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Contribution of Metabolites to P450 Inhibition-Based Drug-Drug Interactions: Scholarship from the IQ DMLG Metabolite Group

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ABBREVIATIONS:

DMLG: Drug Metabolism Leadership Group; IQ: Innovation and Quality Consortium; EMA, European Medicines Agency; FDA, US Food and Drug Administration; DDI, drug-drug interactions; AUC, area under time-concentration curve; P450, cytochrome P450; NCE, new chemical entities; C_{max}, maximum concentration in human plasma; K_i, inhibition potency; MBI, mechanism-based inhibition; K_I, dissociation constant for the enzyme-inactivator complex; k_{inact}, maximum rate for the inactivation; QSAR, quantitative structure-activity relationships.

Abstract

Recent EMA (final) and FDA (draft) drug interaction guidances proposed that human circulating metabolites should be investigated in vitro for their drug-drug interaction (DDI) potential if present at ≥ 25% of parent AUC (FDA) or ≥25% parent and ≥10% of total drug-related AUC (EMA). To examine the application of these regulatory recommendations, a group of scientists, representing 18 pharmaceutical companies of the Drug Metabolism Leadership Group of the Innovation and Quality Consortium, conducted a scholarship to assess the risk of contributions by metabolites to cytochrome P450 inhibition-based DDI. The group assessed the risk of having a metabolite as the sole contributor to DDI based on literature data and analysis of 137 most frequently prescribed drugs, defined structural alerts associated with P450 inhibition/inactivation by metabolites, and analyzed current approaches to trigger in vitro DDI studies for metabolites. The group concluded that the risk of P450 inhibition caused by a metabolite alone is low. Only metabolites from 5 out of 137 drugs were likely the sole contributor to the in vivo P450 inhibition-based DDI. Two recommendations were provided when assessing the need to conduct in vitro P450 inhibition studies for metabolites: consider structural alerts that suggest P450 inhibition potential; and use multiple approaches, including approaches by Yu & Tweedie (2013, a metabolite cut-off value of 100% of parent AUC) and Callegari et al. (2013, the R_{met} strategy), to predict P450 inhibition-based DDI caused by metabolites in the clinic.

Introduction

The recent EMA Guideline on Investigation of Drug Interactions (European Medicines Agency, 2012) and the FDA Draft Guidance on Drug Interaction Studies (US FDA, 2012) have recommended that human metabolites which are present at ≥ 25% of parent AUC (FDA) or ≥25% parent AUC and ≥10% of total drug-related AUC (EMA), should trigger further in vitro inhibition/induction assessment of common drug metabolizing enzymes (mainly P450) to assess these metabolites as possible contributors to drug-drug interactions (DDI). There are a few examples of metabolites being the main contributor to clinically relevant DDI by inhibiting one or more major P450 enzymes. For example, bupropion metabolites, threohydrobupropion and erythrohydrobupropion, have 4- and 12-fold lower K_i values for CYP2D6, respectively, than the parent compound, and are also present at higher concentrations in human plasma than bupropion (Reese et al., 2008). Gemfibrozil glucuronide was identified as an unusual example of a conjugated metabolite which was a considerably more potent inhibitor of CYP2C8 than the parent molecule (Ogilvie et al., 2006). As drug safety (including DDI) is of paramount importance to both regulatory authorities and pharmaceutical companies, these examples clearly highlight the need to thoroughly examine the contribution of metabolites to DDI. To examine the application of these regulatory recommendations, a group of scientists, under the auspices of the Drug Metabolism Leadership Group (DMLG) of the Innovation and Quality (IQ) Consortium, formed the Metabolite-Mediated DDI Scholarship Group. The group, with representation from 18 pharmaceutical companies, conducted a thorough review and summary of the literature on the contribution of metabolites to DDI as well as an assessment of the current practices for in vitro P450 inhibition studies of metabolites in drug development. The Metabolite Scholarship Group focused on the contribution of metabolites to P450 inhibition-based DDI and tackled the issue

from four aspects. First, the group analyzed the risk of DDI caused solely (or mainly) by metabolites based on available literature. Second, the group collected data and analyzed the contribution of metabolites to DDI for the 137 most frequently prescribed drugs in 2012. Third, the group assessed the current literature approaches and common practices among member pharmaceutical companies to trigger in vitro P450 inhibition studies for metabolites to identify their DDI potential prospectively. Last, the group explored the possibility of using structural alerts of metabolites to predict their P450 inhibition/inactivation potential and to trigger in vitro studies. For the risk assessment of metabolites contributing to P450-based DDI, the group focused on identifying cases where a metabolite(s) is the sole contributor to the observed DDI. This manuscript summarizes the recommendations of the Metabolite Scholarship Group.

Risk assessment of contribution of metabolites to P450 inhibition-based DDI using literature data

Several recent publications have assessed the role of circulating metabolites as the perpetrator of DDI, specifically involving inhibition of P450 enzymes through either reversible or mechanism-based inhibition (MBI) (Isoherranen et al., 2009; Yeung et al., 2011). Subsequently, Yu & Tweedie (2013) and Callegari et al. (2013) published strategies that can be adopted by drug researchers in assessing risks of circulating metabolites as P450 enzyme inhibitors. It has been well known that metabolites can be the perpetrators of DDI via P450 inhibition. For example, the observed clinical DDI for verapamil and diltiazem are the combined effects of parent drug and metabolites (Wang et al., 2005; Rowland et al., 2010). A consistent theme from these recent publications was that there is a relatively low risk for clinical DDI (via P450 inhibition) that is

solely attributable to drug metabolites and not the drug itself. In fact, among the 1323 drugs on the US market evaluated by Isoherranen et al. (2009), only 129 drugs (~10% of all drugs) showed clinical DDI via P450 inhibition. The majority (~90%) of the 1323 marketed drugs (likely also including their metabolites) did not inhibit P450 in vivo. Yeung et al. (2011) further analyzed metabolite and parent data from 102 in vivo P450 inhibitors, which were all included in the 129 named drugs in the analysis by Isoherranen et al. (2009) with the exception of one drug. The exposure and K_i data for the parent and metabolites were available for only 24 of the 102 P450 inhibitors. When plasma concentrations and in vitro inhibition K_i values of metabolites were considered, only 3 drugs (amiodarone, bupropion, and sertraline) had clinical DDI via P450 inhibition attributable to metabolites alone (Figure 1). The results are largely consistent with the general understanding that metabolism of drugs usually results in metabolites with increased hydrophilicity relative to that of the parent drugs and decreased affinity for drug metabolizing enzymes. It is worth noting that metabolites may generally have lower plasma protein binding than the parent drug, which results in a higher free fraction. All points considered, metabolites are, in general, unlikely to be more potent P450 inhibitors than their respective parent drugs. Several published quantitative structure-activity relationship (QSAR) models evaluating reversible inhibition of CYP2C and CYP3A families also supported the positive correlation between lipophilicity (logP) and potency for enzyme inhibition (Lewis et al., 2006; Didziapetris et al., 2010). In addition, empirical observations indicate that metabolites are likely to have affinity for the same binding sites as parent (e.g. binding to the pharmacological target of the parent leading to “active metabolites”) and if a metabolite has any affinity for P450 binding sites, the binding pattern tends to be very similar to parent (Humphreys & Unger, 2006).

