, ease of use, cost (as compared to recombinant systems and human hepatocytes) and reasonable in vitro-in vivo extrapolation (IVIVE) (Bohnert, et al., 2016; Gao, et al., 2017; Obach, 2011). When conducting TDI assays in HLM, it is important to consider the activity of multiple microsomal enzymes, the concentration of HLM used, and membrane partitioning of drug into microsomes. Due to the limited thermal stability of HLM especially for specific CYP isoforms as well as incubation conditions (Foti & Fisher, 2004; Nakamura, et al., 2002), incubation times are limited to less than 1 hour. For this and other reasons discussed below, cell based experimental systems have recently been evaluated.

Hepatocytes contain multiple drug metabolizing systems (e.g. microsomal as well as cytosolic enzymes) and drug transporters. Therefore, they may be more physiologically relevant. Cell-based systems are more stable than HLM, allowing for longer primary incubations. Hence hepatocytes have increasingly being used for evaluation of TDI (Albaugh, et al., 2012; Mao, et al., 2012; Mao, et al., 2016; Pham, et al., 2017). Several different cell systems have been used for evaluation of TDI, such as fresh plated primary hepatocytes, cryopreserved plated hepatocytes, cryopreserved HepaRG-plated cells, and suspended hepatocytes with and without plasma. These different systems have their own advantages and disadvantages. Plated cell systems, in general, have the advantage of having long term viability and enzymatic activity over suspensions (Zhao, 2008). For freshly plated hepatocytes, disadvantages include limited availability and donor to donor variability (Grime & Riley, 2006). Further, the pooling of freshly isolated hepatocytes is restricted due to availability. Cryopreserved plated hepatocytes from multiple donors can be combined and used for TDI evaluation. The HepaRG is a unique cell line which can differentiate from an epithelial phenotype to and hepatocyte-like cells with canalicular structures and is routinely used in drug metabolism assays (Andersson, et al., 2012). HepaRG cells are easy to handle and are readily available. It has been shown that HepaRG cells have acceptable levels of CYP (Lübberstedt, et al., 2011). Suspended human hepatocytes either in Dulbecco's Modified Eagle Media or suspended in human plasma have been used for evaluation of TDI (Chen, et al., 2011; Mao, et al., 2013; Mao, et al., 2011, 2012; Mao, et al., 2016; Xu, et al., 2009).

Recombinant CYPs (rCYPs) generally tend to have higher inactivation efficiency (k_{inact}/K_I) than HLM (Ernest, et al., 2005; McConn, et al., 2004; Zhang, Jones, et al., 2009). This

difference may be due to several reasons, including differences in lipid composition and amounts (Brignac-Huber, et al., 2016), and oligomerization of different CYPs (Bostick, et al., 2016; Davydov, et al., 2015). Also, recombinant systems may have different stoichiometries of P450 oxidoreductase and cytochrome b5 to the CYP enzyme. Differences between rCYP and HLM when used for inactivation assays have been reported (Palamanda, et al., 2001; Polasek, et al., 2004; Wang, et al., 2004). Also, rCYPs fail to detect cases in which metabolites generated by one CYP inhibit another CYP. For example, amiodarone is an inactivator of CYP2C8, CYP3A4 (Ohyama, et al., 2000; Polasek, et al., 2004), CYP2C9 (Rougee, et al., 2017) and CYP2J2 (Karkhanis, et al., 2016). It was found that desethylamiodarone inactivates CYP1A1, CYP1A2, 2B6 and 2D6 but not CYP3A4. Finally, it is noteworthy that while rCYPs may not be useful for DDI prediction and IVIVE, they are a very useful tool for deducing the mechanism of inactivation of specific CYP isoforms (Murayama, et al., 2018; Orr, et al., 2012).

ii. Assay design for in vitro TDI—The analysis of in vitro TDI experimental data has evolved over the last few decades. A major use of these assays is to identify inactivators early in drug development in order to prevent clinical DDIs. Two important in vitro parameters are k_{inact} and K_I , and represent the maximal inactivation rate and concentration of inhibitor at half-maximal velocity, respectively.

a. The two-step incubation method for TDI: Typically, CYP TDI assays are conducted in a two-step manner to separate the inactivation step from the remaining activity measurement. In the first step, the enzyme is incubated with inactivator to inactivate the enzyme, and in the second step, remaining activity is measured by adding a probe substrate specific for the enzyme. These two steps can either be performed with or without dilution in the second step. Experimental methods to evaluate TDI range from simple screening assays to assays for determination of TDI parameters (K_I and k_{inact}) (Atkinson, et al., 2005; Berry & Zhao, 2008; Burt, et al., 2012; Fowler & Zhang, 2008; Grimm, et al., 2009; Hollenberg, et al., 2008; Mohutsky & Hall, 2014; Nagar, et al., 2014; Obach, et al., 2007; Obach, et al., 2006; Orr, et al., 2012; Parkinson, et al., 2011; Silverman, 1995; Yates, et al., 2012). To improve accuracy and precision, automation has been used to improve the robustness and quality of the data (Foti, et al., 2011; Zimmerlin, et al., 2011).

In a two-step in vitro incubation, the primary incubation step involves incubating the enzyme (hepatocytes, microsomes, or purified P450 enzymes) with a range of inactivator concentrations. Selection of inactivator concentrations typically relies on either a known clinical maximum unbound plasma concentration of the potential perpetrator or a pharmacologically relevant maximum effective concentration. A range of appropriate inactivator concentrations is then designed for in vitro TDI assays based on the current regulatory guidance for the conduct of DDI studies (Grimm, et al., 2009) (<https://www.fda.gov/media/108130/download>). At different time points, aliquots of the primary incubation mixture are transferred to the secondary incubation mixture with either a dilution step (dilution of the enzyme and the inactivator typically by 10 fold) or without dilution. The secondary incubation mixture has a saturating concentration of the substrate (5 fold K_m) to monitor the remaining activity. Dilution is designed to ensure minimal competitive

inhibition by the inactivator. Determination of K_I and k_{inact} is detailed in the 'Data Analysis' section below.

b. One-step incubations for progress curves: Progress curve incubations involve incubation of several concentrations of inactivator and control with the probe substrate and the enzyme source (e.g. HLM or rCYP) simultaneously ("paired" assay) in phosphate buffer as opposed to the two-step method (Burt, et al., 2012; Fairman, et al., 2007; Stresser, et al., 2014). At various time-points, incubations are analyzed for product (metabolite of probe substrate) formation. Incubations without a probe substrate ("unpaired" assays) can be used to analyze inactivator depletion over time. Incubations can also be analyzed for both product formation and inactivator depletion over time. Measuring loss of inactivator would necessitate a sensitive and precise analytical assay to accurately differentiate small changes in [I]. One advantage of the progress curve assay could be to avoid experimental/human errors in a two-step secondary incubation. Modeling data with progress curve assays is discussed in more detail below.

iii. Considerations for experimental design of TDI assays—A number of publications have addressed critical aspects of the TDI assay experimental design (Ghanbari, et al., 2006; Parkinson, et al., 2011; Stresser, et al., 2014; Van, et al., 2006; Venkatakrishnan, et al., 2007; Yang, et al., 2005, 2007). Some important considerations of the experimental design are briefly discussed below.

a. Experimental matrix size: There is no set guideline for the data matrix size (number of inactivator concentrations and number of pre-incubation times) for a two-step TDI assay. An optimal dataset would include a range of [I] low enough to parameterize high affinity binding constants, and high enough to parameterize plateaus in inactivation. This range is limited by the analytical assay accuracy at one end, and inactivator solubility at the other end. Ultimately, this is an iterative process. We have found that a 4×6 matrix is sufficient for a simple MIC-mediated TDI with MM kinetics with low analytical error (Korzekwa, et al., 2014), while a matrix as rich as 8×14 was used for complex models such as multiple inactivator binding + inhibitor depletion + sequential metabolism (Yadav, et al., 2018).

b. Enzyme concentration and inactivator depletion: In principle, both K_I and k_{inact} are independent of enzyme concentration used during the assay. For the two-step approach, different HLM concentrations in the primary incubation ranging from 0.1–1mg/ml have been used for determination of K_I and k_{inact} (Walsky, et al., 2012). Use of high enzyme concentrations can lead to inactivator depletion due to its metabolism during the primary incubation. This decreases the available inactivator and subsequently decreases the extent of inactivation (Parkinson, et al., 2011). As discussed below, lipid partitioning of inactivator must also be taken into account if using high microsomal concentrations. Given the sensitivity of bioanalytical assays, use of a lower HLM concentration is generally recommended.

