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THE CONDUCT OF IN VITRO STUDIES TO ADDRESS TIME-DEPENDENT INHIBITION OF DRUG METABOLIZING ENZYMES: A PERSPECTIVE OF THE PHARMACEUTICAL RESEARCH AND MANUFACTURERS OF AMERICA (PhRMA)

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Abbreviations: DDI, drug-drug interactions; IVIVE, in vitro-in vivo extrapolation; MBI; mechanism-based inactivation; MIC, metabolite-intermediate complex; NME, new molecular entity; PBPK, physiologically-based pharmacokinetics; SAR, structure activity relationships; TDI, time-dependent inhibition

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Abstract

Time-dependent inhibition (TDI) of cytochrome P450 (CYP) enzymes caused by new molecular entities (NME) is of concern because such compounds can be responsible for clinically relevant drug-drug interactions (DDI). While the biochemistry underlying mechanism-based inactivation of CYP enzymes has been generally understood for several years, significant advances have been made only in the past few years regarding how in vitro time-dependent inhibition data can be used to understand and predict clinical DDI. In this paper, a team of scientists from sixteen pharmaceutical research organizations that are member companies of the Pharmaceutical Research and Manufacturers of America (PhRMA) offer a discussion of the phenomenon of TDI with emphasis on the laboratory methods employed in its measurement. Results of an anonymous survey regarding pharmaceutical industry practices and strategies around TDI are reported. Specific topics which still possess a high degree of uncertainty are raised, such as parameter estimates needed to make predictions of DDI magnitude from in vitro inactivation parameters. A description of follow-up mechanistic experiments that can be done to characterize TDI are described. A consensus recommendation regarding common practices to address TDI is included, salient points of which include the use of a tiered approach wherein abbreviated assays are first employed to determine whether new molecular entities demonstrate TDI or not, followed by more thorough inactivation studies for those that do in order to define the parameters needed for prediction of DDI.

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Introduction

Pharmacokinetic drug-drug interactions (DDIs) can occur when one drug alters the metabolism of a co-administered drug. The outcome is an increase or decrease in the systemic clearance and/or bioavailability, and a corresponding change in the exposure to a co-administered drug. The clinical consequences of DDIs range from lack of therapeutic efficacy of a life saving drug to severe adverse drug reactions, even including fatalities. Significant drug-drug interactions can lead to termination of development of otherwise promising new therapies, withdrawal of a drug from the market, or severe restrictions/limitations on its use (Wienkers and Heath, 2005). Because of the impact on patient health and safety, DDI was the subject of a position paper in 2003 by scientists from member companies of the Pharmaceutical Research and Manufacturers of America (PhRMA) that focused on the in vitro and in vivo assessment and implications of reversible mechanisms of enzyme inhibition (Bjornsson et al., 2003). The U.S. Food and Drug Administration (FDA) has issued a revised draft guidance for the conduct of in vitro and in vivo drug-drug interaction studies (FDA Guidance for Industry, 2006). This draft guidance was broadly written with the intention to assist in the harmonization of approaches and study designs for better comparison between different drugs and data from various laboratories.

Oxidation reactions catalyzed by the cytochrome P450 enzymes (CYPs) are the most prevalent biotransformations of administered drugs, as well as other xenobiotics and some endogenous compounds (Soars et al., 2007). CYP inhibition has been implicated in the majority of reported clinically relevant drug-drug interactions (Bachmann et al., 2003; Thummel et al., 2000). Also, the inhibited metabolic pathway could lead to decreased formation of an active metabolite (or formation of a drug from a prodrug) resulting in decreased efficacy. The increase of plasma concentrations caused by DDIs can be substantial, as reported for the interaction between ketoconazole or itraconazole (strong CYP3A4 inhibitors) and triazolam (a CYP3A4 substrate), in which exposure to triazolam increased by 22- or 27-fold following co-administration with ketoconazole or itraconazole, respectively (Varhe et al., 1994). For drugs

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with narrow therapeutic indices, increases in plasma concentrations can lead to adverse drug reactions.

Inhibition of CYP enzymes can be classified into either reversible or time-dependent inhibition (TDI) (White, 2000). Reversible inhibition involves rapid association and dissociation of drug and enzyme and may be competitive, noncompetitive or uncompetitive. Typically, TDI results from irreversible covalent binding or quasi-irreversible non-covalent tight binding of a chemically reactive intermediate to the enzyme that catalyzes its formation, resulting in loss of enzyme function. In some cases, TDI could result from reversible inhibition from a metabolite(s) generated in situ. The distinction between the terminology TDI and MBI (mechanism-based inactivator) must be appreciated. A TDI is defined as a compound that demonstrates an increase in the extent of inhibition it causes when it is incubated with the enzyme prior to addition of the substrate. As such, this is a kinetic definition only. MBI refers to a subset of TDI in which specific biochemical experiments are conducted that show that the enzyme acts upon the substrate to form a chemically reactive metabolite that subsequently inactivates the enzyme (Silverman, 1988). These two terms will be used throughout this document and are not interchangeable.

Metabolic drug-drug interactions resulting from TDI can display a delayed onset due to the time-dependence in inhibition and can persist even after the inhibitor has been eliminated as enzymatic activity is only restored by de novo protein synthesis. Time-dependent inhibitors of various human CYP enzymes have been well documented from in vitro studies and many have been shown to cause DDI (Table 1). Several comprehensive reviews on TDI have been recently published (Johnson, 2008; Zhou et al., 2005 and 2007; Venkatakrishnan and Obach, 2007).

Drug metabolism sciences have advanced significantly since the publication of the PhRMA position paper (Bjornsson et al., 2003), the 1997 FDA in vitro drug interactions guidance (Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro), and even since the release of the updated FDA draft drug interactions guidance in 2006, with

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reports on drug-drug interactions involving TDI steadily increasing (Ghanbari et al., 2006). Thus, the Drug Metabolism Technical Group of PhRMA assembled a working group of pharmaceutical industry researchers to address the topic of TDI, which builds upon the treatment of reversible inhibition offered in the earlier publication (Bjornsson, et al., 2003). This group devised an anonymous survey to collect data on current practices on TDI within pharmaceutical industry research organizations and developed recommendations regarding the conduct of in vitro TDI studies. The outcome of these efforts is presented in this article.

The Science of Time-Dependent Inhibition and Predicting DDI

Bioorganic Chemistry of Cytochrome P450 Inactivation. The CYP catalytic cycle is illustrated in Figure 1 and the reader is referred to authoritative accounts of the biochemistry of these enzymes (Guengerich, 2001; Ortiz de Montellano and De Voss, 2002). Several steps will be sensitive to inhibition or inactivation including substrate and oxygen binding or electron transfer. The final products of the catalytic cycle can be stable metabolites or reactive intermediates. The reactivity and nature of a formed metabolite will determine further interactions with CYP. The biochemical mechanisms by which compounds function as mechanism-based CYP inactivators can be divided into three categories: quasi-irreversible or metabolite-intermediate complex (MIC) formation, heme alkylation, and protein alkylation. In some cases, inactivation can be caused by protein alkylation by heme fragments (He et al., 1998). Comprehensive reviews of the chemistry and biochemistry of these processes have previously been provided and are beyond the scope of the present discussion (Fontana et al., 2005; Correia and Ortiz de Montellano, 2005; Kalgutkar et al., 2007; Hollenberg et al., 2008).

Briefly, MIC generally form from molecules possessing amine or methylenedioxy-phenyl functional groups. Oxidative bioactivation of these functional groups can lead to intermediate species (e.g., nitroso, carbene, etc.) that form very tight complexes with the heme iron of the CYP. The complex likely disrupts oxygen binding and subsequent catalysis, and produces a

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characteristic absorption spectrum with a Soret maximum of 448-455 nm. Although chemically reversible, MICs are stable under physiological conditions. Several examples of compounds that inactivate CYPs by this mechanism include the macrolide antibiotics (Franklin, 1991), calcium channel blockers such as verapamil and diltiazem (Ma et al., 2000) and the selective serotonin reuptake inhibitor paroxetine (Bertelsen et al., 2003).

Another CYP inactivation mechanism involves covalent attachment of a reactive species to either the prosthetic heme of the CYP or to protein itself. While in some instances heme adducts are not stable, the inactivation is generally considered to be irreversible (Correia and Ortiz de Montellano, 2005). Perhaps one of the best characterized compounds that functions by covalently modifying the heme, is the relatively non-selective CYP inactivator 1-aminobenzotriazole (Ortiz de Montellano and Mathews, 1981). Other functional groups that can be activated to MBI by CYP metabolism are listed in Table 2. The reader is referred to reviews of the chemistry of CYP inactivators (Fontana et al., 2005; Kalgutkar et al., 2007; Riley et al., 2007; Hollenberg et al., 2008.

Enzyme Kinetic Aspects of CYP Inactivation. CYP catalyzed reactions generally are rapid equilibrium interactions between CYP and its substrate, followed by electron transfers (from oxidoreductase/NADPH and cytochrome b_5/NADH) and oxygen incorporation (Guengerich, 2001) leading to oxidized product (metabolite) formation. As illustrated in Scheme 1, a CYP enzyme catalyzes the conversion of a mechanism-based inactivator to its reactive form EI* with the rate constant k_2. The reactive metabolite can either be released as a stable product (k_3), or react with the enzyme (k_4). The interaction of a reactive metabolite with a P450 enzyme results in inactivation of the enzyme (E-X) through covalent protein binding, heme modification or formation of MIC (Silverman, 1995; Correia and Ortiz de Montellano, 2005) as discussed above. The rate of E-X formation is related to k_2, k_3 and k_4.

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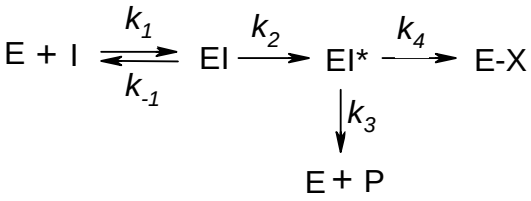

Scheme 1

The rate of CYP enzyme inactivation is proportional to inactivator concentration and can be saturated at high concentration:

$$k_{obs} = \frac{k_{inact} \bullet [I]}{K_I + [I]} \quad \text{Eq. 1}$$

where the k_{obs} is the pseudo first order rate constant of inactivation at inactivator concentration [I], k_{inact} is the maximum inactivation rate (a theoretical value that cannot be experimentally observed), and K_I is the inactivator concentration when the rate of inactivation reaches half of k_{inact}. Further details of the relationships among the micro rate constants and these parameters are beyond the scope of this discussion and can be found elsewhere (Silverman, 1988).

Experimental conditions to determine these kinetic parameters typically involve an "inactivation" incubation (termed in this paper as the "pre-incubation") for specific time periods in the presence/absence of putative inactivator and NADPH, followed by CYP activity measurement by dilution into a secondary incubation. The percentage of CYP enzyme activity loss due to inactivation can be calculated by the equation below (Obach et al, 2007):

$$\%\ \text{activity loss} = 100 \bullet \left[\left(\frac{A_{inactivator}}{A_{vehicle}} \right)_{no\ NADPH} - \left(\frac{A_{inactivator}}{A_{vehicle}} \right)_{+NADPH} \right] \quad \text{Eq. 2}$$

where the A represents measured enzyme activities in the presence and absence of inactivator and NAPDH. $(A_{inactivator}/A_{vehicle})_{no\ NADPH}$ represents the CYP enzyme activity remaining due to the inhibition by the diluted inactivator in the secondary incubation. $(A_{inactivator}/A_{vehicle})_{+NADPH}$ represents the enzyme activity remaining due to pre-incubation loss plus the inhibition by diluted activator in the secondary incubation. For multiple time point pre-incubations, CYP activities

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from 0 min pre-incubation with and without inactivator in the presence of NADPH serve as $(A_{inactivator}/A_{vehicle})_{no\ NADPH}$. Therefore, % enzyme activity loss due to TDI can be expressed as:

$$\%\ \text{activity loss} = 100 \bullet \left[\left(\frac{A_{inactivator}}{A_{vehicle}} \right)_{t_0\ NADPH} - \left(\frac{A_{inactivator}}{A_{vehicle}} \right)_{t_{min}\ NADPH} \right] \quad \text{Eq. 3}$$

The parameters k_{inact} and K_I can be determined by a direct plot of k_{obs} vs. inactivator concentration, and non-least squares fitting using any of a number of commercially available graphing software packages. Historically, a double reciprocal plot of $1/k_{obs}$ vs. 1/[I] was constructed to obtain the inactivation parameters (Kitz and Wilson, 1962). Equations 2 and 3 can be used in abbreviated experiments designed to indicate whether a NME is a TDI, in which the number of preincubation time points and number of concentrations of test compound are greatly reduced. Depictions of these two abbreviated experimental designs for detecting TDI are shown in greater detail in Figures 2 and 3.