Recently, Callegari et al. (2013) evaluated 33 structurally diverse compounds with a total of 115 circulating metabolites from a Pfizer internal database. The authors noted that 94 out of the 115 human metabolites (82%) had circulating concentrations of less than 1 μM, which is below the concentrations that are typically associated with P450 inhibition in clinical studies (Callegari et al., 2013). In addition, for the 12 clinical candidates where concentrations and in vitro K_i values for P450 inhibition were available for both parent and metabolites, the DDI perpetrator risk due to metabolites was considered low for all metabolites based on the I/K_i values (all <0.1).

Collectively, recent publications on assessing perpetrator DDI via P450 inhibition by metabolites all point towards a low risk that DDI potential that is caused by metabolite alone. However, several notable exceptions have been published, including bupropion (Reese et al., 2008), gemfibrozil (Tornio et al., 2008), amiodarone (Nolan et al., 1989; McDonald et al., 2012), and sertraline (Masubuchi & Kawaguchi, 2013), where the perpetrator DDI results could not be sufficiently explained solely based on parent drug data.

In addition to the risk of inhibition of common drug metabolizing enzymes (mainly P450), metabolites may also have increased potential to interact with drug transporters as compared to corresponding parent drugs. DDI due to interactions with transporters or enzyme induction by metabolites are outside the scope of this scholarship. Readers may wish to refer to two recent International Transporter Consortium white papers (Zamek-Gliszczynski et al., 2013; 2014), where the concern of metabolites as both victims and perpetrators of transporter-based DDI was highlighted.

Contribution of metabolites to P450 inhibition-based DDI for the 137 most frequently prescribed drugs

A total of the 137 most frequently prescribed drugs (as of 2012) were selected to evaluate the contribution of their metabolites to in vivo DDI (based on P450 inhibition). These drugs were evaluated because of the high number of patients who use these drugs. The intention of the analysis of the 137 drugs is not to provide a comprehensive review of their DDI profiles. Instead, the authors focused on identifying compounds (within the 137 most prescribed drugs) that have metabolites that could cause DDI that was not predicted by the parent in vitro P450 inhibition properties. A total of 42 drugs out of these 137 drugs overlapped with the drugs analyzed by Isoherranen et al. (129 named drugs, 2009) and Yeung et al. (102 named drugs, 2011). The available data on in vitro P450 inhibition by parent drugs and their abundant metabolites (generally ≥25% of parent AUC and/or ≥10% of total AUC) and in vivo inhibition from clinical studies were collected as follows. These parameters were mainly obtained from the University of Washington Drug Interaction Database and the drug labels from the FDA website and the associated references.

1. *In vitro* inhibition parameters of parent drug towards major human P450 enzymes (IC_{50} and/or K_i (reversible inhibition); K_I and k_{inact} (MBI)).
2. Identification of abundant human metabolites in plasma (≥25% of parent AUC and/or ≥10% of AUC of total drug-related material).
3. *In vitro* inhibition parameters of abundant human metabolites towards major human P450 (IC_{50} and/or K_i values, K_I and k_{inact}).
4. AUC and C_{max} values of parent and abundant metabolites (when available) in human plasma.

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5. C_{max}/K_i values for the parent drug and abundant metabolites (when available).
6. Fold increase of AUC for victim drugs as a result of P450 inhibition by these 137 drugs (when DDI studies were performed). When drug interaction data were available from two or more clinical studies, data from the study with a sensitive P450 probe substrate were selected. Case reports in the University of Washington Drug Interaction Database were generally not used to obtain in vivo drug-interaction data.

The collected parameters (along with other pertinent information, e.g. dose) for all 137 drugs are shown in supplemental Table 1. Based on the in vitro and in vivo parent DDI data, the drugs were divided into five categories using the criteria described below (see Table 1 and Figure 2).

- Category 1 (in vitro inhibition negative and in vivo inhibition negative): parent compound shows no or low inhibition of a P450 isoform in vitro (IC_{50} >10 μM or $I_p/K_i \leq$ 0.1) and does not cause in vivo DDI for this P450 isoform (<1.25-fold change of AUC of the victim drug). If in vivo DDI data with the drug as a perpetrator are not reported, it is assumed that this drug is not an in vivo inhibitor for this P450 isoform due to its extensive use by patients and the lack of reported drug interaction data.
- Category 2 (in vitro inhibition positive, but in vivo inhibition negative): parent compound shows the inhibition of a P450 isoform in vitro (IC_{50} < 10 μM or $I_p/K_i \geq$0.1 or an inactivator) but does not cause in vivo DDI for this P450 isoform (<1.25-fold change of AUC of the victim drug). If in vivo DDI data with the drug as a perpetrator are not reported, it is assumed that this drug is not an in vivo inhibitor for this P450 isoform due to its extensive use by patients and the lack of reported drug interaction data.

- Category 3 (in vitro inhibition negative, but in vivo inhibition positive): parent compound shows no or low inhibition of a P450 isoform in vitro (IC_{50} >10 μM or $I_p/K_i \leq 0.1$) but causes unexpected in vivo DDI for this P450 isoform (>1.25-fold change of AUC of the victim drug).
- Category 4 (in vitro inhibition positive and in vivo inhibition positive): parent compound shows the inhibition of a P450 isoform in vitro (IC_{50} < 10 μM or $I_p/K_i \geq 0.1$ or an inactivator) and causes in vivo DDI for this P450 isoform (>1.25-fold change of AUC of the victim drug).
- Unassigned Category: There are no in vitro and/or in vivo DDI data for the parent drug and/or metabolites reported in the literature or described in the prescribing information.

As shown in Table 1, a total of 102 drugs belong to Categories 1-4 and 35 drugs are in the Unassigned Category. The predictability of the parent in vitro DDI data for in vivo DDI is depicted in Figure 2 for drugs belonging to Categories 1-4. There are 48 drugs in Category 1 (true negatives), 10 drugs in Category 3 (false negatives), 26 drugs in Category 4 (true positives), and 18 drugs in Category 2 (false positives). Therefore, based on the parent $[I]/K_i$ (in vitro) and in vivo DDI data, the true negatives are 83% (48 out of 58 drugs in Categories 1 &3); the false negatives are 17% (10 out of 58 drugs in Categories 1 &3); the true positives are 59% (26 out of 44 drugs in Categories 2 &4), and the false positives are 41% (18 out of 44 drugs in Categories 2 &4). A total of 66 drugs (65% of 102 drugs) in Category 1 and Category 2 did not show any clinical DDI with P450 substrates. This trend is consistent with the findings from Isoherranen et al. (2009) that the majority (~ 90%) of 1323 drugs on the US market did not show P450 inhibition in vivo. A total of 26 drugs (25% of 102 drugs) are in Category 4. These 26 drugs

showed P450 inhibition in vivo, which were predicted qualitatively by the in vitro P450 inhibition data of the parent drugs. Metabolites of clopidogrel (Tornio et al., 2014), diltiazem (Yeung et al., 1993; Zhao et al., 2007), fluoxetine (Yeung et al., 2011), imatinib (Yeung et al., 2011), and omeprazole (Shirasaka et al., 2013) likely have contributed to the observed in vivo P450 inhibition-based DDI based on their clinical concentrations and in vitro P450 inhibition potency. For all other drugs in Category 4, it is challenging to identify the contribution of metabolites to the observed P450 inhibition-based DDI due to the lack of data either on the metabolite concentrations or on their in vitro P450 inhibition potency.