c. Dilution versus non-dilution: The two-step approach with dilution is designed for minimal competitive inhibition (due to the parent inactivator or a metabolite that shows competitive inhibition) during the activity measurement. It is recommended that at least 10-

fold dilution between the two steps is needed to effectively “quench” any further inactivation during the secondary incubation step (Ghanbari, et al., 2006). For potent inactivators like mibefradil, even after 20-fold dilution, significant inactivation has been observed (Foti, et al., 2011). To avoid this problem, a 100-fold dilution was performed while assessing ritonavir inactivation kinetics (Rock, et al., 2014). The non-dilution method requires the addition of substrate in a small volume in order to prevent dilution of the enzyme and the inactivator. Hence, in the non-dilution method, the concentration of inactivator is still high enough to continue inactivating the enzyme in the secondary incubation. Although inactivation can be minimized by adding a high concentration of the probe substrate, complete elimination of inactivation cannot be guaranteed, particularly in the presence of non-MM kinetics. Thus, accounting for the study design becomes important when conducting data analysis (Yang, et al., 2005). Simulations using the numerical method compared the dilution and non-dilution methods (without competitive inhibition from an inactivator metabolite), and no major differences in estimated TDI parameters were observed (Nagar, et al., 2014).

d. Lipid partitioning: The dilution method requires a high concentration of protein in the primary incubation, increasing the non-specific partitioning into microsomes. For highly partitioned compounds, the dilution step causes a re-equilibration of free and partitioned inactivator concentrations, resulting in higher than expected free inactivator concentrations in the secondary incubation (Yadav, et al., 2019). As discussed below, lipid partitioning must be considered when numerical methods are used with a dilution assay protocol.

IV. Data analysis

i. The Replot method—Historically, in vitro TDI datasets have been analyzed under Michaelis-Menten (MM) assumptions, with a replot method. To determine K_I and k_{inact} , the log of percent remaining activity (PRA) versus primary incubation time is first plotted at several inhibitor concentrations, and the slope of each curve at an inhibitor concentration gives the first-order rate constant k_{obs} (observed rate of enzyme loss). These rate constants are then plotted against inactivator concentrations ($[I]$). Non-linear regression of this replot is used to obtain K_I and k_{inact} with the following equation:

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]} \quad \text{Equation 2:}$$

Alternatively, a plot of inactivation half-life ($t_{1/2inact}$) against the reciprocal inactivator concentration can also be used to obtain K_I and k_{inact} (Kitz & Wilson, 1962). The $t_{1/2inact}$ can be obtained from Equation 3:

$$t_{1/2inact} = \frac{0.693}{k_{obs}} \quad \text{Equation 3:}$$

It should be noted that with MM kinetics, the binding constant in a competitive inhibition experiment (K_i) should be identical to the binding constant in a TDI experiment (K_I) (Nagar, et al., 2014). These two values may differ when MM assumptions do not hold.

The data analysis makes the following assumptions: 1) there is negligible metabolism of inactivator in the primary incubation 2) negligible inactivation occurs in the secondary incubation (due to either diluting the inactivator or adding very high concentration of the probe substrate), 3) MM kinetics apply, 4) steady-state conditions apply, and 5) the inactivation is irreversible. These assumptions may not always hold true, which may lead to inaccurate parameter estimation.

As discussed in detail previously (Nagar, et al., 2014), the replot method propagates errors, therefore parameter estimates have high errors (e.g. 1.6-fold average error in K_I) even for low-error (e.g. 10% error) datasets. Importantly, known complexities in CYP TDI such as multiple binding, reversible MIC formation, partial inactivation, and sequential metabolism cannot be analyzed with the replot method (Barnaba, et al., 2016; Korzekwa, et al., 2014). Thus, any complexity in the kinetics of TDI is ignored if the replot method is employed for analysis of rich in vitro datasets. When datasets with very low error (5% or less) and MM kinetics with no complexity are available, the replot method offers two advantages: i) non-specific enzyme loss can be normalized by the zero-inhibitor control data, and ii) the estimated K_I values can simply be corrected *post hoc* for lipid partitioning.

It is noteworthy that TDI data analysis with the replot method can overestimate k_{inact} if non-MM kinetics are observed. When the assumptions of MM kinetics hold, the PRA plot is linear. However, in the presence of kinetics such as reversible MIC formation, partial inactivation, or sequential metabolism, the PRA plot is nonlinear. Utilizing only the linear portion of the PRA plot (i.e. ignoring data for longer primary incubation times) overestimates the k_{inact} , therefore leading to an overprediction of in vivo DDI (Barnaba, et al., 2016; Yadav, et al., 2018).

ii. Numerical methods—The use of ordinary differential equations (ODEs) directly for complex kinetic schemes is proposed to overcome limitations of the traditional replot method (Korzekwa, et al., 2014; Nagar, et al., 2014). The numerical method involves ordinary differential equations (ODEs) that are solved simultaneously to estimate TDI parameters. The advantage of using the numerical method is that no assumptions regarding steady-state, MM kinetics, irreversible inactivation, or initial rates need to be made. Moreover, no assumptions are made regarding the mechanism of inactivation. Hence, models can be modified based on the availability of mechanistic data or the observed kinetics (Barnaba, et al., 2016; Rodgers, et al., 2018). Some assumptions in the development of complex kinetic models described in the sections below include: i) non-specific enzyme loss is modeled as first-order loss from all active enzyme species, and ii) lipid partitioning is assumed to be non-saturable. Different kinetic events like competitive inhibition, inactivation, inhibitor metabolism, substrate metabolism, and enzyme loss can be modeled simultaneously without the need to perform new experiments (Barnaba, et al., 2016; Pham, et al., 2017; Yadav, et al., 2018). The process of obtaining initial estimates for different parameters has been described earlier (Korzekwa, et al., 2014; Yadav, et al., 2018), and is

also discussed below. Improved model identifiability and lower parameter errors with the numerical method compared to the replot method have been described earlier (Nagar, et al., 2014). The numerical approach allows facile modeling of complex TDI characteristics and mechanisms such as non-specific enzyme loss, lipid partitioning, inhibitor metabolism, multiple binding, sequential metabolism, partial inactivation, and reversible MIC formation. These complexities are discussed below.

a. Non-specific enzyme loss: HLM and recombinant enzymes can lose enzyme activity over time in an in vitro incubation. In the replot method, non-specific loss of activity is accounted for by normalizing all inhibitor data to the control (no inhibitor) data. In the numerical method, enzyme loss must be explicitly modeled. The mechanisms of non-specific enzyme loss are not clearly understood. With the assumption that substrate or inhibitor binding can protect the enzyme from non-specific loss (Gonzalez, 2006), we have modeled these processes (unpublished data). Using simulated data, we find that whether or not substrate protects the enzyme, differences in parameter estimates are less than 10%. In a TDI assay, any protection of non-specific enzyme loss by the inactivator cannot be separated from TDI. Therefore, in the absence of mechanistic information about non-specific enzyme loss, we recommend modeling non-specific enzyme loss from all enzyme species. Control data (0 μM inactivator) can be used to obtain an estimate of the first order rate constant for non-specific loss of activity. Often, this parameter can be fixed in TDI models.

b. Multiple inactivator binding (EII models): CYPs are known to exhibit multiple substrate binding kinetics, leading to non-MM kinetics such as biphasic, sigmoidal, or substrate inhibition (Atkins, 2005; Korzekwa, et al., 1998; Marsch, et al., 2018). There has been significant development in terms of mechanistic understanding and inclusion of atypical kinetics in in vitro-in vivo extrapolation (IVIVE) of reversible inhibition (Davydov & Halpert, 2008; Galetin, et al., 2003; Houston & Galetin, 2005; Houston & Kenworthy, 2000; Kenworthy, et al., 2001; Yang, et al., 2012). However, the effect of atypical kinetics on irreversible inhibition has been largely ignored. Two binding events can result in biphasic inactivation, sigmoidal inactivation, or inhibition of inactivation (See Figure 2)(Nagar, et al., 2014). For MM kinetics, the PRA plot displays MM spacing (i.e. hyperbolic k_{obs} versus $[I]$, see Figure 2A). For multiple binding kinetics (EII models), the spacing of the PRA curves can be non-hyperbolic, resulting in a non-hyperbolic replot (see Figures 2B–D). Multiple binding kinetic models to describe inactivation have been developed using the numerical method (Barnaba, et al., 2016; Korzekwa, et al., 2014; Yadav, et al., 2018). An example of a double binding model and the resulting PRA plot is shown in Figure 3 (Nagar, et al., 2014). Model convergence for EII models can be problematic in the absence of rich data. For example, lack of either sufficiently high inhibitor concentrations or longer incubation time points can make it difficult to parameterize k_{inact2} for the second binding site (if $k_{\text{inact2}} > k_{\text{inact1}}$).