Current Status of the Science of Predicting DDI from CYP Inactivation Data. Within the framework of pharmaceutical industrial practices on the assessment of mechanism-based CYP inactivation, a discussion of the utility of generated in vitro parameters for the extrapolation of DDI outcomes must be included. This is not intended to provide a comprehensive review of the literature in this area, but rather to introduce and explain the mathematical models presently in use, the input parameters (in vitro and in vivo) required, and to convey uncertainty associated with these inputs. A more comprehensive review of IVIVE with respect to mechanism-based inactivation has been provided by Venkatakrishnan et al., (2007).

Mathematical models for IVIVE of drug interaction magnitude based on reversible CYP inhibition were known for some time to under predict interactions when the compound producing the DDI was a MBI. A report by Hall and his colleagues described the first viable model that incorporated the kinetic aspects of enzyme inactivation (Mayhew et al., 2000) into extrapolation of DDI. The scaling model essentially stated that because intrinsic clearance (CL_{int}) is inversely

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proportional to exposure (AUC), and directly proportional to enzyme amount, the fractional change in AUC of a victim drug (substrate) in the presence of an inactivator can be described by:

$$\frac{AUC_i}{AUC} = \frac{1}{\left(\frac{k_{deg}}{k_{deg} + \frac{[I] \bullet k_{inact}}{[I] + K_I}} \right)} \quad \text{Eq. 4}$$

where k_{deg} is the natural in vivo enzyme degradation rate constant. The model makes excellent conceptual sense, in that the fraction of enzyme remaining at a steady state (fixed) concentration of inactivator will depend on its natural degradation rate and what amounts to an augmentation of this rate by the rate of inactivation. Several important assumptions must be noted. First, the model assumed that a dose of the affected drug was completely absorbed from the GI tract, and that its clearance was hepatic and dependent only on the affected CYP. Additionally, the model assumed that the inactivator does not alter k_{deg} or the natural enzyme resynthesis rate (k_{syn}). Using the model, the authors extrapolated the anticipated drug interaction between CYP3A substrates and fluoxetine, clarithromycin and N-desmethyldiltiazem and claimed good qualitative agreement with the observed clinical interactions. Predicted values were 1.4, 2.6 and 4.7, respectively, with clinically observed values being 1.26 (Greenblatt et al., 1992), 3.5-9.6 (Yeates et al., 1996; Gorski et al., 1998) and 4.8 (Mousa et al., 2000), respectively. The under prediction of the clarithormycin/midazolam interaction can likely be ascribed to the contribution of intestinal metabolism in limiting the bioavailability of midazolam.

The model was further refined to include the contribution of the affected CYP to clearance ($f_{m,CYP}$ or fraction metabolized), in the event that the substrate has multiple clearance mechanisms.

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$$\frac{AUC_i}{AUC} = \frac{1}{\left(\frac{f_{m,CYP}}{1+\left(\frac{k_{inact} \bullet [I]}{k_{deg} \bullet (K_I + [I])}\right)}\right) + (1 - f_{m,CYP})} \quad \text{Eq. 5}$$

Additionally, potential contributions of gut metabolism to the drug interaction for CYP3A substrates, were considered to yield (Ernest et al., 2005; Wang et al., 2004; Obach et al., 2007):

$$\frac{AUC_i}{AUC} = \frac{1}{F_g + (1\text{-}F_g) \bullet \left(\frac{1}{1+\frac{k_{inact} \bullet [I]_g}{k_{deg(CYP3A,gut)} \bullet (K_I + [I]_g)}}\right)} \bullet \frac{1}{\left(\frac{f_{m,CYP}}{1+\frac{k_{inact} \bullet [I]}{k_{deg} \bullet (K_I + [I])}}\right) + (1\text{-}f_{m,CYP})}$$

Eq 6

The terms are as before, with F_g being the fraction of victim drug (substrate) that escapes gut metabolism in the absence of the inactivator, $k_{deg(CYP3A,gut)}$ is the natural degradation rate constant for CYP3A in the enterocyte and $[I]_g$ is the concentration of inactivator in the gut following oral administration.

For the models discussed, a degree of uncertainty is associated with each input parameter. The inactivation constants K_I and k_{inact} are sensitive to the in vitro study design, and there is considerable variability in experimental designs used (Van et al., 2006; Ghanbari et al., 2006; Yang et al., 2005). The concentration of inactivator at the enzyme active site *[I]*, is not a parameter that can be readily obtained clinically. Several estimates for *[I]* including total C_{max}, unbound C_{max} and estimates of the hepatic portal inlet concentration (unbound and total) have been utilized. The relationship from which the hepatic portal inlet concentration can be estimated is provided below (Ito et al., 1998; Kanamitsu et al., 2000):

$$C_{hepatic,inlet} = C_{max} + \frac{D \bullet k_a \bullet F_a}{Q_h} \quad \text{Eq. 7}$$

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in which D is the oral dose of the inactivator, k_a is its absorption rate constant, F_a is the product of the fractions absorbed and escaping gut metabolism and Q_h is the liver blood flow. When corrected for plasma protein binding, this parameter provided reliable predictions for reversible inhibitors of the CYPs (Obach et al., 2006; Einolf, 2007). However, it was found to over predict interactions for mechanism-based CYP inactivators, and the free systemic C_{max} proved more appropriate (Obach et al., 2007).

The natural degradation rate constant for the affected CYP (k_{deg}) is another important parameter for IVIVE that is not presently ethical to directly measure in humans, in vivo. A summary of the approaches to estimating k_{deg} has recently been provided (Yang et al., 2008), with a guidance to avoid "selective use" of one specific value over another a priori. Both $f_{m,CYP}$, the fraction of systemic clearance associated with a given pathway, and F_g, the fraction escaping gut metabolism (primarily 3A substrates), have high impact on a predicted DDI, based on the models above. Both of these parameters can also be difficult to obtain in vivo. For routinely utilized probe drugs such as midazolam for CYP3A4, estimates of both can be found in the literature (Obach et al, 2006). However, for new compounds in development, estimates of $f_{m,CYP}$ are typically made from a combination of in vitro reaction phenotyping studies and in vivo information from radiolabelled human ADME studies (Venkatakrishnan and Obach, 2007). A review of techniques for the estimation of F_g has recently been provided (Yang et al., 2007; Galetin et al., 2008). Briefly, in vivo estimates of F_g are rarely available during drug development since they minimally require comparison of intravenous versus oral pharmacokinetic data. In fact, this technique provides the product of the absorbed fraction (F_a) and F_g, rather than F_g alone. Other techniques such as the conduct of pharmacokinetic studies in anhepatic patients (Kolars et al., 1991) and administration of grapefruit juice, assuming selective inhibition of intestinal CYP3A4, have also been employed (Ameer and Weintraub, 1997). An in vitro model to approximate F_g has been described (Yang et al., 2007):

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$$F_g = \frac{Q_g}{Q_g + f_{u,g} \bullet CL_{u,int,g}} \quad \text{Eq. 8}$$

where Q_g is the gut blood flow, $f_{u,g}$ is the unbound drug fraction in the enterocyte and $CL_{u,int,g}$ is the intestinal free intrinsic clearance.

The mathematical models described previously are all essentially static models. While they consider the kinetic aspects of mechanism-based inactivation, the predictions they provide are steady state with regard to both inactivator concentration and enzyme amount (after inactivation). Approaches employing physiologically-based pharmacokinetic (PBPK) modeling techniques permit the simultaneous, dynamic simulation of changes to substrate and inactivator concentration and also enzyme amount, with time. A model described by Yang and his colleagues (Yang et al., 2006), examined the potential for inactivation of CYP2D6 by 3,4-methylenedioxymethamphetamine (MDMA), and the potential pharmacokinetic consequences (autoinactivation). The model demonstrated that CYP2D6 was essentially completely inactivated within an hour of an MDMA dose and, therefore, the subsequent administration of additional CYP2D6 inhibitors would not further affect MDMA pharmacokinetics.

PBPK models present several theoretical advantages over static approaches due to their ability to consider changes in concentrations of enzyme, substrate and inactivator with time. For example, a comprehensive comparison of the above described approaches was recently conducted (Einolf, 2007), in this case also using a commercially available population-based ADME simulator (Simcyp®; version 6.4; Jamei et al., 2009). The author noted that for mechanism-based inactivators, the mechanistic static models tended to be more predictive than dynamic models employed in the simulation software. However, it was also noted that the predictions were improved following the substitution of the k_{deg} values in the computational dynamic model with the k_{deg} values used for the static models.

The decision to employ any model to predict DDI magnitude will depend on the availability of reliable in vitro data, the development phase of the putative inactivator or substrate,

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and the need for the prediction in the context of clinical study planning. The static models described above may be more pragmatic than sophisticated PBPK-based approaches, and they have been demonstrated to be of value in early (and perhaps late) drug development for both risk assessment and clinical study planning. With the availability of software tools such as Simcyp®, PBPK-based approaches will undoubtedly become of increasing utility and importance.

Results of the PhRMA Survey

General Comments on the Survey. The survey on PhRMA member industry practices regarding time-dependent inhibition of drug metabolizing enzymes was comprised of 87 questions focused on technical aspects of in vitro experimental study design, the use of the in vitro data generated in making predictions of clinical DDI, and strategic aspects regarding how the data are used in making decisions on progression of NMEs through discovery and development phases. The on-line survey was distributed to 32 R&D organizations of PhRMA member companies, and 17 companies submitted responses (53% response rate) with two-thirds coming from companies employing >10000 staff (mostly companies with multiple research sites). The survey response period was March-April 2008, with administration of the survey and tabulation of the data conducted by staff at PhRMA in order to maintain anonymity of the respondents. A brief list of points for which the survey revealed concordant and disparate responses is in Table 3.