The 10 drugs in Category 3 are the false negatives and of most concern to the prediction of clinical DDI potential. These 10 drugs showed in vivo P450 inhibition, which was not predicted by the in vitro P450 inhibition, inactivation, or IC_{50}/K_i values of the parent drug. Five of these 10 drugs showed ≤ 1.5-fold increase in AUC of the victim drugs, which is generally not considered clinically significant except for victim drugs with a narrow therapeutic window. These five drugs are atorvastatin (midazolam as the CYP3A substrate, McDonnell et al., 2003), venlafaxine (imipramine as the CYP2D6 substrate, Albers et al., 2000), sertraline (pimozide as the CYP3A substrate, Alderman, 2005; desipramine as the CYP2D6 substrate, Kurtz et al., 1997), amlodipine (simvastatin as the CYP3A substrate, Ma et al., 2000), and capecitabine (warfarin the CYP2C9 substrate, Camidge et al., 2005). The in vivo DDI of sertraline may be explained by the more potent inhibition of CYP3A4 by the N-desmethyl metabolite. It is important to note that the in vivo DDI observed with atorvastatin, venlafaxine, and amlodipine cannot be explained by inhibition due to their respective metabolites. The lactone metabolite of atorvastatin is a 100-fold more potent inhibitor of CYP3A4 than atorvastatin (Jacobson et al., 2000). However, the

lactone metabolite can't explain the observed in vivo inhibition of CYP3A4 when solely based on the $[I]/K_i$ ratio (<0.1). The major metabolite of venlafaxine (O-desmethylvenlafaxine) also had an I/K_i ratio less than 0.1. The AUC values of amlodipine metabolites were not available. Some of the metabolites were reported to have similar C_{max} values as amlodipine (Beresford et al, 1988). The P450 inhibition potency of amlodipine metabolites have not been reported in literature. Therefore it is not known whether amlodipine metabolites contributed to the observed weak drug interaction with simvastatin. The AUC values of the metabolites of capecitabine ranged from 0.4-fold to 23.6-fold of the AUC of capecitabine (Twelves et al., 1999). Although the inhibition potency of these metabolites towards CYP2C9 has not been reported, it is believed that the metabolites contributed to the observed drug interaction with warfarin (capecitabine drug label). Bupropion, gemfibrozil, and amiodarone, which are well documented (Reese et al., 2008; Tornio et al., 2008; Nolan et al.,1989; McDonald et al., 2012) to have caused "unexpected" in vivo P450 inhibition, all had metabolite(s) that were more potent inhibitors of P450 than the parent. In addition, the concentrations of their metabolites were approximately equal to or greater than concentrations of the parent drugs. Therefore, in the cases of bupropion, gemfibrozil, and amiodarone, the metabolites are considered the major/sole contributors to the observed clinical DDI. For ciprofloxacin and escitalopram, the "unexpected" inhibition of P450 in vivo is not completely explained in the available literature. Ciprofloxacin was not expected to inhibit CYP1A2 in vivo based on in vitro data (Karjalainen et al., 2008). However it is one of the most potent in vivo CYP1A2 inhibitors in clinical use (FDA DDI 2012 Draft DDI Guidance; Granfors et al., 2004). The most abundant circulating metabolite of ciprofloxacin is oxo-ciprofloxacin, which is present at only ~10% of the AUC of ciprofloxacin (Bergal et al., 1989). Since the in vitro inhibition parameter for this metabolite is not available, it is not known whether the

observed in vivo inhibition of CYP1A2 substrate is due to the oxo-ciprofloxacin metabolite. Pre-incubation of ciprofloxacin in human liver microsomes slightly increased the inhibition potency of CYP1A2, which suggests that ciprofloxacin could be a mechanism-based inhibitor (Karjalainen et al., 2008). In addition, ciprofloxacin may concentrate into hepatocytes due to its lipophilic and basic properties. It remains to be elucidated why ciprofloxacin is a potent in vivo CYP1A2 inhibitor. Similar to ciprofloxacin, escitalopram was not expected to inhibit CYP2D6 in vivo based on in vitro CYP2D6 inhibition data (Skjelbo and Brosen, 1992). Interestingly, it caused a modest 2-fold increase in the AUC of desipramine in humans (Forest Pharmaceuticals, 2005). The abundant human metabolite of escitalopram is N-desmethylcitalopram, which is present at ~36% of the AUC of escitalopram (Rao, 2007). It is worth noting that N-desmethylescitalopram is a 15-fold more potent inhibitor of CYP2D6 than the parent escitalopram (Skjelbo & Brosen, 1992). Therefore, N-desmethylescitalopram may be the major contributor to the modest DDI with desipramine in human. However, when solely based on its $[I]/K_i$ ratio (0.03), N-desmethylescitalopram cannot explain the observed CYP2D6 inhibition. In summary, metabolites were likely the sole contributors to the observed in vivo P450 inhibition for 5 of the 10 drugs in Category 3 (parent in vitro inhibition negative, in vivo inhibition positive). These 5 drugs are amiodarone, bupropion, sertraline, gemfibrozil, and capecitabine. The metabolites of atorvastatin and escitalopram may have also contributed to the observed in vivo DDI. It is not known whether the metabolites of amlodipine, venlafaxine and ciprofloxin contributed to the observed in vivo P450 inhibition.

Review of current literature approaches to trigger in vitro DDI studies for metabolites

Currently there are two approaches in the literature to trigger the in vitro assessment of P450 inhibition potential of metabolites (Callegari et al., 2013; Yu & Tweedie, 2013). These two approaches emphasize the importance of considering both the abundance (AUC or C_{max}) and inhibition potency of metabolites (K_i) in assessing their P450 inhibition potential. Yu and Tweedie (2013) proposed to conduct clinical DDI studies to assess the in vivo inhibition potential for both the parent and metabolites when the parent drug is an inhibitor of one or more P450 enzymes in vitro (i.e. $[I]/K_i > 0.1$, where [I] is the total concentration). When the parent drug is not expected to be an inhibitor of a P450, the proposed default cut-off value to trigger in vitro P450 inhibition studies for metabolites is that metabolite AUC is ≥ 100% of parent AUC. The rationale for the default cut-off value (100% of parent AUC) is based on the generally accepted assumption that metabolites tend to be less potent inhibitors of P450 due to the increased hydrophilicity. In addition to the default cut-off value, lower cut-off values were proposed for exceptions where metabolites are less hydrophilic or contain structural alerts for MBI. For metabolites which are less hydrophilic than the parent molecule, a lower cut-off value (25% of parent AUC) is recommended. For metabolites containing structural alerts for MBI, the cut-off value of metabolite level is considered on a case-by-case basis as it is challenging to ascribe a level of expected inhibition based simply on structure.

Callegari et al. (2013) recommended using an R_{met} strategy to trigger the study of the P450 inhibition by metabolites in vitro, where R_{met} is equal to $C_{max,\ metabolite}/K_{i,\ metabolite}$. When the K_i value of a metabolite is not available, the metabolite is considered a 4-fold more potent inhibitor than the parent, which is generally a conservative scenario. The $K_{i,\ metabolite}$ is therefore assumed to be 0.25 of $K_{i,\ parent}$. The R_{met} strategy was evaluated using metabolite C_{max} and parent K_i data

from Pfizer internal compounds and literature compounds, which successfully identified metabolites that were the main contributors to the in vivo P450 inhibition without introducing a high rate of false positives.