c. Sequential metabolism: Sequential metabolism occurs when the product of one oxidative reaction becomes the substrate of a subsequent oxidative reaction (Sugiyama, et al., 1994). Examples of sequential metabolism TDI include the parent drugs diltiazem, verapamil and erythromycin. The N-desmethyl metabolite of diltiazem is further converted

to the nitroso intermediate, which inactivates CYP3A (Zhao, et al., 2007). Similarly, the primary metabolite of verapamil, norverapamil, inactivates CYP3A via a nitroso intermediate (Wang, et al., 2004). In general, a tertiary amine undergoes at least four oxidative reactions before inactivating the enzyme (Jones, et al., 1999; McConn, et al., 2004; Polasek & Miners, 2008; Zhang, et al., 2008). When intermediates can dissociate from the enzyme active site, sequential metabolism can create a lag in CYP inactivation, resulting in non-hyperbolic inactivation kinetics. Modeling in vitro TDI datasets of such inactivators is aided by collecting parent as well as metabolite inactivation data. When modeling the parent inactivator dataset, kinetic parameters obtained from the metabolite dataset can be fixed. For example, rate constants k_5 and k_6 (Figure 4) can be estimated from the metabolite dataset. These parameters can then be fixed while modeling the parent inactivator dataset. Examples of sequential metabolism and TDI modeling with numerical methods have been previously discussed (Pham, et al., 2017; Yadav, et al., 2018).

d. Partial inactivation: Partial inactivation occurs when the inactivated enzyme retains some catalytic activity (Crowley & Hollenberg, 1995; Hollenberg, et al., 2008). This can be observed when a covalent modification to the apoprotein alters the catalytic activity without completely inactivating the enzyme. Partial inactivation results in concave upward curve PRA plots (Figure 5) (Nagar, et al., 2014). In the event of competitive inhibition in the secondary incubation (even after dilution), the curves at higher $[I]$ will be shifted downward and may have different plateaus. This can be diagnosed by an observed spreading downward of the Y-intercepts. Numerical solutions will differentiate this competitive inhibition and partial inactivation from MIC formation, which also has different plateaus (see below). The clinical outcome for partial inactivation can be complex as the extent of inactivation would be substrate dependent. This may prevent extrapolation of DDI results to other victim-perpetrator pairs.

It is noteworthy that potent inactivators may also exhibit nonlinear PRA plots in the presence of multiple metabolic pathways, as shown in Figure 6. Even if a potent inactivator inactivates an enzyme completely, residual activity due to other enzymes (especially in a microsomal assay) may be observed, depending upon the f_m (fraction metabolized via a specific enzyme pathway) of the substrate. Resulting PRA plots may appear similar to partial inactivation, due to a terminal plateau independent of inactivator concentrations (Figure 6). Assays should therefore be designed such that the lowest fractional remaining activity does not approach the substrate f_m .

e. MIC formation: It was shown by Barnaba et al. (Barnaba, et al., 2016) that aging of the MIC is critical in determining the rate of inactivation. As mentioned earlier, the Fe^{3+} :carbene intermediate can either get reduced to Fe^{2+} :carbene which is terminally inactive, or it can dissociate to regenerate the active enzyme. Furthermore, it was recently shown with CYP2D6 inactivation via 3, 4-methylenedioxymethamphetamine that Fe^{2+} :carbene can be reversible (Rodgers, et al., 2018). The reversible nature of the complexes (Fe^{3+} :carbene complex for CYP3A4 and both Fe^{3+} :carbene and Fe^{2+} :carbene complexes for CYP2D6) leads to concave upward PRA plots. The terminal plateau region (at high inactivator concentration) represents the equilibrium between the active EI complex

and inactive enzyme, and is inactivator-concentration-dependent, i.e. different terminal phases will be observed for different [I] (Figure 7). Kinetic models have been developed using the numerical method based on mechanistic data for MIC formation (Barnaba, et al., 2016; Pham, et al., 2017; Rodgers, et al., 2018; Yadav, et al., 2018). Although spectral binding data were evaluated only for PPT (Barnaba, et al., 2016), the general model of the branched pathway for MIC formation was able to capture the observed in vitro kinetics of inactivation for other compounds (e.g. verapamil, diltiazem, erythromycin, and troleandomycin) (Yadav, et al., 2018). As discussed below, K_I and k_{inact} parameters obtained from the numerical method have resulted in better DDI predictions, showing the utility of the numerical method in capturing complex kinetic mechanisms. For example, the numerical method predicted no DDI between MDZ and PPT in rats, in agreement with the observed AUCr of 1.2 ± 0.3 (Barnaba, et al., 2016). However, the replot method overpredicted DDI (predicted AUCr of 4.5 to 9.3). Even more convincing was the evaluation of 77 clinical DDIs in humans, where the average fold error in prediction between observed and predicted DDI was 3.17 with the replot method and 1.45 with the numerical method (Yadav, et al., 2018). Additional discussion is provided in the section on IVIVE below.

f. Inactivator loss: Inactivator depletion due to metabolism can result in concave upward curvature and atypical distribution (with respect to concentration) in the PRA plots (Funaki, et al., 1991; Nagar, et al., 2014). Inactivator depletion during the primary incubation has been observed previously (Berry, et al., 2013; Parkinson, et al., 2011). As seen in Figure 8, at lower inactivator concentrations, inactivator depletion results in recovery of activity (Figure 8B). An inactivator metabolism step can be incorporated in the model (e.g. k_{13} in Figure 8A) if inactivator depletion is suspected. If inactivator concentrations are measured, these data can be used either as an input for model fitting or to compare the predicted inactivator concentrations from the model. Similar to inactivator depletion data, formation of the metabolite of the inactivator can also be measured and used for model fitting and refining purposes (provided that all significant metabolite pathways are known and can be quantified). The potential for inactivator depletion will be higher for high partition ratio compounds. The partition ratio (ratio of inactivator metabolism to enzyme inactivation) of the inactivator can be determined at low [I] by measuring the percent remaining activity versus the ratio [I]/ $[E_t]$ and extrapolating the low concentration data points to 0% activity to give the partition ratio plus 1 (Silverman, 1995). It should be noted that modeling inactivator loss will also provide the partition ratio as k_{met}/k_{inact} , where k_{met} is the rate of inactivator metabolism.

g. Lipid partitioning (Yadav, et al., 2019): Parameters obtained from in vitro metabolism assays need to be corrected for microsomal partitioning to obtain unbound parameters (e.g. the unbound K_I , or $K_{I,u}$). There are several reports in the literature demonstrating the importance of microsomal partitioning and its effect on predicted pharmacokinetic parameters (Austin, et al., 2002; Kalvass, et al., 2001; Margolis & Obach, 2003; McLure, et al., 2000; Nagar & Korzekwa, 2012; Obach, 1997, 1999; Waters, et al., 2014). Drugs can range from very highly partitioned compounds, e.g. itraconazole and amiodarone (Galetin, et al., 2005; Ishigam, et al., 2001; Isoherranen, et al., 2004) to minimally partitioned compounds, e.g. diclofenac and ibuprofen (Obach, 1999), depending on their

physicochemical properties. In vitro kinetic parameters such as K_m , K_i , etc. can be corrected for partitioning by multiplying with an unbound microsomal fraction (f_{um}). This correction can be used to obtain unbound parameters such as $K_{i,u}$ for reversible inhibition. For TDI with simple MM kinetics with no complexities and low-error data, the standard replot method and a post hoc correction with f_{um} can be used to calculate $K_{i,u}$. As discussed above, for analysis of complex TDI datasets, numerical approaches must be used. For such datasets, when the in vitro assay involves a dilution step, post hoc correction of binding parameters with f_{um} is not accurate (Yadav, et al., 2019). The dilution step is incorporated to reduce inactivator concentrations in the secondary incubation, and this step also dilutes the enzyme concentration by the same fold. For highly partitioned inactivators, dilution causes a shift in the binding equilibrium, leading to less dilution of the inactivator in the secondary incubation. Directly correcting the K_i by simply multiplying by f_{um} can lead to errors. To model lipid partitioning, a rapid association (on rate, k_{on}) can be assumed, and dissociation (off rate, k_{off}) can be calculated from f_{um} . Also, lipid partitioning is assumed to be non-saturable. Since f_{um} is typically determined experimentally, no additional parameters need to be estimated.