Strategic Aspects and Use of TDI Data. The frequency that companies encounter in vitro DDI due to time-dependent inhibition during the drug discovery-development continuum causes it to be thought of as a relatively common issue across various chemistry classes (>5% of compounds tested) for the majority (60%) of the respondents. A smaller percentage of companies felt it was an uncommon or rare issue (29% and 12% of the respondents, respectively). Structural alerts (i.e., substituents in the NME frequently associated with mechanism-based inactivation of CYP enzymes or bioactivation to reactive electrophiles (Kalgutkar et al., 2007; Riley et al., 2007)) are also considered important, especially if the NME has a similar structure to analogs with known

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TDI issues (e.g., macrolide antibiotics). The use of structural alerts is particularly prevalent in the drug discovery phase since respondents reported that many of the potentially offending substituents have been replaced in final lead candidate molecules before entering the development phase. Thus, given the propensity of certain chemical features to be mechanistically associated with TDI, slightly greater than half of the surveyed organizations deliberately avoid these features (e.g., alkynes, 3-substituted indoles, methylenedioxy-phenyls, etc.; Table 2) during lead generation. While not all organizations avoid chemical alerts for TDI, all survey participants (except one) incorporate assays during drug discovery that enable the early detection of TDI. Approximately 1/3rd of respondents screen for TDI prior to lead optimization and the remainder evaluate TDI during lead optimization. The general strategy is to employ an abbreviated experimental format to identify a TDI liability followed by a more rigorous experimental paradigm (later in discovery or early in development) to characterize the magnitude of inhibition. Data from discovery assays appear to be used primarily to flag compounds as high risk, limiting the progression of the compound into development and/or aiding in the design of molecules devoid of the TDI liability. Most respondents (14 of 17) stated that a "cut-off" value is used to flag high risk compounds in the discovery/research phase. The "cut-off" most frequently employed is based on the loss of enzyme activity relative to a positive control (50% of respondents), predicted clinical AUC change (40% of respondents), and comparison of k_{inact} (20% of respondents) or the ratio of k_{inact} to K_I relative to a positive control (20% of respondents) (note some respondents selected multiple measures). During early drug discovery, data from these abbreviated assays is also used to aid lead optimization (SAR) by the majority of respondents (>80%). The general sentiment is that SAR development is possible (75%) with respect to TDI. The remainder of respondents have not looked into SAR or chemical structural constraints for SAR, or felt that SAR around TDI was very challenging.

Survey respondents unanimously indicated that they routinely evaluate compounds for TDI of some human CYP isoforms in the drug development phase. (The specific enzymes most

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commonly studied are discussed below.) An overwhelming majority of participants stated that during drug development in vitro TDI parameters are used for the prediction of in vivo DDI, and the planning of clinical DDI studies. This indicates that the intent behind gathering these data during the drug development phase is for use in developing a clinical DDI study strategy (e.g., timing of these studies relative to Phases 1-4; which probe substrates to test as objects of DDI; information in product labels, etc.). It is also part of an overall risk assessment made for NMEs during early development.

Responses were varied regarding timing of predictions of clinical DDI and ranged from lead optimization to Phase I. Several in vitro parameters are considered important at the initiation of a TDI investigation, and include knowledge of metabolic stability of a compound in the test system, reversible CYP inhibition, and the CYP isoforms that metabolize the compound.

There was no consensus in the models used to predict the clinical DDI impact of TDI. Respondents used one or more of the following methods to predict clinical DDI potential:

(a) simple model to predict fold change in exposure (AUC_i/AUC), assuming that both F_g and $f_{m,CYP} = 1$ (Equation 4; Mayhew et al., 2000);

(b) models that incorporate experimentally estimated F_g and $f_{m,CYP}$ values (Equation 6; Wang et al., 2004);

(c) models that incorporate the pharmacokinetic (PK) parameters of the object and precipitant drugs, such as Simcyp® or PK models developed within research organizations.

The majority of survey respondents indicated that the most important input parameter for accurate predictions is the assessment of in vivo inactivator concentration. However, to develop a prediction, a variety of inactivator concentrations are used. Prior to the acquisition of clinical exposure data, predicted values of inactivator concentration are based on in vitro models, animal pharmacology, or extrapolated human exposures. Various input values for *[I]* are used by the survey respondents. In most cases, early predictions are refined when the knowledge of the therapeutic concentration of the inactivator becomes available.

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Roughly half of the survey respondents have prospectively evaluated the models for prediction accuracy using either literature data alone, or a combination of literature and internal data sets. About half of the respondents claimed that the accuracy of the methods used to quantitatively predict the magnitude of DDI from in vitro TDI data are not adequate, with a majority claiming that the magnitudes of DDI are overestimated. Sixty percent of participants responded that there are not yet enough data to provide confidence in the quantitative predictive modeling of TDI outcomes. However, most (90%) claimed that the models can generally categorize clinical TDI potential as weak (<2-fold increase in AUC), moderate (2-5-fold increase in AUC) or strong (>5-fold increase in AUC). In addition to an assessment of in vivo inactivator concentration, the choice of the appropriate CYP degradation rate constant (k_{deg}) was recognized as very important to the generation of accurate DDI predictions. However, a consensus on the in vivo k_{deg} values for the CYP isoforms has not been reached. Values that respondents use for CYP half-life values ranged from 11.6 to 140 hours, differing slightly from those described in Table 4.

Survey respondents predict the magnitude of a potential DDI from in vitro TDI data in order to decide on whether a NME can be readily developed, to assist in formulating patient exclusion criteria for early clinical trials, and to determine the necessity of clinical DDI studies. A large majority of survey participants indicated that in vitro TDI data are used to predict the magnitude of clinical DDIs and also influence both the timing and study design of in vivo DDI trials. Positive in vitro TDI data is not used alone to discontinue the progression of an NME. The decision of whether to terminate a compound due to a TDI liability depends on several factors including therapeutic area, therapeutic concentration range, market competitiveness, and probable concomitant medications in the patient population. These criteria are consistent with the model proposed for assessing risk of lead compounds based on bioactivation potential (Evans et al., 2004). Seventy percent of respondents indicated that knowing the mechanism of inactivation impacts decision-making. In some cases, this information can help with selection of alternative

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potential drug candidates. For example, development of an NME that causes quasi-irreversible MIC formation is viewed by some as a lower risk than an MBI that causes covalent binding.

Most respondents indicated that their research organizations have progressed a NME with TDI properties into clinical development within the last five years. There was no consensus regarding cutoff values of TDI parameters that would instigate a clinical DDI study, and reported criteria included predicted fold change in AUC of a concomitantly administered drug, percent loss of CYP activity observed in vitro, and in vitro TDI parameters. If the test compound inactivates multiple CYPs, a variety of approaches are used in order to minimize the number of clinical DDI studies. These include "cocktail" studies (i.e. administration of multiple probe substrates), rank-ordering based on either potency (e.g., k_{inact}/K_I), or predicted AUC changes, and prioritizing the most clinically important enzyme(s). Of the respondents who advanced NMEs with TDI potential to clinical trials, half observed clinical DDI findings that corresponded to the in vitro data, while forty-two percent reported that clinical DDI data were not available at the time of the survey. Although predicted DDI magnitude is frequently used to decide whether to conduct clinical DDI studies, thirty-eight percent of respondents have received requests from government regulatory agencies to conduct clinical drug-drug interaction studies even when in vitro CYP TDI and competitive inhibition are weak or negative.

Mechanistic studies are sometimes pursued when initial findings demonstrate TDI, but most respondents indicated that these studies are seldom performed. These mechanistic studies include measurement of MIC via spectrophotometric methods, determination of partition ratios, reversibility assessments, and detection of CYP adducts using LC/MS or radiometric detection. When conducted, results from these mechanistic studies can assist in determining the risk due to reactive intermediate formation.

Only twelve percent of respondents reported performing in vivo DDI studies in animal models. For compounds that display reversible inhibition or induction in addition to TDI, fifty-

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eight percent of respondents felt that it is somewhat or very important to use a cell based assay or an in vivo preclinical DDI study to identify worst-case scenarios.

Experimental Aspects; Drug Development. Overall, there was considerable concordance of responses regarding the technical aspects of conducting TDI studies in the drug development phase. Among the CYP enzymes examined, all respondents replied that compounds are tested as CYP3A inactivators and the vast majority (>70%) also tested CYP2D6, 2C9, 1A2, and 2C19 (Figure 4). About half also tested CYP2C8 and 2B6, and only two respondents stated that they test other CYPs. All respondents use pooled human liver microsomes as the source of enzyme activity, and a few respondents stated that they also use recombinant enzymes and hepatocytes to make these measurements. When hepatocytes were used to make conclusions regarding TDI, it appears that this may be in conjunction with induction assays, in which inactivation can also be detected. In hepatocyte induction assays both mRNA and enzyme activity endpoints can be measured. If there is discordance between these two outputs such that mRNA is increased or unaffected, while enzyme activity decreases, TDI is possible. This is especially true if the activity measurement is made after the test compound has been removed from the system. All replied that substrates used for enzyme activity measurement were selective CYP probe substrates.

Survey respondents stated that TDI assays are done in a format wherein the test compound is incubated with enzyme and NADPH (pre-incubation) followed by dilution into a second mixture containing the CYP selective probe substrate. The dilution factor reported to be used was 6X or greater for all but one respondent. The number of pre-incubation time points ranged considerably among the survey respondents, with a minimum of four points, but all use six or more test compound concentrations when determining inactivation parameters. Concentrations are selected from preliminary range-finding experiments and reversible inhibition data, with considerations for solubility limitations and anticipated/known relevant clinical concentrations. All run control incubations containing NADPH and no test compound in the pre-incubation and a majority also run a control lacking NADPH, but only some normalize the data to

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control values at each time point. Most respondents do not routinely measure the concentration of their test inactivator throughout the pre-incubation period. For those who do this, some adjust the incubation time and repeat the experiment while others will report the data as is or with the caveat that inactivation parameters may be underestimated. In the secondary assay to measure CYP activity, probe substrate concentrations used are high, with 80% of respondents using at least 5 times the K_M. In more definitive experiments conducted in drug development, 2 or 3 replicate incubations are used (either within-day and some between-day). The log-linear phase of decline is used in data analysis to determine k_{obs} values. Most use non-linear regression to determine K_I and k_{inact} values, although some used simple linearized plots (Kitz-Wilson) or global non-linear regression of non-log transformed data. Positive control inactivators at single concentrations are routinely run and in many cases investigators strive for the positive control data to be within 20% of historical response. Frequently employed positive control inactivators include troleandomycin and erythromycin (CYP3A), furafylline (CYP1A2), paroxetine (CYP2D6), and tienilic acid (CYP2C9).

To demonstrate that a compound is not an inactivator, abbreviated experimental designs are used, even in drug development. These approaches use either single test compound concentrations and multiple incubation time points or multiple test compound concentrations at a single incubation time (the "IC_{50} shift" approach) and are described below. Other abbreviated approaches to determining inactivation are generally not used in the drug development phase; when used the number of test compound concentrations and time points range from 2 to 5.

Experimental Aspects for Evaluating TDI in Drug Discovery. Sixteen of 17 respondents of this survey screen for TDI during the drug discovery phase. For those that did screen prior to candidate selection for development, all respondents tested compounds for CYP3A inactivation. Less than half of companies examined CYP enzymes other than CYP3A4 in the discovery phase. The rank order of the most common CYP enzymes examined following CYP3A were CYP2D6 > CYP2C9 > CYP1A2 = CYP2C19 (Figure 4). All but one respondent used pooled human liver

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microsomes to evaluate TDI in drug discovery. Several respondents (6 of 17) also evaluated TDI using recombinant enzymes and 2 out of 17 indicated they have used human hepatocytes or other cellular models. The majority of respondents used selective CYP probe substrate metabolism with LC-MS/MS analysis or radiochemical detection to measure the remaining CYP activity after TDI assays in the discovery stage, with only one using fluorogenic substrates.

Several companies use an IC_{50} shift assay as their first TDI assessment (47%) and a lower percentage of companies are measuring a percent decline or inactivation rate at a single inhibitor concentration (35%). In an "IC_{50} shift" experiment the IC_{50} is determined for a CYP marker activity before and after the test compound has been incubated with enzyme and NADPH for a set pre-incubation time (Bertelsen et al., 2003; Obach et al., 2007). About half of respondents perform IC_{50} shift experiments by conducting the pre-incubation at a higher concentration and then diluting into the activity assessment incubation (like the inactivation experiments described above), while the other half conduct the activity incubation by adding the probe substrate with no dilution step. The majority of respondents stated that the choice of inhibitor concentrations used in the IC_{50} shift experiment was dependent upon the reversible CYP inhibition IC_{50} and solubility limits. Interpretation regarding the magnitude of shift observed is not necessarily clear-cut. Some respondents stated that a clear criterion is not established, while most cited shift values of 1.2 to 3-fold as indicative of a positive finding, with one respondent citing a 10-fold shift as being clearly positive. A smaller percentage (18%) perform an abbreviated K_I - k_{inact} study (defined as abbreviated due to minimal number of inhibitor concentrations and time points). Definitive K_I - k_{inact} studies are not performed as a first assessment of TDI by any respondent company. It was noted, for a couple of companies with different research sites, that different methods were employed within the same company for early screening (e.g. IC_{50} shifts and % decline in activity).