Drugs in Category 3 (parent in vitro inhibition negative, in vivo inhibition positive, see the 137 drugs section above) are of most importance in assessing the need to study P450 inhibition potential of metabolites in vitro. The 10 drugs in Category 3 were tested using the Yu & Tweedie and Callegari et al. approaches with the exception of amlodipine, for which the AUC values of the metabolites are not available. The objective was to evaluate the utility of these two approaches in triggering in vitro P450 inhibition studies for metabolites (Table 2). Using the default 100% of parent AUC cut-off value for metabolites strictly, the Yu & Tweedie approach would lead to the in vitro P450 inhibition studies for the metabolites of atorvastatin, venlafaxine, bupropion, amiodarone, sertraline, and capecitabine (at least one metabolite was predicted for each drug). In addition, since the abundant metabolite of escitalopram was formed via N-dealkylation from a tertiary amine to a secondary amine, which is a structural alert for MBI of P450 (see structural alert section below), the Yu and Tweedie approach would also lead to the study of the P450 inhibition and inactivation potential in vitro for the N-desmethylescitalopram metabolite.

Using the default R_{met} value of 0.1 strictly, the Callegari et al. approach would lead to the in vitro P450 inhibition studies for the metabolites of bupropion, amiodarone, gemfibrozil, sertraline and capecitabine (at least one metabolite was predicted for each drug). If both approaches are

combined, it would have covered 8 out of 10 drugs in Category 3 (only ciprofloxacin was not covered by either of these two approaches and these two approaches were not applied to amlodipine due to the lack of data). It is interesting to note that gemfibrozil glucuronide is not covered by the Yu & Tweedie approach if the 100% of AUC of parent cut-off value is strictly applied; however, it is covered by the Callegari et al. approach using the R_{met} strategy. The opposite is true for the venlafaxine o-desmethyl metabolite, which is not covered by the Callegari et al. approach but covered by the Yu & Tweedie approach. These two approaches appear to be complimentary in that the Yu & Tweedie approach triggers an examination of P450 inhibition by metabolites regardless of parent Ki values whereas Callegari et al. allows a more detailed examination of a particular P450 where there is a measurable parent K_i. Based on the discussion among scientists from the member pharmaceutical companies, it is a common practice to combine multiple approaches when assessing the need to study metabolite DDI potential in vitro. The key points to consider include: a) relative and absolute concentrations of the metabolites; b) potencies of the metabolites for P450 inhibition; c) the presence of structural alerts in metabolites; and d) contribution of metabolites to DDI when un-expected in vivo DDI are observed. Another important tool in predicting and understanding DDI is PBPK modeling. It is recommended to use PBPK modeling to integrate the contributions of parent and metabolites to DDI, especially in complex drug development programs. Investigations are currently underway to generate PBPK models for some drug/metabolite pairs to determine the usefulness of this approach.

Utility of structural alerts in assessing P450 inhibition and inactivation potential of metabolites

Alerts from chemical substructures frequently associated with the risk of P450 inhibition and inactivation are well-established (Halpert, 1995; Orr et al., 2012), especially for lipophilic and nitrogen-containing aromatic heterocyclic compounds and alkylamines. It is common practice to incorporate structural alerts contained in the parent compound in the initial assessment of P450 inhibition potential. Therefore, it is reasonable to also identify such structural alerts in the major circulating metabolites to prioritize in vitro testing for potential risk of P450 inhibition or inactivation. In practice, the chemical structures of major circulating metabolites (>10% of total drug related AUC) are generally elucidated and their plasma concentrations determined quantitatively or semi-quantitatively in early clinical development (e.g. Phase I) to satisfy the recommendation from the FDA MIST and ICH M3 (R2) Guidances (FDA, 2008 and EMA 2009). If the major metabolite retains the structural alert of the parent drug or contains a new structural alert for P450 inhibition as a result of biotransformation, then such information can be used to trigger determination of its P450 inhibition and inactivation in the overall process of assessment of DDI.

Although the intention of this manuscript is not to provide a detailed discussion on different types of P450 inhibition, it is necessary to highlight the mechanisms through which the moieties identified as structural alerts exert their inhibitory effects, as this is essential to understanding and assessing the potential risk of inhibition mediated by drug metabolites. There are three broad categories of P450 inhibition, reversible, quasi-irreversible and irreversible inhibition. There are examples in the literature of metabolites that fit into each of these categories. Reversible inhibition often involves competition for binding to the prosthetic heme iron and lipophilic region of protein within the active site. In general, potent P450 inhibitors are lipophilic

compounds which contain aromatic nitrogen-containing heterocycles such as pyridines, imidazoles, and quinolones. These compounds inhibit P450 through the interaction of the lone pair of electrons with the ferric heme iron of the P450 (Halpert, 1995). A notable example of reversible CYP450 inhibition by compounds is illustrated by itraconazole and its oxidative metabolites, which are as potent as or significantly more potent reversible inhibitors of CYP3A4 than parent (Isoherranen et al., 2004). Both itraconazole and its metabolites are nitrogen-containing aromatic heterocycles. The strong inhibition potencies of itraconazole and its metabolites together provide a reasonable prediction of the clinical DDI (Isoherranen et al., 2004). In addition to reversible P450 inhibition by metabolites, clinically relevant DDI have also been observed with metabolites causing mechanism-based P450 inhibition via irreversible inhibition (interaction with heme or the aproprotein) and quasi-irreversible inhibition. Perhaps the best-understood structural alerts for P450 inhibition are associated with quasi-irreversible inhibition by formation of metabolic-intermediate (MI) complexes, which have a diagnostic Soret peak in the visible spectrum at ~455 nm (Franklin, 1974). Although alkylamine-, arylamine-, and methylenedioxyphenyl- groups are well-known structural alerts for formation of stable MI complexes, the majority of clinical DDI caused by quasi-irreversible inhibitory metabolites are alkylamines (Figure 3). Interestingly, 3 of the 8 drugs in Category 3 (escitalopram, amiodarone, and sertraline) have abundant secondary or primary amine metabolites. More importantly, two of these amine metabolites (from escitalopram and amiodarone) are confirmed to be more potent P450 inhibitors than the respective parent drug. Alkylamine metabolites that inactivate P450 are predominantly secondary alkylamines except for norfluoxetine (a primary alkylamine, Hanson et al., 2010) which was shown to inactivate multiple P450 isoforms (Lutz et al., 2013). Historically, the quasi-irreversible inhibition of

CYP450 by secondary alkylamines is thought to occur via a reaction sequence involving *N*-dealkylation to primary alkylamines, which can be further N-hydroxylated to hydroxylamines, followed by further oxidation and dehydrogenation to nitroso derivatives (Figure 3). Recently, an alternative pathway has been reported in the formation of nitroso metabolites involving exclusively *N*-hydroxylation instead of *N*-dealkylation of secondary alkylamine drugs (Hanson et al., 2010). Regardless of the reaction sequence, it is the nitroso metabolites that bind to the ferrous form of the prosthetic heme iron of P450 with high affinity via coordinate bonds and cause quasi-irreversible inactivation of the enzyme (Franklin, 1991; Kalgutkar et al., 2007). The other well-known structural alert for causing quasi-irreversible inhibition of P450 is the arylamine moiety, which follows a similar mechanism as alkylamines (Figure 3, Kalgutkar et al., 2007; Hollenberg et al., 2008). Finally, the methylenedioxyphenyl groups (as seen in tadalafil and paroxetine), are metabolized to produce carbene intermediates (Figure 3). These carbene intermediates bind to both ferrous and ferric heme iron and cause quasi-irreversible inactivation of P450 enzymes. However, mechanism-based inhibition of P450 by methylenedioxyphenyl-containing compounds is generally covered by assessing the inactivation potential of the parent molecules, because biotransformation leading to retention of the methylenedioxyphenyl group in metabolites is rare.