An advantage of the numerical approach for TDI analysis is that any/all known complexities can be easily incorporated into a single scheme. An example of a kinetic model incorporating several kinetic events discussed above (lipid partitioning, multiple binding, inactivator metabolism, non-specific enzyme loss, and MIC formation) is shown in Figure 9. Examples of fits to experimental data are shown in Figures 10 (-multiple binding with MIC, and lipid partitioning) and 11 (MIC with inactivator depletion, and lipid partitioning).

iii) Estimation of TDI parameters—All binding constants ($K_{i,u}$ values) can be calculated by assuming a k_{on} , and estimating k_{off} ($K_{i,u} = k_{off}/k_{on}$) for each binding event. Errors in K_i and k_{inact} can be calculated by propagating the errors of individual rate constants obtained during model fitting. In the event of multiple binding (e.g. EII), two $K_{i,u}$ values are estimated, one for each binding event. Selection of a ‘relevant’ $K_{i,u}$ value to predict clinical DDIs is complicated. For biphasic or inactivator inhibition kinetics (Nagar, et al., 2014), the lower value (higher affinity) is the obvious choice. However, in the event of sigmoidal kinetics, a single $K_{i,u}$ value may not be appropriate. In this case, a dynamic model (discussed below) with the full TDI ODEs may be necessary. The calculation of an effective k_{inact} will depend on the complexity of the model. For MM models (e.g. Figure 6), k_{inact} is same as k_6 . For complex TDI models, partition analysis and net rate constant concepts can be employed (Cleland, 1975). The standard approach to k_{inact} determination with curved PRA plots has been to ignore longer primary incubation time data, and to use the initial linear portion of the PRA plot. This does not accurately estimate the net inactivation rate, and results in an overprediction of DDI with static methods (Barnaba, et al., 2016; Yadav, et al., 2018). Finally, static methods of DDI prediction (discussed below) require an estimation of k_{inact}/K_i . For many of the complex TDI schemes discussed above, accurate estimation of this ratio is not possible with the replot method, and a numerical approach is necessary.

iv. Covariance in kinetic parameters—At first glance, the schemes in Figures 3–11 appear to have a prohibitive number of rate constants to be parameterized. However, many of

the rate constants are fixed, and others cannot be defined explicitly due to covariance. For example, the simple MM scheme for Equation 1 has 3 rate constants (k_{on} , k_{off} , and k_{cat}) that together define K_m and V_{max} . In the absence of detailed binding data, k_{on} and k_{off} are completely covariant when equilibration is faster than catalysis. When equilibration is slow, k_{on} and k_{cat} are covariant. Practically, one can assume a k_{on} value and solve for K_m . When modeling the CYP schemes discussed above, the values of k_{on} are fixed, greatly decreasing the number of fitted parameters. We have used values between 270 and 810 $\mu M^{-1} min^{-1}$ based on published binding kinetics (Barnaba, et al., 2016; Fersht, 1984). When off rates are very slow, i.e. for very high affinity ligands, $K_m \approx K_d$ (ligand affinity). The numerical method will accurately parameterize the model with the appropriate saturation kinetics, where $K_m = (k_{off} + k_{cat})/k_{on}$.

A second common covariance occurs when maximum velocities are not defined. If inactivator concentrations are not high enough to define k_{inact} , only k_{inact}/K_I can be defined (equivalent to the V_{max}/K_m region in MM kinetics). A common example of poorly defined k_{inact} is the second binding event in a biphasic reaction. For sequential metabolism, the kinetics of the metabolite will not be well characterized without measuring the inactivation parameters for the metabolite. For all these cases, either k_{inact} or K_I can be fixed, and the other optimized. Thus, only the parameterized ratio k_{inact}/K_I is meaningful, and the individual values of each parameter are meaningless.

Finally, sigmoidal inactivation kinetics can occur when either i) the second binding event (EII) occurs with higher affinity than the first binding event (EI), or ii) the inactivation rate from EII is faster than from EI (Nagar, et al., 2014). Again, either the binding constants can be fixed and inactivation rates parameterized, or vice versa. Unfortunately, there seems no obvious or facile method to further characterize this system.

Covariance can be identified from the covariance matrix resulting from the model fit. Highly correlated parameters will also show high parameter standard errors. It should be noted that the quality of the dataset can greatly impact the covariance matrix. If the dataset is small or contains substantial experimental error, random covariance can be misleading. Covariance observed with rich datasets and/or datasets with low error can be informative with respect to model identifiability and the need for additional experimental data. For example, covariance between K_I and k_{inact} could suggest that saturation has not been achieved, and additional data at higher inactivator concentrations are required.

v. Step-by-step approach to analyze in vitro TDI datasets with a numerical approach

1. Determining non-specific enzyme loss—The solvent control data (no inhibitor) is first used to evaluate if enzyme loss (independent of inhibitor) must be included in all models for the data set. In the absence of further mechanistic insight, this enzyme loss pathway should be modeled to occur for every enzyme species. The no-inhibitor data is assumed to exhibit 1st order enzyme loss, therefore the slope of the log percent remaining activity versus pre-incubation time provides the rate-constant for non-specific enzyme loss (k_9 in Figure 9).

2. Plotting a diagnostic PRA plot—The full in vitro TDI dataset (e.g. an 8×10 dataset, 8 [I] each at 10 pre-incubation times in Figure 4B) is now plotted as a PRA plot (log percent remaining activity versus pre-incubation time). This plot is generally corrected for non-specific enzyme loss using the no-inhibitor data as 100% for each time point.

The shape of the PRA plot will provide information on possible kinetic schemes.

- a. Concave upward curvature can be due to a number of kinetic mechanisms, as follows.
 - i. Reversible MIC formation can result in biphasic PRA plots. Barnaba et al (Barnaba, et al., 2016) have proposed that the first phase (at early pre-incubation times) is due to the formation of the Fe^{3+} complex, which is reversible on a time-scale of minutes. The second phase (at later pre-incubation times) is proposed to be due to the reduction of the Fe^{3+} complex to the Fe^{2+} complex. Although the Fe^{2+} complex was proposed to be essentially irreversible for CYP3A4, new evidence suggests that this complex may also be reversible for CYP2D6 (Rodgers, et al., 2018). The characteristic feature of this mechanism is that the second phase of the PRA plots is parallel for different [I] (see Figures 4B and 7B).
 - ii. Partial inactivation will also result in concave upward PRA plots. However, in this case, the terminal phase of all [I] plots will converge at a residual activity level, with a slope approaching zero (Figure 5B). The lines will not converge if competitive inhibition is observed in the secondary incubation.
 - iii. A concave upward PRA plot similar to partial inactivation may also be seen when multiple CYP isoforms catalyze metabolism of the probe substrate. As seen in Figure 6B, all curves converge to an activity equal to $1-f_m$, where f_m is the fraction of probe substrate metabolized by the CYP isoform that is inactivated in the assay. Care should be taken to design the experiment such that the maximum inactivation does not approach this f_m .
 - iv. In the event of inhibitor depletion, concave upward PRA plots will be observed at low [I], but not at high [I] (Figure 8B).

Kinetic schemes can be developed to include all the above scenarios, as seen in Figures 4–8.

- e. An initial lag (i.e. concave downward instead of linear decrease) observed in the PRA plot is consistent with sequential formation of an inactivating metabolite. An example is depicted in Figure 4B.
- f. When multiple inactivators can bind simultaneously to the active site of the enzyme (EII formation), inactivation kinetics may not be hyperbolic. Diagnosis of such non-MM kinetics is possible with the help of both the PRA plot and the replot of k_{obs} vs. [I]. In the case of MM kinetics, the slope (k_{obs}) of the PRA plot approaches k_{inact} in a hyperbolic manner (Figure 2A). In comparison, the

approach to k_{inact} for EII models can be either biphasic, sigmoidal, or show inhibitor inhibition (Nagar, et al., 2014) (Figure 2). This ‘non-MM spacing’ of k_{obs} vs. $[I]$ can most easily be observed in the replot of k_{obs} vs. $[I]$ (Figure 2B–D, middle panels). For biphasic kinetics (Figure 2B), the replot slopes continue to increase, since saturation of the 2nd binding site is not observed. For sigmoidal kinetics, the replot is S-shaped because the spacing of the PRA slopes at low as well as high $[I]$ is compressed (Figure 2C). Finally, for inactivator inhibition, the k_{obs} values decrease at higher $[I]$, evidenced as a reversal in the trend in the PRA slopes (Fig 2D). Figure 3 also shows an example of EII with biphasic kinetics. Any residual competitive inhibition will complicate interpretation of the PRA plot, since the PRA lines do not have the same Y-intercept and slopes cannot be easily compared (e.g. Fig 3B).

- g. In some cases, initial activity (i.e. at low pre-incubation times) can be >100% at higher $[I]$, followed by a decrease in activity (unpublished results). This is presumably due to the inactivator initially activating the metabolism of the probe substrate. Probe substrate activation can be modeled as ESI formation (Pham, et al., 2017; Yadav, et al., 2018).