Unresolved Scientific Issues. Virtually all participants in the survey agree that the purpose of preclinical in vitro TDI studies is to use the data to predict in vivo drug-drug interactions and to propose a clinical DDI study strategy. However, as mentioned above, many respondents

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expressed the opinion that there are not enough clinical data to have confidence in predictive modeling of TDI outcomes and maintain that the various methods used to predict the magnitude of DDI from in vitro TDI data are not accurate enough and often overestimate the DDI outcomes.

Several factors may contribute to the lack of quantitative prediction from in vitro TDI data and as such represent issues in need of resolution with further investigation. These include: (1) Models used to predict the clinical DDI impact of TDI may not be satisfactory, partly due to the limited knowledge and uncertainty of parameters used for the prediction (described in more detail below). (2) CYP enzymes, such as CYP3A4, demonstrate complicated biochemical behaviors that are not fully understood at a molecular level. (3) In vivo drug disposition involves numerous complex physiological processes. Furthermore, CYP enzymes, although important, may not be the only biochemical entities determining the outcome of clinical DDI as other factors such as active tissue uptake mediated by drug transport proteins could impact the magnitude of DDI. Deriving a prediction of an in vivo outcome from a simple in vitro experiment requires knowledge of numerous in vivo processes, many of which may be unknown for a given drug. These complicating factors are not restricted to compounds that are TDI as they apply to reversible inhibitors as well.

Although all these factors could contribute to the difficulty of quantitative DDI prediction, it is apparent that accurate information and knowledge to make the prediction better are not always possessed. The general equations used to calculate the potential for a TDI to cause an increase in AUC as a result of inactivation of hepatic CYP (cited above) contain 5 important terms: $[I]_{in\ vivo}$, K_I, k_{inact}, k_{deg} and $f_{m,\ CYP.}$ For CYP3A4, one may also add a term for the effect on CYP3A in the intestine. For each of these 5 parameters, the question as to what values are the most appropriate can be posed, as the use of different reported values will give different predicted AUC increases.

$[I]_{in\ vivo}$: The majority of survey respondents indicated that an important parameter for accurate predictions is the assessment of in vivo inactivator concentration, $[I]_{in\ vivo}$. However, as

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important as it is, there is no consensus as to what $[I]_{in\ vivo}$ value should be used for prediction of clinical DDI. Although unbound maximum plasma concentration ($C_{max,u}$) is the most commonly used value, other parameters, such as $C_{max,total}$, unbound hepatic inlet C_{max}, average total or unbound plasma concentration, and enzyme site concentration from PK model predictions, have also been used. All values obtained from systemic concentrations, however, are only surrogates for those at the enzyme active site. In addition, the best $[I]_{in\ vivo}$ value can be dependent on the compound (e.g. potentially compensating for active hepatic uptake). Thus, there are considerable uncertainties on the $[I]_{in\ vivo}$ values used for DDI prediction.

K_I and k_{inact}: In well-designed experiments, K_I and k_{inact} can be accurately determined. However, it is well-known that these values can vary depending on the enzyme source (HLM, human hepatocytes and recombinant human CYP). The variability can be due to differences in the availability of the inhibitor or substrate to the CYP in each system (non-specific microsomal or hepatocyte binding, excessive metabolism, etc.). In addition, the well-accepted biochemical method in which inactivation parameters are generated (i.e., pre-incubation with inactivator and enzyme followed by dilution into substrate) may not reflect the in vivo situation in which inactivator and substrate are simultaneously present (i.e., the presence of the substrate could slow the rate of inactivation by competitive inhibition).

k_{deg}: The choice of the appropriate in vivo CYP degradation rate constant (k_{deg}), derived from CYP half-life values, is very important to the generation of accurate DDI predictions. However, the survey revealed no consensus on the in vivo k_{deg} values for human CYP isoforms. Both in vitro and in vivo systems, using various approaches, have been used to estimate the turnover half-lives of human hepatic CYPs. Reported half-lives for all human CYPs range from 23 to 140 hours (Table 4). In some reports, large variability in half-life can be found in the same experiment for a single CYP. For example, individual CYP3A4 half-life estimates range from 2 to 158 hours in one experiment and 85 to 806 hours in another experiment (Yang et al., 2008).

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The influence of such a large difference in enzyme half-life on the recovery of enzyme levels from *de novo* biosynthesis can be enormous.

$f_{m,CYP}$: The fraction of metabolism contributed by a specific CYP to the total clearance ($f_{m,CYP}$) is another important parameter used for the prediction of clinical DDI. The calculated AUC increase can be very sensitive to $f_{m,CYP}$, particularly when the value approaches unity. There are few reports in the literature describing experimental determinations of $f_{m,CYP}$, despite the importance of this parameter in DDI prediction. These can sometimes be derived from genetic polymorphism studies or they can be estimated by combining data from human ADME studies in which metabolic pathways are determined and data from in vitro reaction phenotyping experiments. Sometimes, an $f_{m,CYP}$ of 1 is assumed as a worst case scenario, but this assumption can contribute to overestimations of predicted magnitude of clinical DDI.

It is very clear from the current knowledge on CYP biochemistry, physiological aspects of drug metabolism and distribution, and the models available for DDI prediction, that uncertainties exist in several critical areas. More research is needed to improve the models. Despite these limitations, the majority of survey respondents felt comfortable that the current models can generally categorize clinical DDI potential as weak, moderate or strong from in vitro TDI data.

Experimental Approaches for TDI Studies: Recommendations

The detection of mechanism-based inactivators needs to satisfy a set of criteria that have evolved for characterization of this type of inhibition (Silverman, 1995). This, together with the difficulty in quantitatively predicting the mechanism-based inactivation potential of NME from chemical structure is consistent with the U.S. FDA recommendation of minimally examining NME for time-dependent inhibition following a thirty minute pre-incubation prior to assessment of enzyme activity (FDA Draft Guidance for Industry on Drug Interaction Studies, 2006). Hence, TDI experiments have evolved as a surrogate method for detection of mechanism-based inactivators. The published literature on mechanism-based inactivation studies shows significant

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variation in experimental design, which can result in considerable difference in the estimated inactivation kinetic parameters (Ghanbari et al., 2006). This limited guidance together with the numerous published methods for detecting mechanism-based inactivation of CYP enzymes prompted the PhRMA Drug Metabolism Technical Group to sponsor development of consensus recommendations on best practices for in vitro assessment of mechanism-based inactivation of CYP enzymes. Also, a result from the survey indicated that about 80% of respondents would welcome a consensus set of TDI assays that are acceptable to the FDA. Therefore, a standardized approach would help to minimize inter-laboratory variability for detection and assessment of potency of mechanism-based inactivators.

Examination of published mechanism-based inactivators of CYP enzymes identified functional groups on compounds responsible for mechanism-based inactivation via MIC formation and covalent binding to heme and/or apoprotein (Franklin, 1991; Murray, 1997; Fontana et al., 2005; Kalgutkar et al., 2007; Riley et al., 2007; Hollenberg et al., 2008). However, not all compounds with such functional groups are mechanism-based inactivators, but nonetheless such a structural alert knowledge base can be useful for triggering screening of compounds for mechanism-based inactivation of CYP enzymes. Time-dependent inhibition of CYP enzymes is often used in the pharmaceutical industry to distinguish between reversible inhibition and mechanism-based inactivation, and to screen out compounds with this type of CYP enzyme inactivation property. Hence, the methods for detecting time-dependent inhibition of CYP enzymes, if not properly designed, can result in false positive results due to reversible inhibition from a metabolite(s) generated in situ and false negative results from inadequate resolution of the method to separate potent reversible inhibitors from potent time-dependent inhibitors. The recommended methods to identify a time-dependent inhibitor include a standard dilution method and IC_{50} shift method. Once the NME tests positive for TDI of CYP enzymes, further experiments would be required before it can be concluded to be a mechanism-based inactivator. These follow-up mechanistic experiments include determination of the partition ratio, demonstration of

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irreversibility of inhibition, protection by substrate or competitive inhibitor, and demonstration of inactivation of the catalytic site prior to release of reactive intermediates. However, these mechanistic follow-up experiments are not needed for making predictions of DDI and are therefore not required. The experimental approaches and recommendations for all these methods will be discussed below.

Standard Dilution Method and Determination of K_I and k_{inact}. The most commonly used method to evaluate the time-dependent inhibition potential is the dilution method as described elsewhere (Waley, 1985; Silverman, 1988 and 1995; Guengerich, 1999; Tucker et al., 2001). Although human liver microsomes, hepatocytes, purified CYP enzymes, and cDNA expressed recombinant CYPs have been used for time-dependent inhibition studies in different laboratories, pooled human liver microsomes seem to be the preferred source of enzyme activity for time-dependent inhibition studies. Briefly, human liver microsomes (1-2 mg/ml or lower, if possible) are pre-incubated at 37°C with various concentrations of NME in buffer containing NADPH. (In some cases, addition of EDTA (ethylenediaminetetraacetic acid) can reduce the baseline level of activity decline that can occur when enzyme is pre-incubated in the presence of NADPH and in the absence of NME.) At various time points, aliquots of the pre-incubation mixtures are diluted (≥ 10-fold) with fresh assay buffer containing NADPH and probe substrate at a saturating concentration (≥ 4-fold K_M) to monitor the remaining enzyme activities. The 10-fold or greater dilutions together with using a saturating probe substrate concentration minimize the contribution of reversible inhibition from test compound and/or its metabolite to residual enzyme activities.

A simplified two time-point screening approach is frequently employed by pharmaceutical companies to support drug discovery efforts. First, the % of activity remaining in the presence of one or two concentrations of the test compound (e.g., 10 and 50 μM) before or after 30 minutes pre-incubation is determined at a saturating probe substrate concentration after dilution. The enzyme activity of a sample containing vehicle without pre-incubation can serve as

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control activity. Since this is an initial experiment to assess whether TDI could possibly occur, the high concentration is recommended to avoid false negatives. To assess TDI potential of a test compound, the % of activity loss is calculated by the equation:

$$\%\ \text{activity loss} = 100 \bullet \left(1 - \frac{\left(A_{inactivator}\right)_{t0,NADPH}}{\left(A_{inactivator}\right)_{t30,NADPH}} \right) \quad \text{Eq. 9}$$

It should be noted that this equation represents a degenerate version of equation 3 in which the level of decline in activity of the vehicle control is assumed to be negligible. If there is a considerable decrease in activity in the vehicle control, then correction for this factor is advocated.

Alternately, pre-incubation of test compounds with and without NADPH for 30 minutes followed by assessment of residual enzyme activity after dilution into an incubation mixture containing standard probe substrate at a saturating concentration is another simplified method frequently used to evaluate time-dependent inhibition. Recent improved methods can reliably detect time-dependent inhibition of CYP enzymes (Atkinson et al., 2005; Lim et al., 2005; Obach et al., 2007). Increasing the single concentration for testing from 10 to 50 μM (Riley et al., 2007), evaluation of TDI at concentration corresponding to 10 x IC_{25} (Obach et al., 2007) or titration with multiple concentrations (Lim et al., 2005) improved detection of time-dependent inhibitors including weak time-dependent inhibitors. However, using a single concentration (10 or 50 μM) has the advantage of higher throughput and is ideally suited for evaluating time-dependent inhibition during the drug discovery stage (Wang et al., 2002).