Additional structural alerts for P450 inactivation are included in Table 3. Although they are not expected to be as important as the structural alerts outlined in Figure 3 in terms of P450 inactivation potential, it is important to consider assessing the P450 inactivation potential of these structural alerts proactively, if an abundant metabolite contains one or more of these structural alerts. It is also noteworthy to point out that many structural alerts are potentially

"masked" in the parent molecule, for example, substituted alkylamines, arylamines and aminophenols, and metabolism of these parent molecules may lead to "unmasking" of such structural alert in the metabolites thereby leading to enhanced potential for P450 inhibition.

The interesting results from mechanistic studies of the gemfibrozil and cerivastatin DDI (Backman et al., 2002), where the DDI was attributable in part to gemfibrozil acyl-β-glucuronide but not gemfibrozil, has raised the concern of acyl glucuronides being P450 inhibitors. Jenkins et al. (2011) evaluated acyl glucuronides of 11 compounds as direct-acting and metabolism-dependent inhibitors of CYP2C8. Lai et al. of Eisai Pharmaceuticals (personal communication) also assessed the P450 inhibition potential for the glucuronide metabolites (ether and acyl glucuronides) of several structurally-diverse drugs. The results from both studies show that MBI of CYP2C8 by gemfibrozil acyl-β-glucuronide appears to be specific to gemfibrozil and not likely generalizable to other glucuronide conjugates. However, a recent case of clinical DDI between cerivastatin and clopidogrel led to the identification of clopidogrel acyl-β-glucuronide as a potent time-dependent inhibitor of CYP2C8 (Tornio et al., 2014). Further investigation may be needed to address the potential risk of P450 inactivation (especially CYP2C8) by acyl glucuronides as a class of reactive metabolites and whether these conjugates should be added to the list of structure alerts for metabolite mediated DDI.

Discussion

The EMA (final) and FDA (draft) drug interaction guidances proposed that human circulating metabolites should be investigated in vitro for their drug-drug interaction (DDI) potential if present at ≥ 25% of parent AUC (FDA) or ≥25% parent and ≥10% of total drug-related AUC

(EMA). Based on the data from Callegari et al. (2013), it is estimated that approximately 2 metabolite per development compound (60 metabolites from 25 drugs were present at ≥ 25% of parent AUC) would meet the FDA criterion, which is more stringent than the EMA criterion. Besides the metabolite abundance requirement (≥ 25% of parent AUC and ≥ 10% of total AUC), the EMA guidance focuses on studying the DDI potential of Phase I metabolites, which can decrease the number of metabolites that need to be evaluated for DDI potential. For example, in the Callegari et al. paper (2013), only 26 out of a total of 115 circulating metabolites for 33 drugs were Phase I metabolites. Despite the difference in the cut-off criteria for metabolites, the FDA and EMA guidances highlighted the importance of including metabolites in the overall assessment of P450 inhibition-based DDI for development drugs. Early work by Isoherranen et al. (2009) and Yeung et al. demonstrated that circulating metabolites are often present with inhibitors of P450 enzymes and in vivo P450 inhibition-based DDI may only be explained by considering the metabolite in vitro P450 inhibition data for 3 drugs.

The Metabolite Scholarship Group performed a comprehensive risk analysis of P450 inhibition-based DDI that are caused solely by metabolites based on work by Isoherranen et al. (2009) and Yeung et al. (2011) and our own analysis of 137 most-frequently prescribed drugs, assessed the utility of current approaches in the literature as well as common practice within the pharmaceutical industry to trigger in vitro drug-metabolism studies for metabolites, and identified structural alerts of metabolites that may suggest their P450 inhibition/inactivation potential. Overall, the risk of metabolites as the sole contributor to P450 inhibition-based clinical DDI appears to be relatively low. Metabolites of 3 drugs (amiodarone, bupropion, and sertraline out of 102 drugs, which are the in vivo P450 inhibitors identified from 1323 drugs on

the US market) were identified as the sole contributor to the observed clinical DDI by Isoherranen et al. (2009) and Yeung et al. (2011). Metabolites of 5 drugs (amiodarone, bupropion, sertraline, gemfibrozil, and capecitabine, out of 137 most frequently prescribed drugs) were identified as the sole contributor to the observed clinical DDI by the Metabolite Scholarship Group. The difference between these two sets of analysis is that the metabolites of gemfibrozil and capecitabine were also identified as the sole contributor to the observed DDI by the Metabolite Scholarship Group. Gemfribrozil glucuronide is an MBI of CYP2C8 (Ogilvie et al., 2006). Several metabolites of capecitabine are highly abundant and believed to inhibit CYP2C9 (capecitabine drug label). Since DDI potential is an important part of drug safety, it is highly important to proactively manage the DDI risk of metabolites. The combination of the two literature approaches (Callegari et al., 2013; Yu & Tweedie, 2013), which involved a metabolite cut-off value of approximately 100% of AUC of parent, and consideration of metabolite C_{max}/K_i, was able to flag the metabolites of 8 of 10 drugs in Category 3 for investigating metabolite P450 inhibition potential in vitro. Structural alerts of metabolites can also be used proactively in planning and prioritizing in vitro DDI studies for metabolites, as in the case of escitalopram and amiodarone.

Similar to the literature analyses (Isoherranen et al., 2009; Yeung et al., 2011), our analysis of the 137 most-frequently prescribed drugs has also been limited by the lack of P450 inhibition data for some of the parent drugs and the lack of P450 inhibition and exposure data for most of the circulating metabolites. Due to these limitations, our approach focused on identifying compounds for which the parent drug did not show in vitro P450 inhibition, but caused P450 inhibition in vivo. Our analysis did not consider transporter mediated DDIs, which may

complicate the parent and metabolite in vitro-in vivo correlation of P450 inhibition. Additionally, our analysis did not account for the fact that metabolites can be enriched in the liver, resulting in higher intracellular free metabolite concentrations that are not reflected by the plasma concentration.

To summarize the considerations in addressing DDI risks of metabolite, a decision tree is proposed in Figure 4. The key intention of the decision tree is to propose the criteria to initiate in vitro inhibition assessment of metabolites based on the exposure of parent and metabolites in Phase I studies (very early in clinical development). The objective is to provide an early alert for "surprise" DDIs as a result of the formation of potential inhibitory metabolites. Briefly, if the parent compound is likely to inhibit P450 in vivo based on in vitro inhibition data and therapeutic exposure, conduct clinical DDI studies to assess the inhibition potential of both the parent and the metabolites. It is important to consider the pharmacokinetic properties of parent and metabolites to ensure steady state concentrations are achieved for the parent and metabolites in the clinical DDI studies. On the other hand, if the parent compound is not likely to inhibit P450 in vivo, consider in vitro P450 inhibition studies for abundant metabolites. If a metabolite does not contain a structural alert for P450 inhibition/inactivation, calculate R_{met} (using $C_{max,\ metabolite}$ and 0.25 of $K_{i,\ parent}$) and determine the abundance of the metabolite. If R_{met} is less than 0.1 and the abundance of the metabolite is less than 100% of parent AUC, the metabolite is probably not going to inhibit P450 in vivo (based on the amiodarone, gemfibrozil, sertraline, and bupropion examples) . Therefore in vitro P450 inhibition/inactivation studies are generally not needed. On the other hand, if R_{met} is > 0.1 or the abundance of the metabolite is above 100% of parent AUC, conduct in vitro P450 inhibition/inactivation studies for the metabolite. For

metabolites containing structural alerts for P450 inhibition/inactivation (e.g. alkylamine), extra caution should be exercised in assessing the need to conduct in vitro P450 inhibition/inactivation studies. However, given that a structural alert is not necessarily predictive of the extent of P450 inactivation, the in vivo abundance (C_{max} and AUC) of the metabolite may be a more important determinant of the need for in vitro P450 inhibition and inactivation studies. A reasonable starting point may be that when a metabolite with a structural alert is present at ≥25% of parent AUC and ≥10% of total AUC, consider in vitro P450 inhibition/inactivation studies for this metabolite. Once the in vitro P450 inhibition parameters are determined for the metabolite, similar approaches used to predict the parent in vivo DDI potential can be used to predict the in vivo DDI potential for the metabolite. If the metabolite is predicted to cause in vivo inhibition, a clinical DDI study is warranted to confirm the prediction.