3. Development of kinetic scheme and ODEs—Based on the above analyses, a kinetic scheme is constructed, and ODEs are derived for each species in the scheme. The base scheme will be either a single binding (MM) or a double binding (EII or ESI) scheme. Added to this base scheme will be any number of kinetic complexities identified from the diagnostic plots. For example, an EII kinetic scheme can be expanded to include MIC formation, non-specific enzyme loss, and inhibitor depletion, as shown in Figure 9. Additional examples are shown in Figures 10 and 11.

4. Modeling lipid partitioning—Particularly for highly partitioned compounds (low f_{um}), it is essential to incorporate lipid partitioning in the kinetic scheme (Yadav, et al., 2019) (see Figure 9). Although model fits may be unaffected for compounds with low partitioning, incorporation of an experimentally determined f_{um} value does not add additional parameters to be estimated, and in most cases will provide better parameter estimates.

5. Obtaining initial estimates—For schemes with a single inhibitor binding event, an initial estimate of K_I can be obtained from the highest primary incubation time data. For EII models, the PRA plot can provide information on the best initial estimates. For biphasic as well as inactivator inhibition kinetics, the first phase of the replot provides an initial estimate of $K_{I,1}$ and $k_{\text{inact},1}$. If the second phase of the replot approaches a plateau, it can provide an initial estimate of $K_{I,2}$ and $k_{\text{inact},2}$. However, this is rarely the case, and only an estimate of the ratio $k_{\text{inact},2}/K_{I,2}$ can be determined. In this case, $K_{I,2}$ can be fixed at $K_{I,2} \gg K_{I,1}$, and $k_{\text{inact},2}$ can be estimated. In other words, $K_{I,2}$ and $k_{\text{inact},2}$ cannot be independently estimated. For sigmoidal kinetics, there may not be a single solution, since sigmoidal kinetics can result from either $K_{I,2} < K_{I,1}$, or $k_{\text{inact},1} < k_{\text{inact},2}$ (Korzekwa, et al., 1998).

An initial estimate of k_{inact} can be obtained from the observed half-life at the highest inhibitor concentration. At saturating inhibitor concentration, $k_{\text{inact}} = 0.693/t_{1/2}$. An estimate

of the rate of product formation from ES (e.g. k_3 in Fig. 9) can be obtained from the product formation at zero inhibitor concentration and zero primary incubation time.

6. Competitive inhibition—If competitive inhibition is observed in the PRA plot (intercepts decreasing on the Y-axis, e.g. Fig. 3B), an estimate of one K_i can be obtained from the zero primary incubation time data. When determining a K_i value from the Y-intercept data, the data should be corrected for lipid partitioning, especially for highly partitioned compounds. The unbound concentration of inactivator should be calculated from the f_{um} value. This is necessary only for initial estimates, since including lipid partitioning in the final model scheme (e.g. Fig. 9) will automatically account for unbound concentrations. As discussed previously (Nagar, et al., 2014), the competitive inhibition K_i should equal the inactivation K_I provided that MM kinetics apply. Even if there is significant enzyme inactivation during substrate incubation, the numerical method will correctly parameterize the inhibition constant ($K_I = K_i$) when MM kinetics apply. When the numerical method cannot correctly model such data with an MM scheme, an EII model should be tested. Another possibility is competitive inhibition by an inactivator metabolite. We have simulated the formation of a metabolite with a K_i 10-fold lower than that for the inactivator, with MM TDI and no kinetic complexity (unpublished data). As expected, for a dilution experiment, the inhibitory metabolite had no impact on the TDI parameter estimates. For a non-dilution experiment, the K_I estimate with a replot method is not affected by competitive inhibition since the Y-intercepts of the PRA plot are not included in the analysis. For the numerical method, a scheme with two binding events would be necessary.

7. Model fitting and analysis—All binding on-rates can be fixed based on published literature values (e.g. $60 - 810 \mu\text{M}^{-1} \text{min}^{-1}$) (Barnaba, et al., 2016; Guengerich, Wilkey, Glass, et al., 2019; Guengerich, Wilkey, & Phan, 2019; Yadav, et al., 2018; Yadav, et al., 2019). Any software package that can fit non-linear ODE models e.g. Mathematica, Matlab, can be utilized. Generally, $1/y$ weighting can be used. Goodness of fit is evaluated by checking model fitted parameter errors, the correlation matrix, residuals, and objective functions such as R^2 . When comparing models with the same dataset, an objective function such as the corrected Akaike information criterion can be used to help select the best model. As with any modeling exercise, initial estimates should be varied to determine a global minimum, and sensitivity analysis should be performed on fixed parameters.

vi. Progress curve methods

Progress curve analysis for inactivation has been previously described for non-CYP enzymes (Duggleby, 1986; Gray & Duggleby, 1989) which was used to investigate pre steady-state kinetics of CYP1A2 inactivation (Fairman, et al., 2007). Burt et al., (Burt, et al., 2012) further adopted a modified progress curve method for in vitro TDI data analysis to overcome some of the limitations of the replot method. While the authors compared the progress curve method with the traditional two-step TDI assay, they did not collect experimental data for a two-step TDI assay. Use of a richer dataset in one case (product formation as well as inactivator depletion in the progress curve modeling) cannot be compared directly with two-step TDI assays where only product formation is typically measured. Others have utilized a single incubation (substrate and inactivator are added to the enzyme in a single step) and

have analyzed substrate metabolism (Fairman, et al., 2007; Salminen, et al., 2011). Progress curves have also been used to evaluate aldehyde oxidase kinetics (Abbasi, et al., 2019).

In order to assess whether progress curve datasets provide better TDI estimates compared to the two-step incubation, we conducted simulations ($n = 100$ per group) by generating the following datasets, each with 5% error: a two-step TDI incubation, an ‘unpaired’ progress curve with no addition of substrate, and a ‘paired’ progress curve with simultaneous addition of substrate and inactivator. All datasets were 6×6 (6 inactivator concentrations, each at 6 time points). A simple EII model was used to generate biphasic, inhibitor inhibition, or sigmoidal datasets (Table 1). Model fitting was conducted for all cases with a single dataset (i.e. product formation for two-step and paired progress curve assays, and inactivator loss for unpaired progress curves) to allow comparison. Further, for the biphasic case, additional model fitting was conducted with paired datasets simultaneously (i.e. both product formation and inactivator loss were fit for two-step as well as paired progress curve assays). As can be seen in Table 1, the progress curve assay offers no advantage over the two-step assay when comparing TDI parameter estimates and errors. In our simulations, the two-step assay often provides TDI parameter estimates with markedly reduced absolute average fold errors over the progress curve assay. Whether this is true for other kinetic schemes besides EII remains to be evaluated.

e. In vitro – in vivo extrapolation (IVIVE) to predict TDI mediated DDIs—In vitro-in vivo extrapolation (IVIVE) to predict TDI mediated DDIs utilize static and dynamic pharmacokinetic models. These methods are discussed below.

i. Static Modeling: DDI predictions with static models use information such as the natural degradation/synthesis rate of the enzyme (k_{deg}), in vitro TDI parameters (K_I and k_{inact}), and fraction metabolized by tissue- and isoform-specific CYPs of the victim and perpetrator drugs. There are various static models used in the literature for prediction of TDI mediated DDI (Blanchard, et al., 2004; Brown, et al., 2006; Galetin, et al., 2008; Kanamitsu, et al., 2000; Ohno, et al., 2008). These models range from basic models to complex mechanistic models. The ratio of k_{inact}/K_I is usually used as an indicator of inactivator potency (Grime, et al., 2009; Obach, et al., 2007; Zimmerlin, et al., 2011).

Mayhew et al developed a static model for DDI predictions assuming only hepatic elimination of low extraction ratio drugs (Mayhew, et al., 2000). However, models typically underpredict DDI if intestinal inhibition is not considered. Higher AUC ratios (AUC_r, victim drug plasma concentration-time area under the curve in the presence of inhibitor versus in absence of inhibitor) have been observed when substrates were dosed orally as compared to intravenously, suggesting significant contribution of intestinal inhibition to DDI (Gorski, et al., 1998; Kharasch, et al., 2004; Olkkola, et al., 1993; Wang, et al., 2005; Zhang, Quinney, et al., 2009). Wang further modified the model to include gut metabolism for prediction of TDI mediated DDI (Wang, et al., 2004).