Ultimately, the use of kinetic parameters of inactivation, k_{inact} and K_I, is recommended for predicting human drug interaction potential of time-dependent inhibitors since it is well established (Mayhew et al., 2000; Wang et al., 2004; Obach et al., 2007). Further determination of the inactivation kinetic parameters is recommended when a NME causes more than 20-25% loss of activity after 30 min pre-incubation in a simplified experiment since it has been shown that inactivators causing less than this degree of change are not associated with DDI. At least five

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pre-incubation time points should be used to establish pseudo first-order rate constants for inactivation (k_{obs}) which are calculated from the negative slopes of the lines using linear regression analysis of the natural logarithm of the remaining activity as a function of time. (Importantly, if a biphasic decline in activity is observed, then only the data points representing the initial rate should be used.) To determine k_{inact} and K_I values, at least 5 inhibitor concentrations (plus vehicle control) are recommended. The k_{inact} and K_I values can be derived by nonlinear regression analysis using equation 1. Good enzyme kinetic practice dictates that the kinetic parameters should only be reported if the inhibitor concentrations adequately encompass K_I. Ideally, the selected concentration range for testing permits the time-dependent inhibition process to reach saturation. These two parameters can also be generated by the double reciprocal Kitz-Wilson plot ($1/k_{obs}$ vs $1/[I]$) where k_{inact} is estimated from the reciprocal of the y-intercept and K_I from the negative reciprocal of the x-intercept, however it should be noted that modern methods of nonlinear regression provide better estimates of kinetic parameters than those generated following linearization of the data. In some cases, limitations on the aqueous solubility of inhibitor will limit the upper concentrations that can be tested and in these cases the use of the linearized Kitz-Wilson plot will be necessary to estimate the kinetic parameters.

The following are recommended general aspects of experimental approaches:

(1) The preferred enzyme source is pooled human liver microsomes at 1-2 mg/mL during pre-incubation (or lower concentrations if possible based on limits of detection of analytical methods). Non-specific microsomal protein binding may be an issue for certain NME and correction may be needed for better estimation of K_I.

(2) At least a 10-fold dilution after pre-incubation is necessary to quench any further inactivation during the incubation step and minimize further inhibition of probe substrate by the NME and its metabolites. If the NME is also a potent reversible inhibitor, more than 20-fold dilution and a longer pre-incubation time would improve the method for the evaluation of a time-dependent inhibitor.

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(3) CYP3A4 is the most frequently affected CYP enzyme for TDI and should always be assessed. The recommended probe substrates and positive control TDI for CYP3A4 together with other CYP isoforms are listed in Table 5.

(4) Use of a concentration of the probe substrate that approaches saturation (≥ 4-fold K_M) is recommended to minimize further inhibition of probe substrate by test compound and its metabolites.

(5) Vehicle and minus NADPH control samples should be included.

(6) Low concentrations of organic solvent vehicle (final solvent concentration in the pre-incubation ≤1%) should be employed to minimize inhibition of CYP activities (Iwase et al., 2006).

IC_{50} Shift Method. The time-dependent IC_{50} shift approach (Figure 5) is another method used in the discovery stage for rigorous comparison of several lead candidates for nomination of a compound into development. The data obtained from this method can also be useful for setting up the concentration range for determination of inactivation kinetic constants. The IC_{50} shift method has the capability for simultaneous detection of both reversible inhibitors and time dependent inhibitors, plus the added benefit for potentially predicting drug-drug interactions (Obach et al., 2007). In this assay, human liver microsomes (0.25-1 mg/mL) are pre-incubated for 30 min with various concentrations of NME that surround 10-fold (or greater) of their reversible inhibition IC_{50} value in the presence and absence of NADPH. The pre-incubation samples are then diluted 10-fold (or greater) into a solution containing probe substrate and NADPH for determination of the remaining enzyme activity. Probe CYP substrate concentrations around their K_M values are used. A left shift of the IC_{50} curve from samples pre-incubated with NADPH compared to those without NADPH suggests a potential for enzyme inactivation (Figure 5). However, this left shift of the IC_{50} curve could also theoretically be due to generation of metabolites during the first incubation that are highly potent reversible inhibitors of the enzyme. Furthermore, the lack of a

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time-dependent shift of the IC_{50} curves cannot exclude the possibility that a weak irreversible inhibition may be masked by potent reversible inhibition from test compound. Also, the resolution of the IC_{50} shift method in detection of compound that is both a potent reversible inhibitor and a potent mechanism-based inactivator can be improved by using a saturating probe substrate concentration (≥ 4-fold K_M) and longer pre-incubation time to generate more of the reactive intermediate. The shifted IC_{50} instead of the degree of shift of IC_{50} was found to correlate well with the ratio of k_{inact}/K_I obtained from the standard dilution assay (Obach et al., 2007). Compounds that are known to be TDI showed IC_{50} shifts of ≥1.5X with a 30 min pre-incubation (Berry and Zhao, 2008), suggesting the use of such a values as a cutoff for identifying a compound as a TDI.

The potential contribution of potent reversible inhibition by metabolites generated in situ to shifts in IC_{50} can be assessed by generation of a parallel set of pre-incubation samples in the IC_{50} shift experiment, extraction of these by acetonitrile precipitation, evaporation of the supernatant, and reconstitution in incubation buffer (Li et al., 2005). Then fresh microsomes, along with probe substrate and cofactors are added to measure reversible inhibition. The maximum degree of separation derived from the difference due to loss of enzyme activity in the first pair of IC_{50} curves is attributed to enzyme inactivation plus reversible inhibition by parent compound and its metabolites. In comparison, the maximum difference between the second pair of IC_{50} curves is only due to different extents of reversible inhibition of the enzyme by parent compound vs its metabolites (with an assumption of negligible inactivation during activity measurement). The net enzyme inactivation is calculated from the difference between the differences of these two pairs of IC_{50} curves. The potency of time-dependent inhibition can then be defined as a ratio of the % maximum net loss of activity over the inhibitor concentration that resulted in % maximum net loss of activity. A good correlation of this ratio with the ratio of k_{inact}/K_I obtained in the dilution assay was observed for 9-marketed drugs (Li et al., 2005).

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Other Experiments That Can Yield Mechanistic Insight. Upon observation of TDI and generation of the parameters needed to make predictions of DDI, there are other follow-up experiments that can be done to permit a greater understanding of the underlying mechanism for TDI. It is not proposed that such experiments are required. Rather, these are described here to provide the reader with a greater understanding of other experiments that can yield mechanistic insight into the TDI caused by any given NME or chemical series to which it belongs.

Partition Ratio: The partition ratio (r) is defined as the number of moles of inactivator metabolized to product for each mole of inactivated enzyme (Silverman, 1995). While the partition ratio itself is not useful for predicting DDI, it has been used as a measure of the biochemical efficiency of the mechanism-based inactivation process and can be used to compare potency of NME in early drug research. The kinetic definition of the partition ratio is:

$$r = \frac{k_{cat}}{k_{inact}} = \frac{k_3}{k_4} \quad \text{Eq. 10}$$

where the kinetic constants k_3 and k_4 have previously been defined in Scheme 1.

Factors like reactivity of the reactive intermediate and the rate of diffusion of the reactive intermediate out of the active site of the CYP enzyme can influence the magnitude of the partition ratio of a compound. A highly efficient mechanism-based inactivator will have a low partition ratio because relatively few of the reactive intermediate molecules formed will be able to leave the active site without binding covalently to the enzyme or complexing with the heme iron. Conversely, a higher proportion of the less reactive intermediate molecules will be metabolized to product and leave the active site resulting in a high partition ratio for weak mechanism-based inactivators.

Methods reported for determination of partition ratios of mechanism-based inactivators of CYP enzymes included using k_{cat}/k_{inact} ratio (Jones et al., 1999), from substrate depletion (Kunze and Trager, 1993) and from titration of enzyme with inactivator (Kent et al., 1998). Purified CYP enzymes, cDNA expressed recombinant CYPs and human liver microsomes have been used for

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determination of partition ratio by these three methods. It should be noted that there may be differences in the apparent partition ratio generated using human liver microsomes compared to the intrinsic partition ratio determined using recombinant CYP enzyme due to depletion of the test compound caused by metabolism by the target CYP or other CYP enzymes. The titration method is commonly used for estimation of partition ratio of mechanism-based inactivators of CYP enzymes based on a literature survey (Ghanbari et al., 2006), which indicated that the titration method used recombinant CYP enzymes twice as frequently as compared to use of human liver microsomes. A detailed description of the methods used to determine partition ratios is beyond the scope of this paper and can be found other references (Kent et al., 1998; Lim et al., 2005; Atkinson et al., 2005; Hollenberg et al., 2008).

Dialysis and Microsomal Washing: Following demonstration that a compound is a time-dependent inhibitor, the nature of the inhibition can be assessed by dialysis (Ma et al., 2000) or microsomal washing (Schlezinger et al., 1999) experiments to investigate if the decrease in activity is due to tight reversible or covalent binding of metabolites to the heme and/or protein or due to formation of relatively more unbound and/or more potent metabolites. Small volumes (e.g., 1 mL) of microsomal samples pre-incubated with vehicle control, test compound, and positive control for the specific P450 of interest (known MBI, e.g., mibefradil for CYP3A) can be dialyzed against a large volume (e.g., 1 L) of buffer at 4^{o}C with stirring overnight. Typically, the samples will not show significant volume shift because of the low microsomal protein concentration used (e.g., 0.5 mg/mL). However, it is recommended to determine the protein concentration after dialysis. Activities after dialysis are compared to the dialyzed vehicle control sample to see if there is recovery of activity in the test compound sample.

Repeated microsomal washing is another method to test if enzyme activity can be recovered following treatment with an inactivator. Pre-incubated samples are spun in an ultracentrifuge at 100,000g for ≥30 min and the protein pellet is resuspended with a dounce to provide a homogeneous microsomal suspension. This procedure is usually repeated 2-3 times

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and each time, aliquots of samples are set aside. After this washing procedure, activities are measured along with the protein concentrations. The protein normalized enzyme activities are compared to the pre-incubation vehicle control. If the washed microsomal samples of the NME and negative control show recovery of activities while the positive control does not, the NME can be classified as a time-dependent inhibitor that is not a mechanism-based inactivator.

Ferricyanide Treatment of MIC: The quasi-irreversible MIC generated by coordinate bond formation between the reactive intermediate and the ferrous iron of the heme can be readily identified from the absorbance maximum at the Soret peak at around 450-455 nm by difference spectra scanning (Franklin, 1991). Thus the formation of MIC can be measured in the samples containing one or two concentrations of test compound (e.g., 50 or 100 μM) by scanning from 380 to 500 nm using a dual-beam spectrophotometer to monitor the formation of an absorbance maximum at 455 nm. Human liver microsomes or recombinant CYP are incubated with the test compound in the sample cuvette and vehicle in the reference cuvette. The reaction is initiated by the addition of NADPH.

The MIC can be dissociated, as indicated by a reduction in the Soret absorbance maximum, upon oxidation with ≥50 mM potassium ferricyanide (Bueing and Franklin, 1976). Alternatively, the MIC formed during pre-incubation can be dissociated from further incubation with potassium ferricyanide prior to another 5-fold or greater dilution for residual activity assessment using ≥ 4-fold K_M probe substrate concentration in the presence of a minimum 50 mM potassium ferricyanide (Lim et al., 2005; Watanabe et al., 2007). Oxidation with potassium ferricyanide only dissociates nitrogen-based MICs (Franklin, 1991), therefore oxidation with ferricyanide is not recommended for detection of other MIC forming compounds.