The chemical synthesis of metabolites can present challenges. A semi-quantitative and resource-sparing approach (without the need to synthesize a metabolite standard) can be considered for cases where a metabolite is the major component of the mixture (e.g. ≥ 80%) after the incubation of the parent with either liver microsomes or hepatocytes. If CYP inhibition by the mixture is weak, the metabolite is unlikely to be a potent inhibitor of P450s.

The scholarship presented in this manuscript is intended to provide a useful framework for rational risk assessment during drug development and enable productive scientific exchanges with regulators. It should be pointed out that this and other analyses have focused on P450 inhibition-based DDI where data are relatively abundant. However, there are insufficient data on the evaluation of metabolites in P450 mediated induction, other enzyme systems (e.g. UGT's) or

transporter-mediated DDI. Additional data on metabolite contribution to DDI, when applicable, will need to be collected over the next few years to help drug metabolism scientists and clinicians to better understand the contribution of metabolites to DDI. The Metabolite Scholarship Group encourages collecting and sharing experiences with clinicians and regulators with metabolites as contributors to DDI to help gain a better understanding of this topic.

In conclusion, the in vivo P450 inhibition potential can be generally predicted by the in vitro P450 inhibition parameters of the parent drug. The risk for an unexpected in vivo DDI as a result of not assessing in vitro P450 inhibition by metabolites is considered low. However, the contribution of metabolites to DDI should be considered in light of the totality of data (in vitro K_i values and systemic concentrations) of both the parent drug and the metabolites, and strategies for evaluating metabolites in DDI after obtaining the exposure of parent and metabolite in Phase I studies have been proposed in this manuscript.

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Authorship Contributions

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Footnotes:

1. Robert L. Walsky also represented AstraZeneca.

2. Bo Wen also represented Roche.

Figure legends:

Figure 1. Role of metabolites as perpetrators of DDI via P450 inhibition based on literature data (Isoherranen et al., 2009 and Yeung et al. 2011)

Figure 2. Distribution of the 137 drugs in Categories 1-4

Figure 3. Main structural alerts for metabolites associated with inactivation of P450 enzymes (alkyl amine, aryl amine and methylenedioxyphenyl)

Figure 4. A proposed decision tree to investigate the P450 inhibition potential of metabolites

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Table 1 Summary of the 137 drugs in 5 different categories

Category 1 (in vitro -/ in vivo -) (48 drugs)		**Category 2 (18 drugs) In vitro +/ in vivo -**	**Category 3 (10 drugs) In vitro -/ in vivo +**	**Category 4 (26 drugs) In vitro +/ In vivo +**	**Unassigned (35 drugs) No in vitro and/or in vivo inhibition data**	
Amphetamine (2D6)	Metoprolol (2D6)	[3]Atomoxetine (2D6)	Amiodarone (2C9)	Atazanavir (2C8, 3A)	Alendronate	Lamotrigine
Amitriptyline (2C19 and 2D6)	Mometasone (2B6, 2C8)	Budesonide (3A)	Amlodipine (3A)	Azithromycin (3A)	Alfuzosin	Latanoprost
Anastrozole (2C9)	Moxifloxacin (2B6)	Diclofenac (3A)	Atorvastatin (3A)	Bicalutamide (3A)	Bisoprolol	Levalbuterol
Aripiprazole (2D6)	Olanzapine (1A2)	Ezetimibe (3A)	Bupropion (2D6)	Celecoxib (2D6)	Darbepoetin alfa	Meropenem
Bosentan ([1]major P450s)	Olmesartan (2C9)	Fenofibrate (2C8)	Capecitabine (2C9)	Clopidogrel (2B6)	Desloratadine	Metformin
Candesartan (3A)	Pemetrexed (1A2, 2C9, 2D6, 3A)	Fluticasone (3A)	Ciprofloxacin (1A2)	Cyclosporin (3A)	Donepezil	Oseltamivir
Carvedilol (2B6, 2C8)	Pioglitazone (2C8)	Irbesartan (2C9)	Escitalopram (2D6)	Diltiazem (3A)	Dorzolamide	Ramipril
Cefdinir (1A2, 2C19, 2D6, 3A)	Pramipexole (2D6)	Lansoprazole (2C19)	Gemfibrozil (2C8)	Duloxetine (2D6)	Doxazosin	Risedronate
Cetirizine (3A)	Pravastatin (2C9)	Levofloxacin (2C9)	Sertraline (2D6, 3A)	[2]Efavirenz (3A)	Enalapril	Rivastigmine
Docetaxel (3A)	Pregabalin (major P450s)	Montelukast (2C8)	Venlafaxine (2D6)	Erlotinib (3A)	Enoxaparin	Sevoflurane
Famotidine (2C19, 2D6, 3A4)	[1]Quetiapine (major P450s)	Ondansetron (3A)		Esomeprazole (2C19)	Erythropoietin	Somatostatin
Fentanyl (3A)	Ranitidine (1A2, 2C8, 2C9, 2D6, 3A)	Pantoprazole (2C19)		Fluconazole (2C9, 2C19, 3A)	Estrogen	Somatropin
Gabapentin (2B6, 2C9, 2D6, 3A)	Risperidone (2D6)	Rabeprazole (2C9, 2C19)		Fluoxetine (2C19, 2D6)	Eszopiclone	Sumatriptan
Glimepiride (2C9)	Ropinirole (1A2)	Raloxifene (2C9)		Fluvastatin (2C9)	Fexofenadine	Temozolomide
Irinotecan (3A)	Rosuvastatin (2C8)	Rosiglitazone (2C8)		Imatinib (3A)	Filgrastim	Teriparatide
Letrozole (3A)	Salmeterol (2C8)	Sildenafil (3A)		Lopinavir (3A)	Finasteride	Tiotropium
Levetiracetam (3A, 2C9)	Simvastatin (3A)	Tadalafil (3A)		Modafinil (2C19)	Goserelin	Valacyclovir

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Levothyroxine (2C8)	Telmisartan (2C9)	Tamoxifen (2D6)		Nefazodone (3A)		Zoledronate
Lidocaine (2D6, 3A)	Thalidomide (3A)			Nifedipine (3A)		
[1]Linezolid (major P450s)	Topiramate (2C9)			Omeprazole (2C19)		
Losartan (2C9)	Valsartan (2C9)			Oxcarbazepine (2C19)		
Meloxicam (2C9)	Vardenafil (3A)			Paroxetine (2D6)		
Memantine (2D6)	Ziprasidone (2D6)			Terbinafine (2D6)		
Mofetil (3A)	Zolpidem (1A2, 2D6, 3A)			Valproate (2C9)		
				Voriconazole (2B6, 2C8, 2C9, 3A)		
				Zileuton (1A2)		

1 Major P450s: major drug metabolizing P450s (1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and CYP3A4)

2 Efavirenz induction masks in vivo inhibition

3 Atomoxetine: category 1 for 3A substrates

Table 2 Application of the Yu & Tweedie and Callegari et al. approaches to trigger in vitro studies for metabolites from 9 drugs in Category 3