Obach et al. proposed a prediction model on the basis of IC_{50} values following a primary incubation (Obach, et al., 2007). A comparison of DDI predictions was performed using k_{inact}/K_I or IC_{50} estimates from in vitro TDI assays. It was found that the IC_{50} model

significantly underpredicted the extent of in vivo DDI. Fahmi et al (Fahmi, et al., 2009; Fahmi, et al., 2008) further modified the model and developed a net effect model which incorporated different mechanisms like reversible inhibition, irreversible inhibition and induction. Kirby and Unadkat (Kirby & Unadkat, 2010) modified the model further to include the extraction ratio of the victim drug in the DDI prediction. Table 2 lists some commonly used IVIVE equations for static modeling. The US FDA guidance on the conduct of in vitro DDI studies specifies the modeling and interpretation of TDI experiments using model 4 in Table 2 (<https://www.fda.gov/media/108130/download>).

Static methods suffer from the following deficiencies: 1) Static models utilize a single drug concentration instead of concentration-time profiles of the victim and perpetrator, 2) static models are not able to differentiate between different victim-perpetrator dosing regimens, 3) the impact of active transport on intracellular concentrations of the victim or perpetrator are typically ignored, 4) for sequential metabolism, metabolite concentrations cannot be included in static IVIVE methods, and 5) models accounting for complex kinetics (e.g. multiple binding sites) are not easily incorporated into static equations.

ii. Dynamic modeling: Although static modeling offers a simple approach for predicting TDI based DDI, it often leads to overprediction (Fahmi, et al., 2009; Fahmi, et al., 2008; Xu, et al., 2009; Zhou & Zhou, 2009). The use of compartmental as well as physiologically-based pharmacokinetic (PBPK) models in the prediction of DDI is increasingly reported. Dynamic modeling takes into consideration the victim as well as inactivator concentration-time profile upon a specified dosing regimen, as well as the change in enzyme levels with time. Some literature reports suggest that dynamic models may be better than static models for DDI predictions (Einolf, et al., 2014; Iga & Kirayama, 2017; Sekiguchi, et al., 2011; Zhang, et al., 2010).

One advantage of compartmental modeling is that it does not require numerous physiological inputs, and accurately reproduces the time-dependence of drug concentrations, and changes in enzyme concentrations can also be predicted (Zhang, Quinney, et al., 2009). PBPK modeling requires physiological inputs e.g. tissue blood flow, partition coefficients, etc. One of the main advantages of current commercial PBPK software is that it incorporates population variability and genetic polymorphisms (Wang, 2010; Yang, et al., 2005).

A semi-PBPK model (2-compartmental PK model with physiological models for the gut and liver) was used for prediction of the nonlinear disposition of DTZ and its interaction with MDZ (Zhang, Quinney, et al., 2009). It was found that neither DTZ nor N-desmethyl DTZ alone could account for the observed increase in MDZ AUC. However, with the simultaneous inactivation by DTZ and N-desmethyl DTZ, the fold increase of MDZ AUC after oral and intravenous administration was accurately predicted (Zhang, Quinney, et al., 2009). This study was further modified to include inactivation of CYP3A by N-desmethyl diltiazem in the gut (Yeo, et al., 2010). Similar models have been developed to describe inactivation of CYP3A by verapamil (Wang, et al., 2013) and erythromycin (Zhang, et al., 2010).

Both static and dynamic models with MM assumptions tend to overpredict in vivo DDI. In general, static modeling predictions show accuracy comparable to predictions from dynamic PBPK models (Einolf, 2007; Guest, Rowland- Yeo, et al., 2011; Wang, 2010). It is noteworthy that both static and dynamic models described to date assume MM kinetics for TDI. Incorporation of complex TDI schemes into dynamic DDI models with the numerical method is facile since both PBPK models and numerical TDI models consist of collections of ODEs, and may improve the predictability of these models. Therefore, any complexity in TDI kinetics can be incorporated into dynamic models. For example, a reversible MIC, while not truly an irreversible inactivator, may still be an in vivo inhibitor when co-administered with a victim drug. The extent of inhibition can be predicted using numerical methods and a dynamic model.

iii. Accuracy of IVIVE in predicting in vivo DDI due to TDI: AUC_r values are used to measure the degree of DDI. Per a recent US FDA guidance (<https://www.fda.gov/media/108130/download>), a strong inhibitor increases the AUC of a sensitive index CYP substrate 5-fold, a moderate inhibitor increases the AUC of a sensitive index CYP substrate by 2 to < 5-fold, and a weak inhibitor increases the AUC of a sensitive index CYP substrate by 1.25 to < 2- fold.

The US FDA Guidance on Clinical Drug Interaction Studies proposes two approaches to assess a no-effect boundary (the interval within which a change in a systemic exposure measure is considered not significant enough to warrant clinical action). No-effect boundaries can be based on concentration-response relationships and knowledge of metrics such as the maximum-tolerated dose. The first approach is based on the change in exposure. If the 90 % confidence interval for the measured change in systemic exposures falls completely within these no-effect boundaries, no clinically significant DDI is expected. The second approach uses the default no-effect boundary of 80 to 125%. The 80 to 125% boundaries represent a conservative standard for drugs that have wide safety margins, so the first approach is preferred for evaluating the impact of DDI on the safety and efficacy of the substrate drug.

There is a tendency in reported studies to deem a DDI prediction successful if the predicted AUC_r is within 2-fold of the observed (Einolf, 2007; Fahmi, et al., 2009; Galetin, et al., 2006; Garcia, et al., 2018; Guest, Rowland- Yeo, et al., 2011; Peters, et al., 2012). However, It was suggested by Guest et al. (Guest, Aarons, et al., 2011) that the 2-fold criterion may result in a potential bias toward successful prediction of weak DDI (AUC_r is <2). Moreover, a change in the AUC_r may be difficult to identify due to variability in clinical data. Therefore, a new measure of prediction accuracy which incorporates variability in the pharmacokinetics of the victim drug was proposed (Guest, Aarons, et al., 2011). These new criteria have been used in recent publications (Kenny, et al., 2012; Vieira, Kim, et al., 2014; Wagner, et al., 2015).

Predictions with static models tend to over-predict TDI mediated DDI from in vitro systems (Figure 12) (Fujioka, et al., 2012; Galetin, et al., 2006; Greenblatt, 2014; Ito, et al., 2004; Kenny, et al., 2012; Venkatakrishnan & Obach, 2005). This can be due to many factors, including use of inappropriate in vivo inactivator concentrations (Obach, et al., 2007; Vieira,

Kirby, et al., 2014), variable intestinal factors (e.g. blood flow, rate of absorption, and fraction of drug absorbed) (Brown, et al., 2005; Galetin, et al., 2007; Lin, et al., 1999), the synthesis and degradation rate (k_{deg}) of the enzyme, and inaccurate TDI parameters. Issues around k_{deg} and TDI parameters are discussed below.

The hepatic and intestinal enzyme synthesis rate constants ($k_{deg,h}$ and $k_{deg,g}$ respectively) are important parameters in DDI prediction equations. In the literature, k_{deg} has been measured using both in vitro and in vivo approaches. Since CYP3A is a major drug metabolizing enzyme, much effort has focused on the estimation of CYP3A half-life. There is wide variability in the reported values for CYP3A $k_{deg,h}$ in the literature. The reported values for CYP3A half-life range from 28 to 140 hours (Table 3). One reason for poor DDI prediction may be the use of inaccurate $k_{deg,h}$ and $k_{deg,g}$ values. A number of studies have discussed the impact of k_{deg} on DDI predictions (Galetin, et al., 2006; Venkatakrishnan & Obach, 2005; Wang, 2010).

The $k_{deg,h}$ values derived from in vitro systems (e.g. hepatocytes) tend to be higher (resulting in lower half-life, $t_{1/2} \sim 36$ hours) (Chan, et al., 2017; Pichard, et al., 1992; Ramsden, et al., 2015; Takahashi, et al., 2017) than $k_{deg,h}$ values derived from in vivo studies ($\sim t_{1/2}$ 80 hours) (Bahr, et al., 1998; Fromm, et al., 1996; Hsu, et al., 1997; Rostami-Hodjegan, et al., 1999), indicating system/method specific bias in the estimation of k_{deg} . Recent literature reports (Fahmi, et al., 2008; Mao, et al., 2011; Peters, et al., 2012) have used in vitro derived values for $k_{deg,h}$ ($k_{deg} = 0.00032 \text{ minutes}^{-1}$, or $t_{1/2} = 36$ hours).

Despite experimental error inherent in the determination of in vivo $k_{deg,h}$, these values are possibly closer to the real enzyme synthesis rate than in vitro measurements. Analysis of the static equation for DDI prediction reveals that using a high $k_{deg,h}$ value (i.e. short enzyme half-life) decreases the DDI prediction. Wang reported that using a $k_{deg,h}$ value of 0.03 h^{-1} (which corresponds to a $t_{1/2}$ of 23 hours) improves prediction of TDI mediated DDI. The RMSE and GMFE for DDI predictions with k_{deg} of 0.0077 hr^{-1} were always higher than k_{deg} of 0.03 hr^{-1} (Wang, 2010). Since TDI parameters obtained from replot analysis often overpredict k_{inact} , use of a shorter CYP3A half-life would mitigate the overprediction. This may explain the trend to use a higher $k_{deg,h}$ value (i.e. a shorter half-life than is determined with in vivo experiments) for DDI prediction.