Protection by Substrate or Competitive Inhibitor: Another criterion for a mechanism-based inactivator is the demonstration of decrease in the observed rate of inactivation when a substrate or competitive inhibitor is included during pre-incubation (Silverman, 1988). The

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observed rate of inactivation in the presence of substrate or a competitive inhibitor is compared to the rate in the absence of substrate or a competitive inhibitor to determine if protection from inactivation is afforded. It should be noted that multiple substrate binding sites have been reported for CYP2C9 (Kumar et al., 2008) and CYP3A4 (Kenworthy et al., 1999); therefore, the fulfillment of this criterion may not always be possible for some inactivators of these two CYPs.

Inactivation of Catalytic Site Prior to Release of Reactive Intermediates: Another criterion of a mechanism-based inactivator is that inactivation of the enzyme occurs prior to release of the reactive intermediates from the active site of the enzyme (Silverman, 1988). It is informative to differentiate between enzyme inactivation due to nonspecific covalent modification of outside the active site vs that which occurs at the active site. This type of inactivation will also increase in a time-dependent manner with increasing nonspecific covalent modification of the enzyme from buildup of concentration of the reactive species in solution. This can be addressed by determining whether inactivation is reduced when electrophile scavenging reagents such as reduced glutathione (1-5 mM) or reactive oxygen species scavengers like catalase and superoxide dismutase are included (Kunze and Trager, 1993; Regal et al., 2000).

Recommendations. A decision flow chart is presented in Figure 6. An abbreviated version of the standard dilution approach, such as the measurement of a change in the amount of inhibition observed when the NME (at 1-2 concentrations) is pre-incubated with enzyme and cofactor (Figures 2 and 3) is recommended. The concentration tested should be sufficiently high (e.g. 10-50 μM) to ensure that weak TDI are identified (Riley et al., 2007), with the potential limitation of solubility taken into consideration for some compounds. False positive results (i.e. inactivation observed at a high concentration that turns out to be irrelevant for DDI in vivo will be captured when K_I and k_{inact} are measured in follow-up studies. Alternately the IC_{50} shift method (Figure 5) can be used for initial testing of NME for time-dependent inhibition. Human liver microsomes are the preferred source of enzyme activity. The selection of the specific CYP enzymes to be tested can be based on reaction phenotyping information (i.e., the individual enzymes that are

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responsible for the turnover of the NME) and/or the isoforms that are competitively inhibited by the NME. However, there can be exceptions to using only these criteria for selection of CYP enzymes to test. For example, some sponsor companies have observed TDI in the absence of significant compound turnover. As such, it is generally good practice to evaluate TDI potential for all of the major human CYP enzymes important in the clearance of drugs during the development phase, especially CYP3A4 (Table 5). While many known P450 TDI act upon CYP3A, there are several drugs known to inactivate other important human P450 enzymes (Table 1) and cause DDI. If the results indicate no observable time-dependent inhibition, then this result should obviate any further investigation. Identification of the threshold that defines whether a NME is a TDI is important and should be established for the experimental paradigm being employed, using known positive control inactivators (Table 5). The reason for this is that comparison to different types of control incubations (i.e., vehicle control with pre-incubation done in the presence of cofactor (Figure 3) or a control in which NME is included but the pre-incubation is done in the absence of cofactor (Figure 2)) could yield different conclusions. Furthermore, the typical limits on intra and inter-assay precision (i.e., 10-15%) need to be appreciated when defining what degree of time dependent inhibition is detectable vs simple experimental variability. In assays in which single concentrations of NME are tested, a decrease of 20-25% activity over 30 min pre-incubation (corrected for vehicle control) represents a reasonable cutoff to identify those compounds that are TDI meriting further study. For the IC_{50} shift approach, a decrease of 1.5 to 2-fold for IC_{50} with a 30 minute pre-incubation represents a significant enough change to categorize a NME as a TDI.

For those NME demonstrating TDI using an abbreviated experimental method, further follow-up experiments using a standard dilution method with multiple time points and multiple inhibitor concentrations to determine the kinetic parameters for inactivation (K_I and k_{inact}) are recommended for verification that the NME is a time-dependent inhibitor. These parameters are also essential input values needed for the prediction of human DDI potential from in vitro data.

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For those NME in which K_I and k_{inact} values, along with other input parameters (e.g., $[I]_{in\ vivo}$, $f_{m,CYP}$) suggest the likelihood of a point estimate DDI greater than 2-fold, verification with a clinical DDI study using an appropriate probe substrate (Bjornsson et al., 2003) should be considered. Confidence in such predictions of DDI magnitude is dependent on the confidence of individual input parameters (e.g. $[I]_{in\ vivo}$, k_{deg}) and the known performance of the algorithm employed at scaling in vitro TDI data to the magnitude of DDI caused by known CYP inactivators. Since there is no consensus on k_{deg} values to use in the prediction of DDI and no method to definitively determine such values in humans, individual laboratories should take an empirical approach to determine specific k_{deg} values that accurately scale DDI for positive control compounds using inactivation kinetic parameters generated in their own laboratories. As an alternate shorter approach, determination of k_{obs} at a single high $[I]$ can provide an estimate of k_{inact} and when used in the prediction equations will yield an upper estimate of the magnitude of DDI. The selection of the single high value for $[I]$ will need to be scientifically defensible.

Certainly the science around CYP inactivation will continue to develop. Algorithms in which in vitro data are used to predict in vivo DDI will likely improve. Methodologies used to generate the in vitro data will likely become more automated and streamlined. Also, as medicinal chemists venture into unknown chemical space, it will likely be the case that new structural motifs will be identified as CYP inactivators. Other drug metabolizing enzymes may also increase in importance, and any concerns for inactivation of these will become relevant to the discovery and development of new drugs. The basic concepts underlying the recommendations described in this paper for CYP enzymes should be applicable to other enzymes, although experimental details will differ. As these advances are made, revisiting these recommendations will be needed and adaptations made.

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Figure Legends

Figure 1. Cytochrome P450 catalytic cycle.

Figure 2. A graphical presentation of an abbreviated TDI experiment . In the left panel, the data are shown that would be generated if a complete set of time points were measured. The panel on the right shows the abbreviated TDI experiment with the same data represented with dashed lines. In this experiment, the test compound is examined at just one concentration (i.e. concentration "C" in this example) and is compared to vehicle control, with pre-incubations carried out for 30 min in the presence and absence of NADPH. The notations of activity, i.e. "A" are as described in equation 2. In this case, had a time zero time point been taken, a noticeable decline in activity of 6% would have been observed in the absence of inactivator and NADPH (i.e. $(A_{vehicle})_{no\ NADPH}$). In the example of this experimental set-up, the calculation of the % activity loss would be

$$\%\ \text{activity loss} = 100 \bullet \left[\left(\frac{\text{A}_{\text{inactivator}}}{\text{A}_{\text{vehicle}}} \right)_{no\ NADPH} - \left(\frac{\text{A}_{\text{inactivator}}}{\text{A}_{\text{vehicle}}} \right)_{+NADPH} \right]$$

$$\%\ \text{activity loss} = 100 \bullet \left[\left(\frac{0.82}{0.94} \right)_{no\ NADPH} - \left(\frac{0.20}{0.73} \right)_{+NADPH} \right] = 60\%$$

Thus, even though the inactivator caused an 80% decline in activity, the actual loss attributable to the inactivator at a concentration of "C" is 60%.

Figure 3. A graphical presentation of an abbreviated TDI experiment. In the left panel, the data are shown that would be generated if a complete experiment was done to determine K_I and k_{inact}. The panel on the right shows the abbreviated TDI experiment with the same data represented with dashed lines. In this experiment, the test compound is examined at just one concentration (i.e. concentration "C") and is compared to vehicle control, with pre-incubations in the presence of NADPH carried out for 30 min. The notations of activity, i.e. "A" are as described in equation 3. In this case, there is a detectable decline in activity even without the inactivator such that 21% of

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the activity is lost (i.e. ($A_{vehicle)t30,NADPH}$). Also, even without preincubation, the inactivator causes a decline in activity of 13%, i.e. ($A_{inactivator})_{t0,NADPH}$). These factors need to be accounted for when calculating the percent decline that occurs:

$$\%\ \text{activity loss} = 100 \bullet \left[\left(\frac{\text{A}_{\text{inactivator}}}{\text{A}_{\text{vehicle}}} \right)_{t0,NADPH} - \left(\frac{\text{A}_{\text{inactivator}}}{\text{A}_{\text{vehicle}}} \right)_{t30,NADPH} \right]$$

$$\%\ \text{activity loss} = 100 \bullet \left[\left(\frac{0.87}{1.0} \right)_{t0,NADPH} - \left(\frac{0.20}{0.79} \right)_{t30,NADPH} \right] = 62\%$$

Thus, even though the inactivator caused an 80% decline in activity, the actual loss attributable to the inactivator at a concentration of "C" is 62%.

Figure 4. Percentage of respondents who assess new chemical entities for their ability to cause time-dependent inhibition for specific human cytochrome P450 enzymes in drug discovery and development phases.

Figure 5. Illustration of IC_{50} Shift Data and Change in Inhibition at a Single Concentration (IC_{25})

Figure 6. Schematic Flow Chart for Identification and Characterization of CYP TDI. (Refer to the text for specifics around decision points and threshold critera.)

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Table 1. Drugs and other compounds known to cause time-dependent inhibition of drug-metabolizing human cytochrome P450 enzymes.

1-Aminobenzotriazole (several)	Methoxsalen (CYP2A6)
Amiodarone (CYP3A)	3,4-Methylenedioxymethamphetamine (CYP2D6)
Amprenavir (CYP3A)	3-Methylindole (CYP2F)
Azamulin (CYP3A)	Mibefradil (CYP3A)
Azithromycin (CYP3A)	Midazolam (CYP3A)
Bergamottin (CYP3A)	Mifepristone (CYP3A)
Cannabidiol (CYP3A)	Nefazodone (CYP3A)
Carbamazepine (CYP1A2)	Nelfinavir (CYP3A)
Chlorgyline (CYP1A2)	Nicardipine (CYP3A)
Cimetidine (CYP2D6)	Paroxetine (CYP2D6)
Clarithromycin (CYP3A)	Phencyclidine (CYP2B6)
Clopidogrel (CYP2B6)	Phenelzine (several)
Delavirdine (CYP3A)	Pioglitazone (CYP3A)
Diclofenac (CYP3A)	n-Propylxanthate (CYP2B6)
Dihydralazine (CYP1A2)	Raloxifene (CYP3A)
6,7-Dihydroxybergamottin (CYP3A)	Resveratrol (CYP3A)
Diltiazem (CYP3A)	Rhapontigenin (CYP1A1)
Disulfiram (CYP2E1)	Ritonavir (CYP3A)
Efavirenz (CYP2B6)	Rofecoxib (CYP1A2)
EMTPP (CYP2D6)	Rosiglitazone (CYP3A)
Enoxacin (CYP1A2)	Roxithromycin (CYP3A)
Erythromycin (CYP3A)	Rutaecarpine (CYP1A1, 1B1)
Ethinyl estradiol (CYP3A)	Saquinavir (CYP3A)
2-Ethynylnaphthalene (CYP1A)	Silybin (CYP3A)
Fluoxetine (CYP3A)	Suprofen (CYP2C9)
Furafylline (CYP1A2)	Tabimorelin (CYP3A)
Gemfibrozil glucuronide (CYP2C8)	Tamoxifen (CYP3A)
Gestodene (CYP3A)	ThioTEPA (CYP2B6)
Glabridin (CYP2B6)	Ticlopidine (CYP2B6, 2C19)
Hydrastine (several)	Tienilic acid (CYP2C9)
4-Ipomeanol (CYP3A)	Troglitazone (CYP3A, 2C8, 2C9)
Irinotecan (CYP3A)	Troleandomycin (CYP3A)
Isoniazid (several)	Verapamil (CYP3A)
Lopinavir (CYP3A)	Zafirlukast (CYP3A)
Menthofuran (CYP2A6)	Zileuton (CYP1A2)

EMTPP = (1-[(2-ethyl-4-methyl-1H-imidazol-5-yl)methyl]-4-[4-(trifluoromethyl)-2-pyridinyl]piperazine

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Table 2. Chemical substituents frequently associated with cytochrome P450 time-dependent inhibition.