Drug	Metabolites	Inhibited P450	$AUC_{metabolite}$ /AUC_{parent}* 100%	R_{met}: $C_{max,met}$/ ($K_{i,parent}$/4)	Tweedie & Yu Predict	Callegari et al. Predict
Atorvastatin	Atorvastatin lactone	CYP3A4	89	0.004	No	No
	2-OH-atorvastatin	CYP3A4	123	0.007	Yes	No
	2-OH-atorvastatin lactone	CYP3A4	261	0.01	Yes	No
Venlafaxine	O-desmethyl-venlafaxine	CYP2D6	286	0.06	Yes	No
Bupropion	Hydroxybupropion	CYP2D6	10600	0.76	Yes	Yes
	Threohydro-bupropion	CYP2D6	413	0.44	Yes	Yes
	Erythrohydro-bupropion	CYP2D6	72	0.08	No	No
Amiodarone	N-desethyl-amiodarone	CYP2C9	150	0.12	Yes	Yes
Gemfibrozil	Gemfibrozil glucuronide	CYP2C8	65	4.7	No	Yes
Escitalopram	N-Desmethyl-citalopram	CYP2D6	36	0.007	[1]Yes	No
Sertraline	N-Desmethyl-sertraline	CYP3A4, CYP2D6	259	0.3	Yes	Yes
Ciprofloxacin	Oxociprofloxacin	CYP1A2	10	0.02	No	No
Capecitabine	5-deoxy-5-fluorocytidine	CYP2C9	284	0.41	Yes	Yes

	5-deoxy-5-fluorouridine		235	0.376	Yes	Yes
	Dihydro-5-fluorouracil		40	0.16	No	Yes
	α-fluoro-β-analine		2360	1.164	Yes	Yes

Yes: in vitro P450 inhibition studies triggered by the Yu & Tweedie approach or the Callegari et al. approach.

No: in vitro P450 inhibition studies not triggered by the Yu & Tweedie approach or the Callegari et al. approach.

1: Covered due to the N-dealkylated metabolite (structural alert)

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Table 3 Additional structural alert for P450 inactivation

Structural alert	Example	Reference
Alkene	secobarbital	He et al., 1996a; He et al., 1996b
Alkyne	17α-ethynylestradiol and erlotinib	Lin et al., 2002; Li et al., 2010
Hydrazine	1-aminobenzotriazole	Ortiz de Montellano and Watanabe 1987
Cyclopropylamine	*N*-(2-phenylcyclopropyl)amine	Bondon et al., 1989; Cerny and Hanzlik 2005; Shaffer et al., 2002; Kalgutkar et al., 2007
Dihaloalkane	chloramphenicol and halothane	Pohl et al., 1978; Orr et al., 2012
Furan	methoxsalen, bergamottin, 4-lpomeanol	Koenigs and Trager 1998; Lin et al., 2012; Orr et al., 2012
Thiophene	tienilic acid, ticlopidine, suprofen	Koenigs et al., 1999; Orr et al., 2012
Phenol and aminophenol	trazodone, dasatinib, tacrine	Baer et al., 2007; Hollenberg et al., 2008; Wen et al., 2009