Another possible reason for DDI overprediction is the use of inaccurate in vitro TDI parameters. The replot method considers only the initial rate of inactivation, ignoring the curvature in the PRA plots. This can lead to an overestimation of k_{inact} . Further, since the replot method assumes MM kinetics, it is unable to account for atypical kinetics. This can lead to differences in estimates of $K_{I,u}$ with the two approaches (Nagar, et al., 2014). The numerical method is able to capture non-linearity in PRA plots, and atypical kinetics can be modeled. For example, estimates of $k_{inact}/K_{I,u}$ for MIC forming compounds were 2.4 to 46 fold lower than the replot method and led to improved DDI predictions with the static method (Yadav, et al., 2018). The TDI parameters obtained from the numerical method were able to predict the observed DDI with CYP3A substrates within an average of 1.66-fold as compared to 2.97-fold with the replot method. For 77 clinically observed DDI, a majority of DDI predictions are within 2-fold by using parameters from the numerical method (Figure

12) (Yadav, et al., 2018). Other research groups have also shown that TDI parameters from the replot method tend to overpredict DDI (Fahmi, et al., 2009; Fahmi, et al., 2008; Peters, et al., 2012; Vieira, Kirby, et al., 2014).

4. Conclusions and future directions

It is clear that many CYP-mediated oxidations are not well represented by MM kinetics, making TDI data analysis from a replot difficult. Numerical methods provide a flexible and powerful approach to model TDI. Current software packages allow for the facile solution of complex TDI schemes directly from the ODEs. Both MM and non-MM CYP TDI models can be parameterized with the numerical method without the need for simplifying assumptions. Events occur within an in vitro TDI experiment, e.g. dilution, can be readily incorporated into the models.

Static and dynamic models for DDI prediction currently use MM assumptions for TDI kinetics. Instead of limiting the models to the explicit MM equation, the numerical approach allows for many of the CYP complexities to be added to DDI prediction models. ODEs for enzyme kinetic models can be seamlessly added to PK models including compartmental, PBPK, and hybrid models. We anticipate that more accurate in vitro TDI models will result in better DDI predictions.

We also speculate that the trend to use lower k_{deg} values in IVIVE has occurred in an attempt to balance the overprediction of DDI when using in vitro TDI parameters from a replot analysis. As analysis of in vitro data improves, we anticipate that in vivo estimated k_{deg} values will be used for IVIVE. A complexity not yet addressed is the possibility that enzyme synthesis rates may not equal the enzyme degradation rates in the presence of an inactivator. Inactivators can stabilize CYP3A from degradation, or increase the degradation rate by tagging the protein for ubiquitination (Chien, et al., 1997; Kim, et al., 2016).

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Abbreviations

AUC	area under the curve
AUCr	area under the curve ratio of AUC of victim drug in the presence of inhibitor versus absence inhibitor
CO	carbon monoxide
CYP	cytochrome P450
DDI	drug-drug interaction
DTZ	diltiazem

E_t	total enzyme concentration
FAD	flavin
f_m	fraction metabolized
f_{um}	unbound microsomal fraction
HLM	human liver microsome
IC₅₀	inhibitor concentration at 50% inhibition
[I]	inactivator concentration
IVIVE	in vitro-in vivo extrapolation
k_{cat}	catalytic turnover rate
K_d	ligand affinity constant
k_{deg}	rate of enzyme degradation/synthesis
K_i	competitive inhibition binding constant
K_I	inactivation constant
k_{inact}	rate of inactivation
K_{I,u}	unbound inactivation constant
K_m	Michaelis-Menten constant
k_{met}	rate of inactivator metabolism
k_{obs}	observed rate of enzyme loss
k_{on}	rate of association, on rate
k_{off}	rate of dissociation, off rate
MDZ	midazolam
MBI	mechanism-based inactivator
MIC	metabolite intermediate complex
MM	Michaelis-Menten
ODE	ordinary differential equations
PBPK	physiology-based pharmacokinetic models
PPT	podophyllotoxin
PRA	log percent remaining activity versus primary incubation time plot
rCYP	recombinant cytochrome P450

TDI	time-dependent inactivation, time-dependent inactivator
t_{1/2}	half-life
t_{1/2inact}	inactivation half-life
V_{max}	maximal velocity

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Figure 1. Plausible fate of a TDI inactivator. 1. Product formation, 2. MIC formation with heme, 3. Heme destruction, 4. Modification of apoprotein, 5. Release of metabolite and GSH trapping. CYP: Cytochrome P450, D: Drug molecule which is an inactivator, P: Product, GSH: Glutathione.

Figure 2.

Single (MM) and multiple (EII) binding kinetics. Datasets were simulated with no error, and fit to MM and EII models. Diagnostic PRA plots (top) and replots (middle), and model-fitted activity plots (bottom) are shown for A) single binding (MM) hyperbolic inactivation, B) biphasic inactivation, C) sigmoidal inactivation, and D) inhibitor inhibition.

Figure 3.

A. Kinetic scheme for inactivators showing double binding (EII) kinetics. B. Simulated dataset with 1% error shown in dots. Solid lines are the fit of kinetic scheme in A. E: enzyme, I: Inhibitor, L: lipid, P: product, S: substrate, E^* : inactive enzyme, k: rate constants.

Figure 4.

A. Kinetic scheme for inactivators showing lag in inactivation by MIC formation. Part of the scheme shown in red accounts for lag in PRA plots. B. Simulated dataset with 1% error shown in dots. Solid lines are the fit of kinetic scheme in A. E: enzyme, I: inhibitor, L: lipid, M: inhibitor metabolite, P: product, S: substrate, k: rate constants.

Figure 5.

A. Kinetic scheme for partial inactivators. B. A simulated dataset with 1% error shown in dots. Solid lines are the fit of the kinetic scheme in A. E: enzyme, I: inhibitor, L: lipid, P: product, S: substrate, k: rate constants.

Figure 6.

A. Kinetic scheme for potent CYP3A inactivators showing MM kinetics in HLM. B. Simulated dataset with 1% error shown in dots. Solid lines are the fit of kinetic scheme in A. E: enzyme, I: inhibitor, L: lipid, P: product, S: substrate, k: rate constants.

Figure 7.

A. Kinetic scheme for MIC forming inactivators (multistep inactivation shown in green). B. Simulated dataset with 1% error shown in dots. Solid lines are the fit of kinetic scheme in A. E: enzyme, I: inhibitor, L: lipid, P: product, S: substrate, k: rate constants.

Figure 8.

A. Kinetic scheme incorporating inhibitor depletion (shown in blue). B. The impact of inhibitor depletion on PRA plot. Simulated dataset with 1% error shown in dots. Solid and dashed lines are the fits of scheme in A with and without inhibitor depletion (k_{13}) respectively. E: enzyme, I: inhibitor, L: lipid, P: product, S: substrate, k: rate constants.

Figure 9.

A. Kinetic scheme for MIC forming inactivator with multiple binding kinetics showing different components. Secondary incubation shown in black, lipid partitioning shown in yellow, non-specific enzyme loss shown in brown, first binding event shown in red, second binding event shown in violet, inhibitor depletion shown in blue and inactivation shown in green.

Figure 10.

Kinetic scheme for CYP3A inhibition by troleandomycin (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0 μM) in HLM. (A) Kinetic scheme for the MIC-EII-IL model. E, enzyme; I, inhibitor; L, lipid; P, product; S, substrate; k, rate constant. (B) Experimental (points) and MIC-EII-IL-model-fitted (solid lines) PRA plots. (C) Plot of k_{obs} vs [I] for the standard replot method with linear data points ($n = 4$ points). Reproduced with permission from Yadav et al, *Molecular Pharmaceutics* 2018, 15(5):1979–1995, copyright © 2018, American Chemical Society.

Figure 11.

Kinetic scheme for CYP2D6 inhibition by paroxetine (40, 20, 10, 5, 2.5, 1.25, 0.625, 0 μM) in HLM. A: Kinetic scheme for the MIC-M-IL model. B: Experimental (points) and MIC-M-IL model fitted (solid lines) PRA plots. C: Plot of k_{obs} versus [I] for the standard replot method with linear data points ($n = 4$ points in Fig. 4B). E: enzyme, I: inhibitor, L: lipid, M: inhibitor metabolite, P: product, S: substrate, k: rate constants. Non-specific enzyme loss (E^{***} formation) was modeled from all active enzyme species as first-order degradation. All parameter estimates are listed in Supplementary Materials. Reproduced with permission from Yadav et al, Drug Metabolism and Disposition 2019, 47(7), 732–742.