Substituent	Putative Responsible Entity	Example
Aliphatic Amine	Nitroso	Clarithromycin
Alicyclic Amine	Iminium Ion	Phencyclidine
Cyclopropylamine	Radical	Tranylcypromine
Methylenedioxy-phenyl	Carbene	Paroxetine
Furan	α,β-unsaturated carbonyl or epoxide	Methoxsalen
Thiophene	S-oxide or epoxide	Tienilic Acid
Alkenes	Cation radical	Secobarbital
Alkynes	Ketene or oxirene	Gestodene
2-Alkylimidazole	Imidazomethide	Furafylline
3-Alkylindole	α,β-unsaturated imine	Zafirlukast
Dihaloalkane	Acylhalide	Chloramphenicol
Hydrazine	Radical	Dihydralazine
Aminophenol	Quinoneimine	Nefazodone
Phenol	Quinone	Raloxifene

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Table 3. Similarities and differences in the practice and use of time-dependent inhibition studies in drug discovery and development.

Common Practices (≥75% concordance in responses)	Divergent Practices (<75% concordance in responses)
Strategic Aspects	
- All assess TDI during drug discovery/development continuum - TDI data are used for predicting DDI	- Timing of definitive assays for clinical DDI predictions ranges from lead optimization through phase 1 - No common cut-off values for TDI data for further progression of NMEs - Use of various study designs for TDI assessment in drug discovery (e.g., IC_{50} shift vs % activity loss at single NME concentration, etc.) - No common consideration of structural alerts
Technical Aspects	
- Pooled human liver microsomes (100%) - Major P450 enzymes tested (Figure 4) - LC-MS/MS for measurement of probe substrates (100%) - Solvent control at each time point (- test article + NADPH) are used (100%) - Determine the log-linear phase of enzyme inactivation (100%) - Conduct control incubations without NADPH - Replicate determinations of K_I and k_{inact} are conducted - Positive controls are included - Test article depletion not measured	- Fold dilution used during IC_{50} shift determinations range from no dilution to greater than 10-fold - Number of NME concentrations used to determine inactivation parameters (6 or greater) - Number of time-points used (4 to >6) - Data Analysis ▪ Log-linear regression (k_{obs}) followed by non-linear fitting to determine K_I and k_{inact} parameters ▪ Reciprocal plot (e.g., Kitz-Wilson) to estimate K_I and k_{inact} ▪ Global non-linear regression
Use of TDI Data	
- Current models cannot accurately predict DDI due to TDI - Existing models can categorize compounds as weak, moderate or potent clinical DDI risks - DDI predictions to decide whether to conduct a DDI study and inform its design	- Various models (static vs. dynamic, inclusion of gut first-pass vs. no gut first pass etc.) are used for predicting DDI risk based on K_I and k_{inact} values. - Various values used as surrogates for $[I]_{in\ vivo}$ (e.g. C_{max}, free vs total, etc) - Microsomal and plasma protein binding corrections used by some

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Table 4. Summary of estimates of turnover half-lives for cytochrome P450 enzymes in humans (adapted from Yang et al., 2008).

Enzyme	Range of $t_{1/2}$ values (hr)	
	Estimated from In Vitro Data	Estimated from In Vivo Data
CYP1A2	36-51	39-105
CYP2B6	32	no data
CYP2C8	23	no data
CYP2C9	104	no data
CYP2C19	26	no data
CYP2D6	70	51
CYP2E1	27	50-60
CYP3A4	26-79	36-140

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Table 5. Recommended in vitro probe substrates and inactivators for evaluation of TDI (including MBI) of P450 enzymes.

Isoform	Substrate	Positive Control TDI	Reference
CYP1A2	phenacetin ethoxyresorufin	furafylline	Kunze and Trager, 1993
CYP2B6	bupropion	ticlopidine thioTEPA	Richter et al., 2004 Richter et al., 2005
CYP2C8	paclitaxel amodiaquine	gemfibrozil-glucuronide phenelzine	Ogilvie et al., 2006 Polasek et al., 2004
CYP2C9	diclofenac tolbutamide S-warfarin	tienilic acid	Jean et al., 1996
CYP2C19	S-mephenytoin omeprazole	ticlopidine	Ha-Duong et al., 2001
CYP2D6	dextromethorphan bufuralol	paroxetine	Bertelsen et al., 2003
CYP3A4	testosterone midazolam	mifepristone verapamil troleandomycin erythromycin	He et al., 1999 Wang et al., 2004 Atkinson et al., 2005 McConn, et al., 2004

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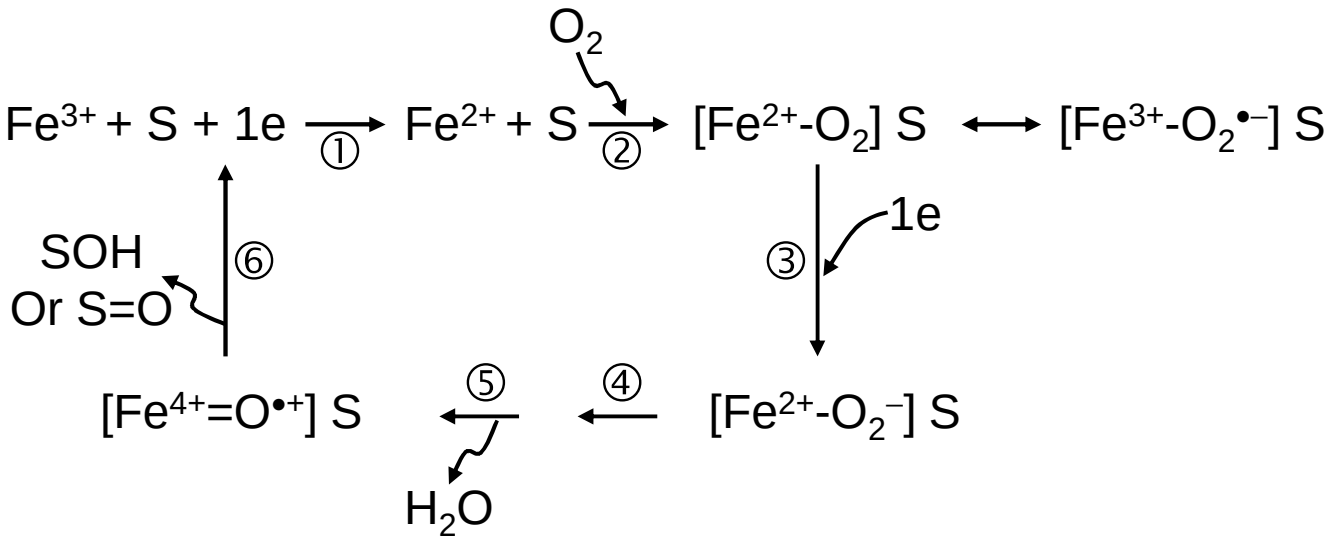

Figure 1

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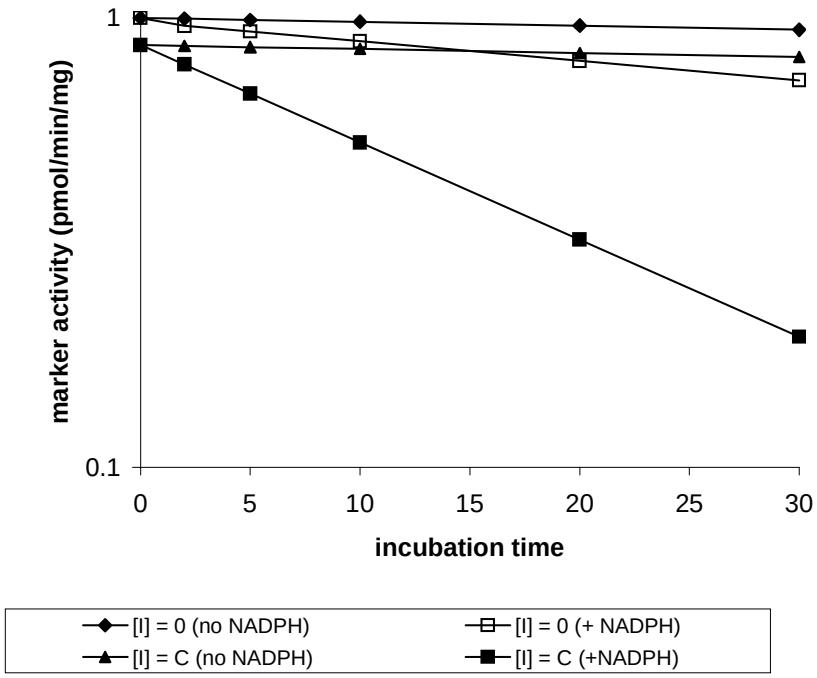