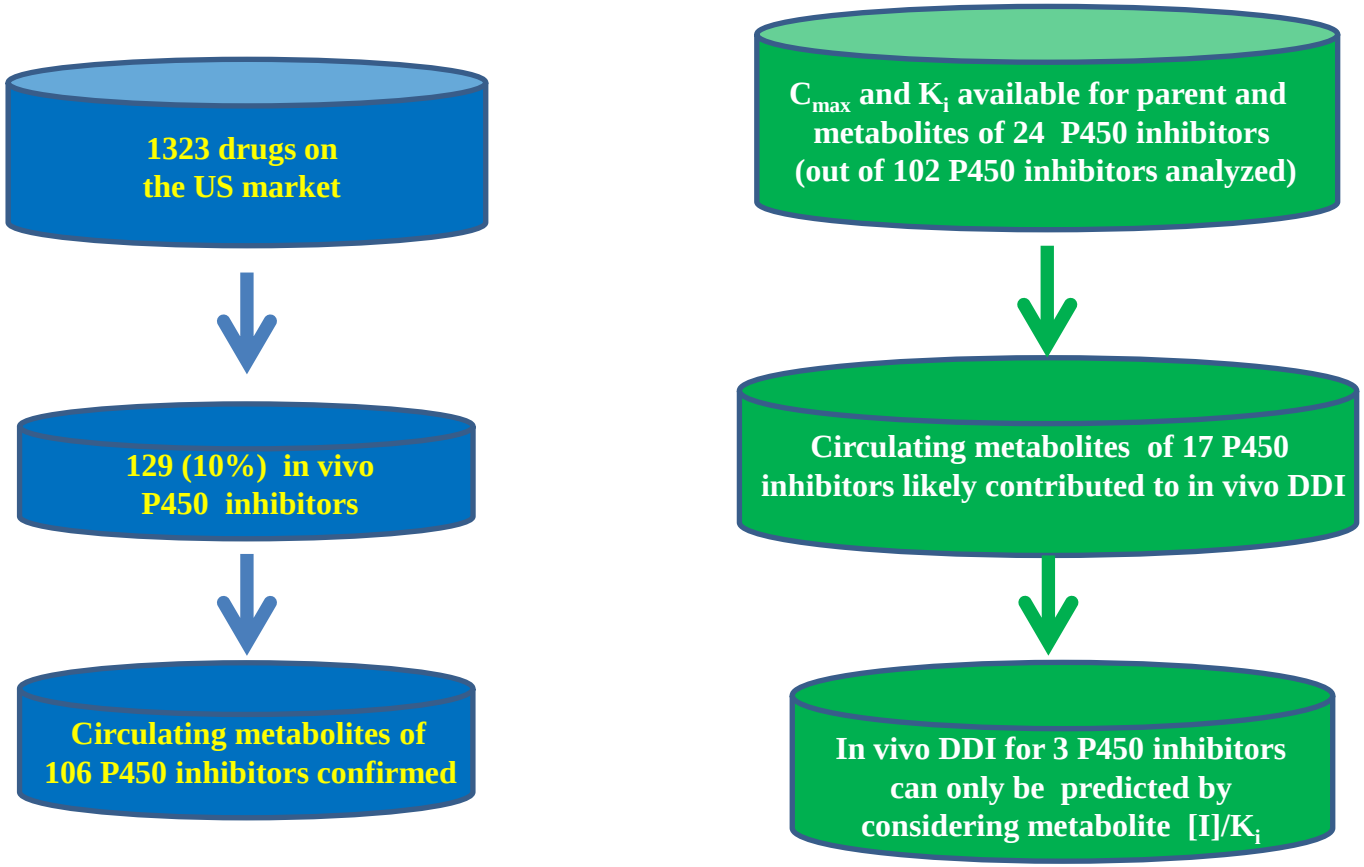

Figure 1

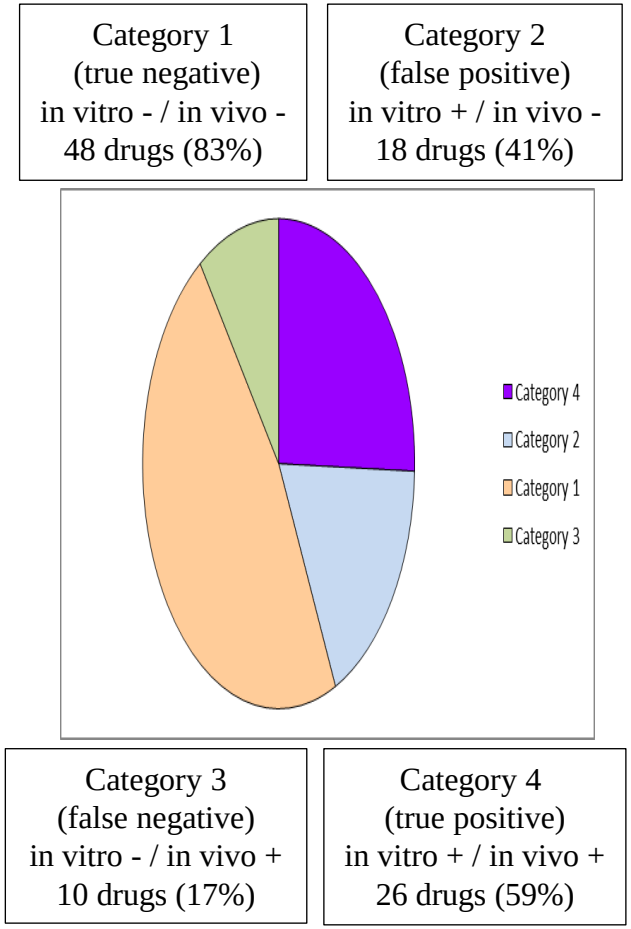

Figure 2

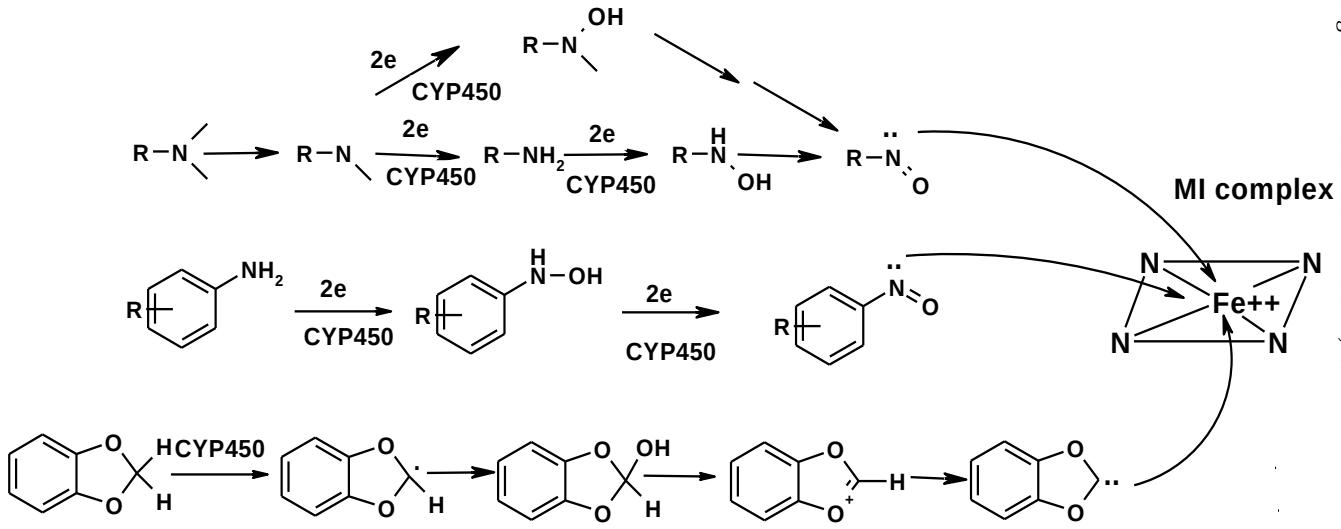

Figure 3

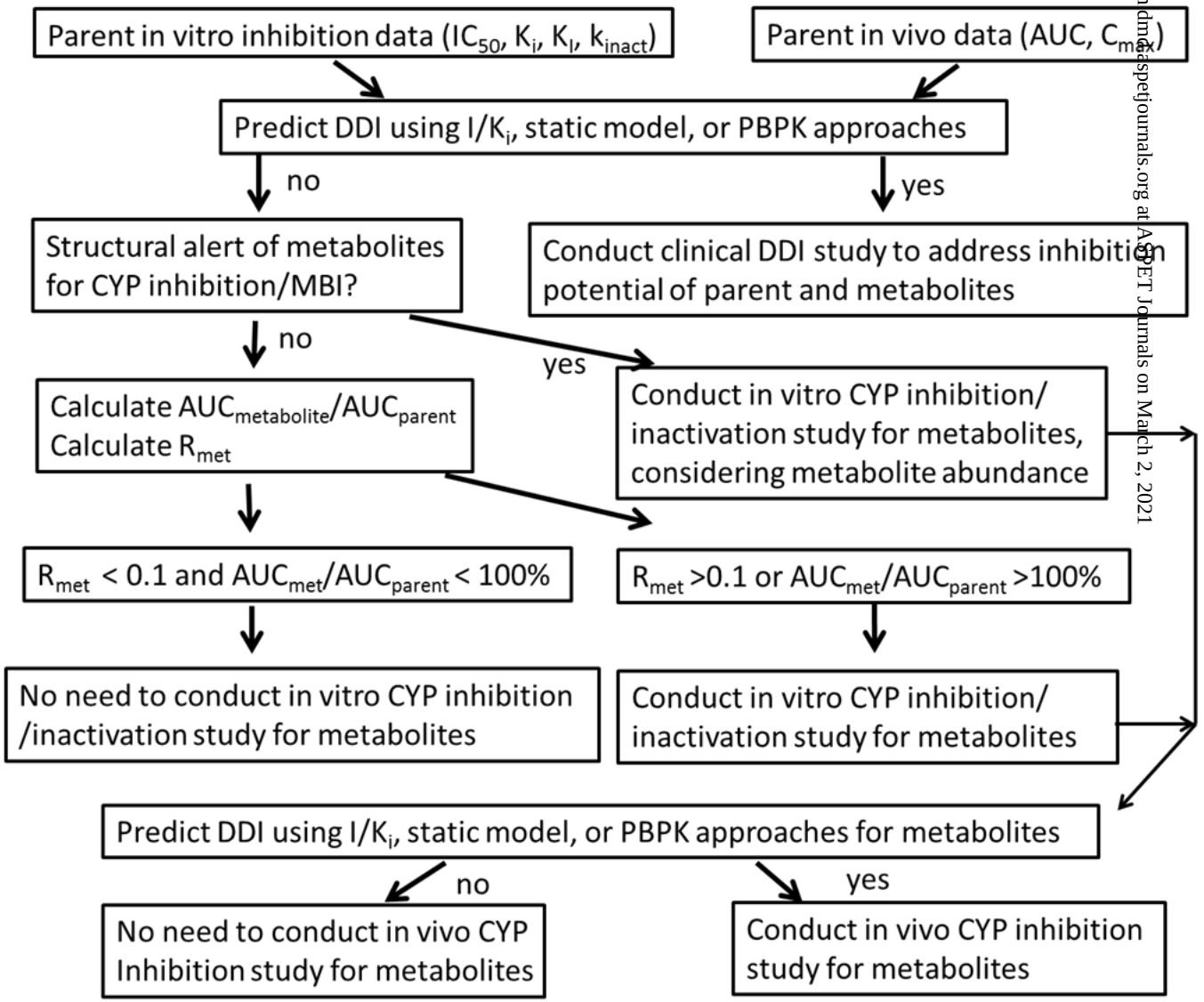

Figure 4