Figure 12.

Observed versus predicted DDIs using TDI parameters with replot (red) or numerical (blue) methods. Results with different hepatic k_{deg} are shown. Data from 77 clinical studies was used. (A) $k_{deg} = 0.00015 \text{ min}^{-1}$ ($t_{1/2} = 79 \text{ h}$). (B) $k_{deg} = 0.00032 \text{ min}^{-1}$ ($t_{1/2} = 36 \text{ h}$). The solid lines are the lines of unity, the dashed lines (—) are the 1.25-fold lines, and dot-dashed lines (— · — · —) are the 2-fold lines. Reproduced with permission from Yadav et al, *Molecular Pharmaceutics* 2018, 15(5):1979–1995, copyright © 2018, American Chemical Society.

Table 1.
Simulations to compare TDI parameter estimation from standard non-dilution 2-step TDI incubations versus progress curve incubations.

Simulated data were generated with simple EII models, normally distributed 5% error, and 100 simulated datasets were generated for each modeling exercise. Simulated estimate $\pm$ SE is reported, along with absolute average fold error in parentheses. Greater than 1.30 AAFE is marked in bold. ND: not determined.

Biphasic					
Parameter (value in simulated datasets)	2-Step, fit only [P]	Progress curve paired, fit only [P]	Progress curve unpaired, fit only [I]	2-Step, fit [P] and [I]	Progress curve paired, fit [P] and [I]
$K_{I,1}$ (10 μ M)	9.83 ± 0.02 (1.06)	10.1 ± 1.2 (1.07)	8.12 ± 0.15 (1.25)	9.80 ± 0.76 (1.05)	9.83 ± 0.44 (1.04)
$k_{inact,1}$ (0.02 min^{-1})	0.020 ± 0.002 (1.08)	0.03 ± 0.01 (1.59)	0.04 ± 0.01 (1.70)	0.02 ± 0.01 (1.09)	0.02 ± 0.01 (1.37)
$k_{inact,2} / K_{I,2}$ (0.0004 $\mu\text{M}^{-1} \text{min}^{-1}$)	0.00039 ± 0.00004 (1.06)	0.0004 ± 0.0001 (1.29)	0.0003 ± 0.0001 (3.65)	0.0004 ± 0.0002 (1.06)	0.0004 ± 0.0002 (1.49)
Inhibitor inhibition					
$K_{I,1}$ (3 μ M)	3.1 ± 0.3 (1.13)	2.9 ± 0.4 (1.20)	2.4 ± 0.2 (1.25)	ND	ND
$k_{inact,1}$ (0.025 min^{-1})	0.026 ± 0.004 (1.19)	0.027 ± 0.009 (1.40)	0.029 ± 0.007 (1.26)	ND	ND
$K_{I,2}$ (15 μ M)	15.1 ± 2.7 (1.23)	16.6 ± 2.8 (1.27)	19.1 ± 4.5 (1.28)	ND	ND
$k_{inact,2}$ (0.005 min^{-1})	0.005 ± 0.004 (1.65)	0.007 ± 0.009 (3.38)	0.008 ± 0.006 (3.68)	ND	ND
Sigmoidal					
$K_{I,1}$ (10 μ M)	9.91 ± 0.24 (1.03)	9.88 ± 0.57 (1.04)	9.92 ± 0.41 (1.01)	ND	ND
$k_{inact,1}$ (0.0025 min^{-1})	0.005 ± 0.003 (2.19)	0.015 ± 0.017 (7.06)	0.011 ± 0.02 (ND) *	ND	ND
$K_{I,2}$ (10 μ M)	Fixed	Fixed	Fixed	ND	ND
$k_{inact,2}$ (0.025 min^{-1})	0.024 ± 0.003 (1.13)	0.022 ± 0.010 (1.37)	0.025 ± 0.008 (1.13)	ND	ND

* Not determined, as many values approach zero.

Table 2:

Static model for DDI predictions

Model	Equations	Reference
1 ^a	$\frac{AUC_{po}}{AUC'_{po}} = \frac{k_{deg}}{k_{deg} + \frac{[I]k_{inact}}{[I] + K_I}}$	(Mayhew, et al., 2000)
2 ^{a, d, e, h}	$AUCr = \left(\frac{F'_g}{F_g} \right) \left(\frac{1}{\frac{f_m}{1 + \sum_{i=1}^n \left(\frac{k_{inact,i}[I]_{u,i}}{k_{deg}(K_{I,i})} \right)} + (1 - f_m)} \right)$ $\frac{F'_g}{F_g} = \frac{1}{F_g + (1 - F_g) \left(1 + \sum_{i=1}^n \frac{k_{inact,i}I_{g,i}}{k_{deg}(K_{I,i} + I_{g,i})} \right)}$	(Wang, et al., 2004)
3 ^{a, d, e, h}	$AUCr = \left(\frac{1}{\left[1 + \frac{[I]_g}{0.5 IC_{50}} \right] (1 - F_g) + F_g} \right) \left(\frac{1}{\frac{f_m}{1 + \left(\frac{[I]_u}{0.5 IC_{50}} \right)} + (1 - f_m)} \right)$	(Obach, et al., 2007)
4 ^{a, b, d, e, h}	$AUCr = \left(\frac{1}{\left[A_g \times B_g \times C_g \right] (1 - F_g) + F_g} \right) \times \left(\frac{1}{\left[A_h \times B_h \times C_h \right] f_m + (1 - f_m)} \right)$ $A = \frac{1}{1 + \frac{[I]_u}{K_{i,u}}}$ $B = \frac{k_{deg}}{k_{deg} + \frac{[I]_u \times k_{inact}}{[I]_u + K_I}}$ $C = 1 + \frac{d E_{max} [I]_u}{[I]_u + EC_{50}}$	(Fahmi, et al., 2009)
5 ^{c, f, g}	$\frac{AUC'_{IV}}{AUC_{IV}} = \frac{1}{f_{hep} \left(\frac{\frac{1}{EH}}{\left(\frac{1}{EH} - 1 \right) \left(\frac{1}{\sum_{n=1}^p f_{m,n} \left(f_{CL_{int,n}}^{Hep} \right) + (1 - \sum_{n=1}^p f_{m,n})} \right) + 1} \right) + (1 - f_{hep})}$	(Kirby & Unadkat, 2010)

^a[I]_u is the unbound inhibitor concentration in plasma

^b A, B and C are the effect of reversible inhibitions, TDI, and induction respectively.

^c EH is hepatic extraction ratio.

^d F_g is the fraction available after intestinal metabolism

^e f_m is the fraction of systemic clearance of the victim drug.

^f f_{hep} is the fraction of systemic clearance that is subject to the hepatic blood flow limitation.

^g f_{CLint}^{Hep} is the product of the fraction of intrinsic clearance remaining as a result of inhibition, inactivation and induction which are similar to A, B and C.

^h Subscripts 'h' and 'g' denote liver and gut respectively.

Table 3. k_{deg} values reported in the literature for human CYP3A

Half-Life (hr)	k_{deg} (hr^{-1})	Method	References
36.6	0.0189	In vitro	(Chan, et al., 2017)
40.5	0.0171	In vitro	
28.9	0.0240	In vitro	(Ramsden, et al., 2015)
29.6	0.0023	In vitro	(Takahashi, et al., 2017)
44	0.0158	In vitro	(Pichard, et al., 1992)
40	0.0173	In vitro	
36	0.0193	In vitro	(Renwick, et al., 2000)
79	0.0088	In vitro	
40.5	0.0171	In vitro	(Chan, et al., 2018)
49.5	0.0140	In vitro	
26	0.0267	In vitro	(Maurel, 1996)
26.6	0.0261	In vitro	(Dixit, et al., 2015)
49	0.0141	In vitro	
21.5	0.0322	In vitro	
43	0.0161	In vitro	
31.1	0.0223	In vitro	
56	0.0124	In vitro	
140	0.0050	In vivo	(Bahr, et al., 1998)
94	0.0074	In vivo	(Rostami-Hodjegan, et al., 1999)
36 – 50.4	0.0193–0.0138	In vivo	(Fromm, et al., 1996)
36	0.0193	In vivo	
85	0.0082	In vivo	(Hsu, et al., 1997)
70	0.0099	In vivo	(Magnusson, et al., 2008)
96	0.0072	In vivo	(Yang, et al., 2008)
72	0.0096	In vivo	
86.6	0.008	In vivo	(Reitman, et al., 2011)
27.7	0.025	In vivo	(Quinney, et al., 2010)