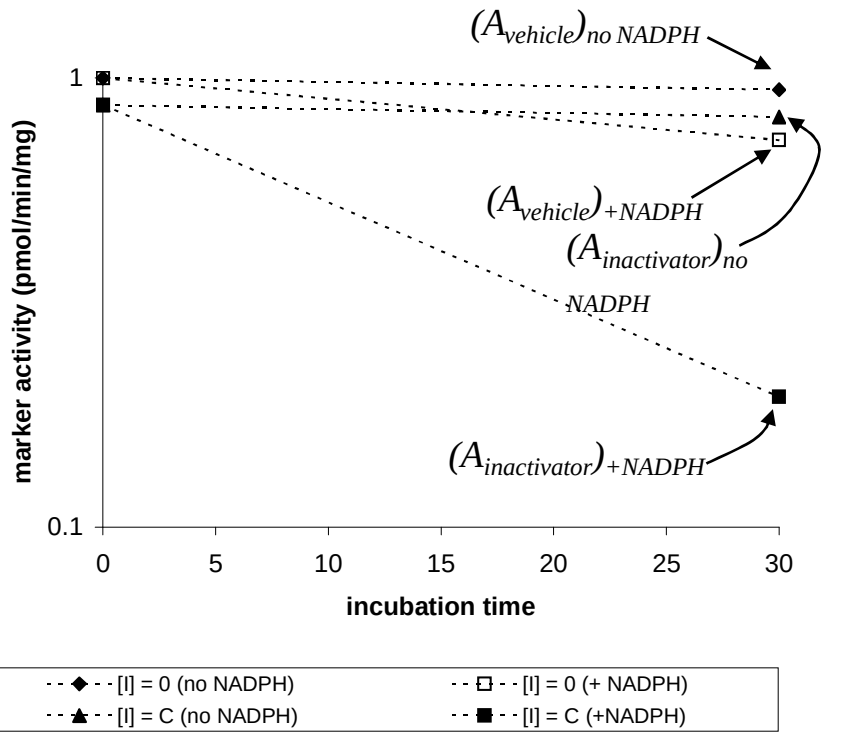

Figure 2

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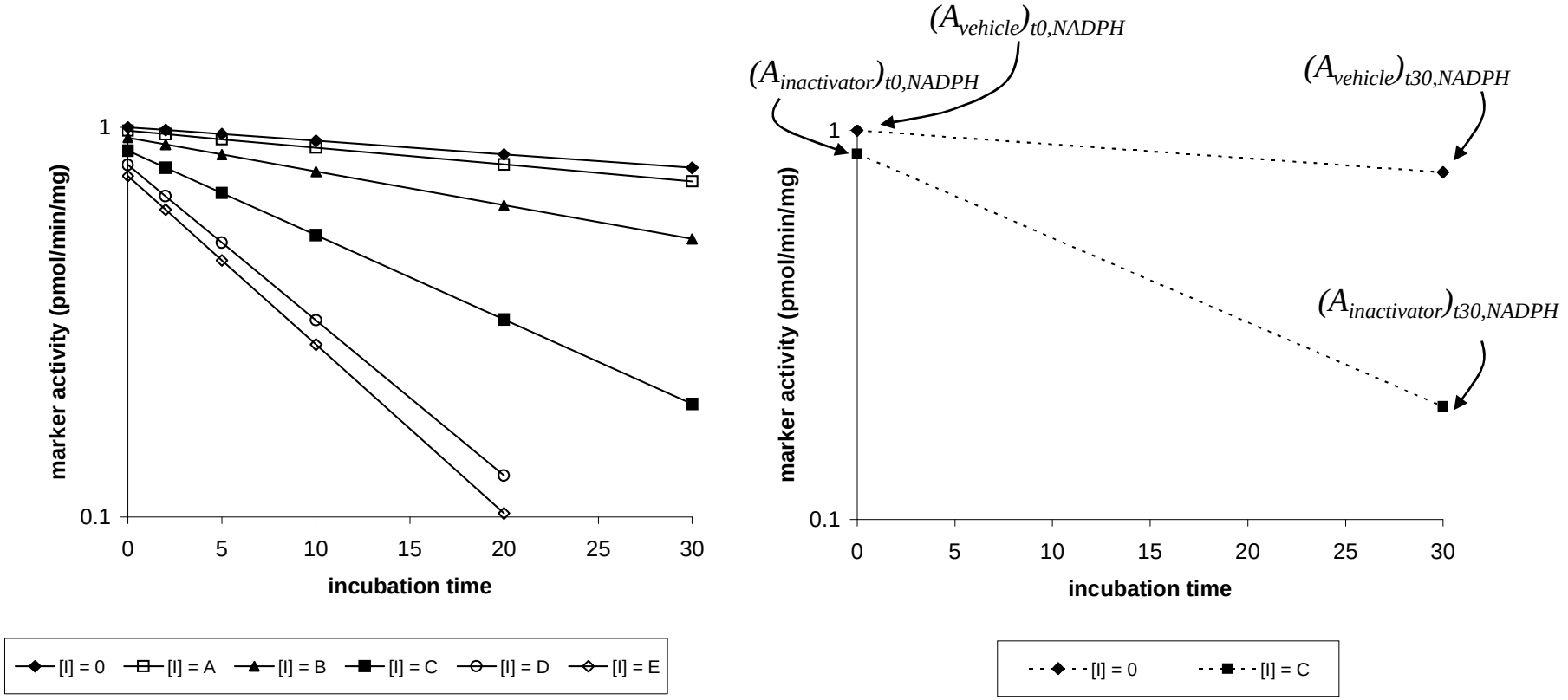

Figure 3

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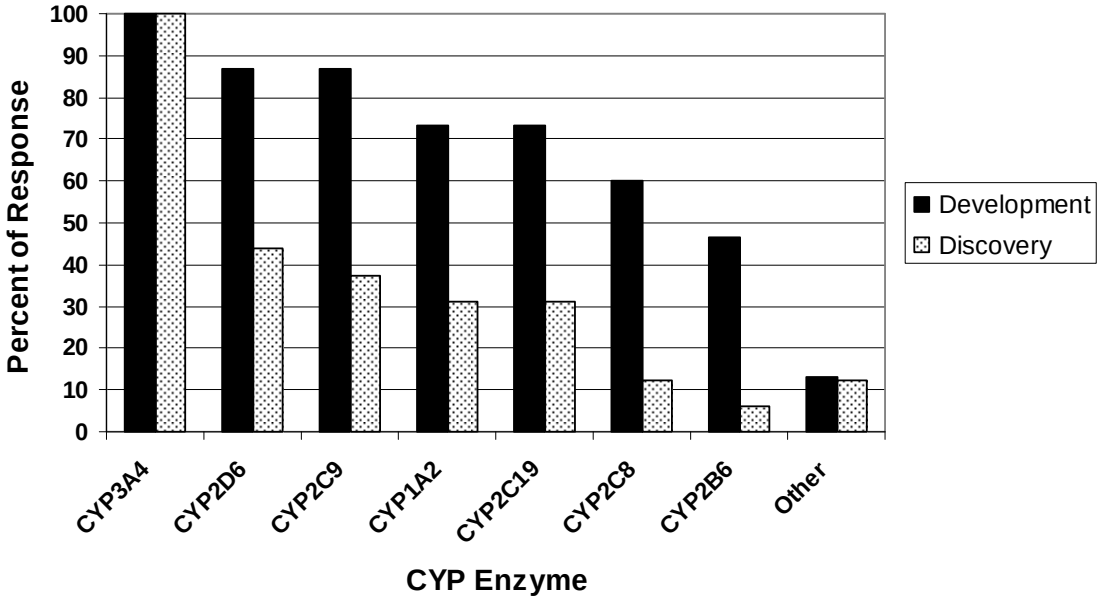

Figure 4

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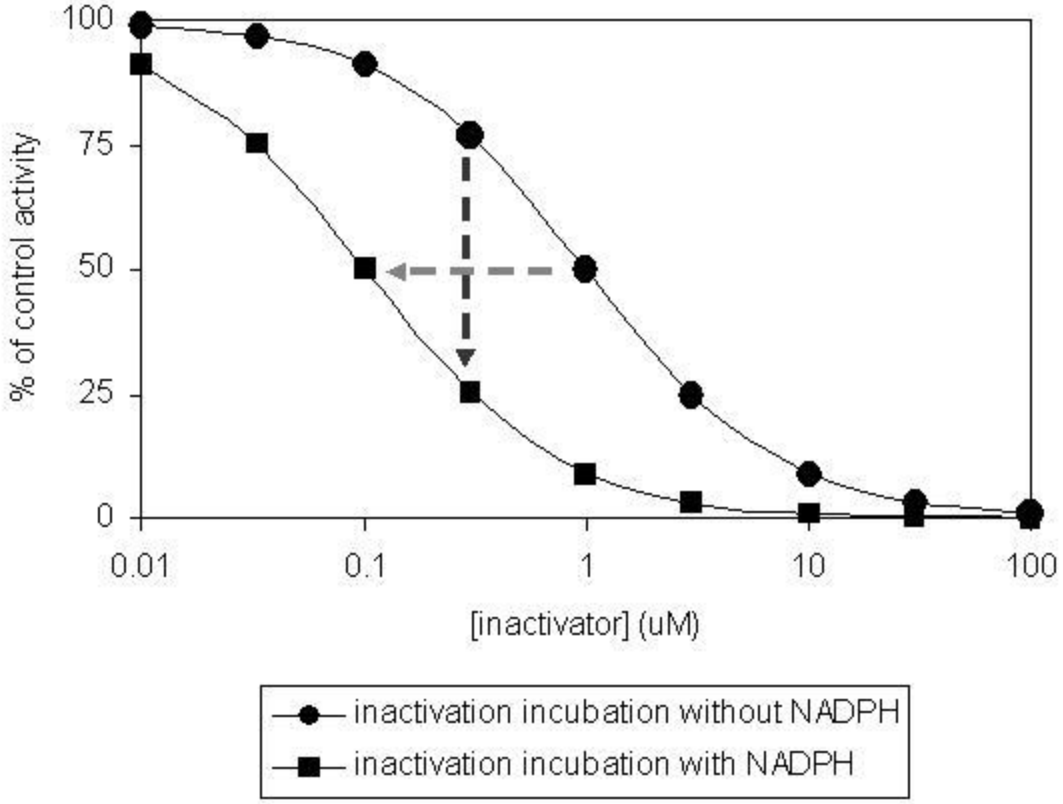
100
75
50
25
0
% of control activity
0.01
0.1
1
10
100
[inactivator] (uM)
inactivation incubation without NADPH
inactivation incubation with NADPH

Figure 5

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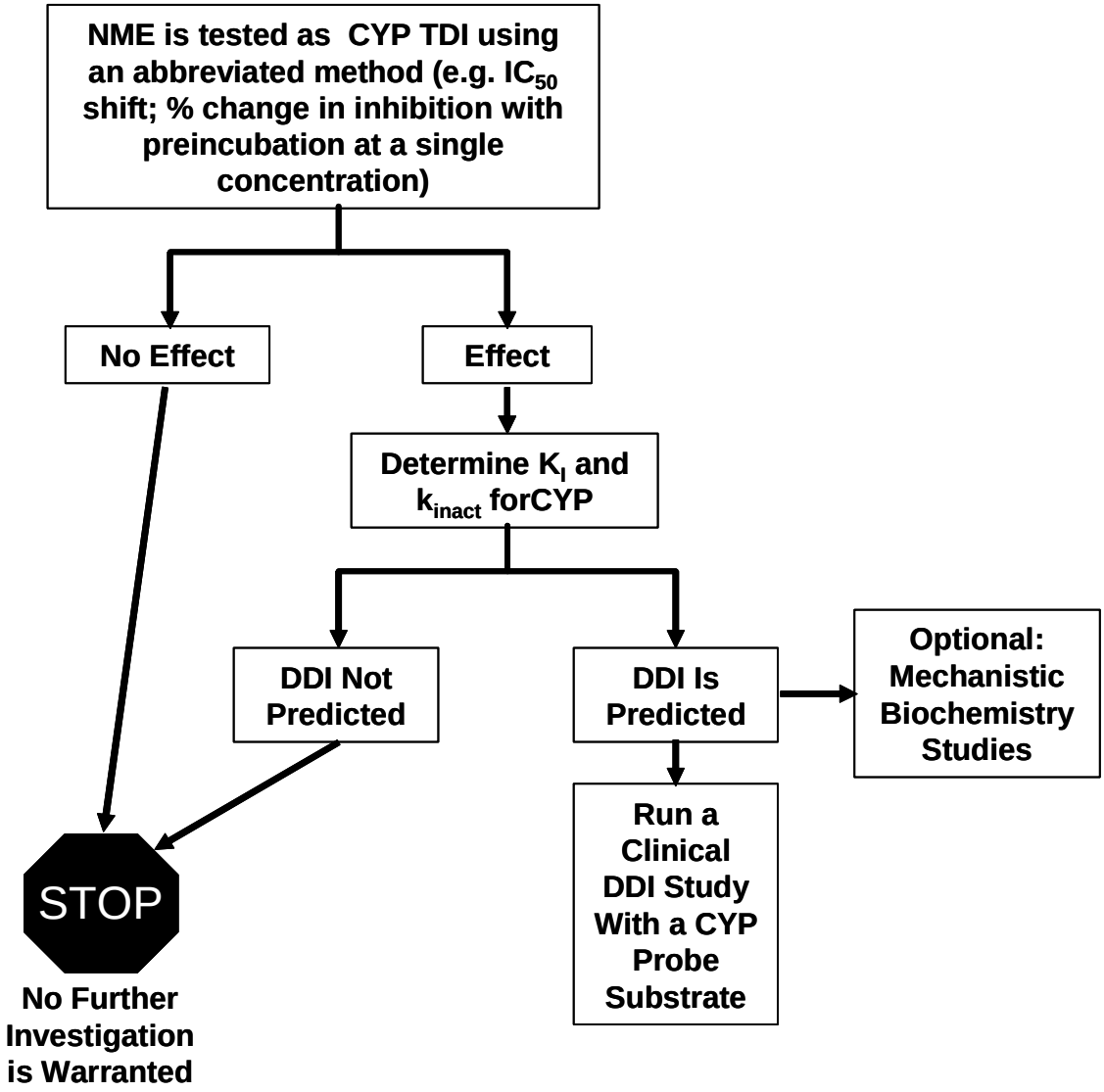

Figure 6

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