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The role of metabolites in predicting drug-drug interactions: Focus on irreversible P450 inhibition

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Abstract

Irreversible inhibition of cytochrome P450 enzymes can cause significant drug-drug interactions (DDIs). Formation of metabolites is fundamental for the inactivation of P450 enzymes. Of the 19 inactivators with a known mechanism of inactivation, 10 have circulating metabolites that are known to be on path to inactive P450. The fact that inactivation usually requires multiple metabolic steps implies that predicting *in vivo* interactions may require complex models, and *in vitro* data generated from each metabolite. The data reviewed here suggest that circulating metabolites are much more important in *in vivo* P450 inhibition than is currently acknowledged.

Keywords

mechanism-based inactivation; cytochrome P450 enzymes; circulating metabolites; drug-drug interactions; irreversible inhibition; *in vitro-in vivo* predictions

1. Introduction

Inhibitory drug-drug interactions (DDIs) are a considerable concern in drug development and in patients undergoing polytherapy. Inhibitory drug-drug interactions can be life-threatening by increasing the exposure to narrow therapeutic index drugs. Of the current drugs on the US market, 129 are *in vivo* inhibitors of P450 enzymes, and of these inhibitors, 80% have circulating metabolites[1]. However, the quantitative role of inhibitory metabolites in *in vivo* DDIs is rarely known. Several examples of reversible P450 inhibitors, which have circulating metabolites that are predicted to contribute to *in vivo* interactions do, however, exist. For example, the CYP2C9 inhibitor sulfinpyrazone and CYP3A4 inhibitor itraconazole have circulating metabolites that also inhibit CYP2C9 and CYP3A4, respectively. Based on plasma concentrations and *in vitro* K_i values, CYP2C9 inhibition after sulfinpyrazone administration can be attributed to the circulating sulfide metabolite that has a 15-fold lower K_i in HLMs than the parent drug and circulates at comparable concentrations with the parent[2,3]. Three circulating metabolites of itraconazole are predicted to contribute approximately 50% of the total CYP3A4 inhibition *in vivo*[4].

In addition to reversible interactions, many clinically important pharmacokinetic drug-drug interactions result from inhibition of metabolic clearance via mechanism-based inactivation (MBI) of cytochrome P450 (CYPs) enzymes. By definition, MBI involves metabolism of the inhibitor to a reactive metabolite, which modifies the P450 enzyme and results in irreversible

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loss of enzyme activity[5]. Enzymatic activity can only be restored by *de novo* protein synthesis. Even though metabolites play a fundamental role in the MBI of P450's, and MBI of P450 enzymes has been studied for multiple decades, in many instances the reactive metabolite responsible for inhibition of the P450 enzyme is unknown. General understanding of the biological fate of metabolites, especially reactive metabolites, has surfaced as an important part of the drug discovery process, and increasing attention has been paid to predicting and identifying circulating human metabolites[6-9]. Despite this increasing interest, known circulating metabolites are often not characterized for MBI of P450 enzymes and are usually only tested for pharmacological activity. As a result, the mechanism and extent of P450 inhibition by inhibitory metabolites is not well established. This review presents the current knowledge of the role of metabolites in MBI of P450 enzymes, focusing on the circulating metabolites that contribute to P450 inactivation and the complex metabolic pathways that lead to inactive enzyme. For detailed review on the chemical and biochemical mechanisms of MBI by the various compounds presented the reader is referred to Kalgutkar et al 2007[10]

2. Classification of mechanism-based inhibitors

From the literature, 31 *in vitro* mechanism-based inhibitors were indentified. A drug was considered an MBI if any reports existed of irreversible or time-dependent P450 inhibition by the given drug, regardless of other reports, which may have determined reversible inhibition parameters for the same drug. Generally, MBI of P450s can be classified into two groups: protein and/or heme alkylation and metabolic-intermediate (MI) complex formation.

The general pathways to formation of inactive P450 via metabolites are shown in Figure 1 and a typical pathway for MI complex formation is indicated by (a). An MI complex refers to a tight, coordinate bond between the metabolite and the reduced state of the P450 heme iron. Although the interaction between the final ligand and the heme iron is strong, no covalent bond is formed and the complex can be reversed under experimental conditions by the addition of potassium ferricyanide. The MI complex is catalytically inactive. MI complexes have distinct spectral properties and are measured by difference spectroscopy using the UV/Vis detectable Soret peak centered on λ_{max} of 452-457 nm[11]. MI complexes are believed to be reasonably stable, and have been observed in microsomes prepared from animals and liver biopsy specimens from humans treated with troleandomycin[12,13].

Protein and heme adducts to P450 enzymes are generally characterized by a covalent bond forming between a bioactivated, electrophilic drug and a nucleophilic amino acid in the P450's active site or the heme prosthetic group. As shown in Figure 1, pathway b, the reactive intermediate may bind to the P450 enzymes, forming an irreversible protein adduct, or the reactive intermediate may be released into the media and inactivated, in many instances by reacting with glutathione. The resulting glutathione and other conjugates can often be detected in plasma or urine. P450 protein adducts are usually measured by detection of radioactive drug binding to the P450 or by identification of adducted peptides via mass spectroscopy. In addition, detection of the deactivated metabolites is used as support for determining the mechanism of inactivation.

Nine of the total 31 inhibitors (29%) were classified as irreversible inhibitors by protein alkylation (Table 1). Protein alkylation was defined based on reports of a covalent bond forming to an amino acid of the P450 enzymes, trapping of an electrophilic, reactive intermediate with glutathione, or evidence of a covalent adduct to the P450 heme pyrrole nitrogen. Fourteen inhibitors (45%) were classified as MI complex forming inhibitors (Table 2) based on reports of time- and NADPH-dependent formation of a Soret peak between 452-456 nm. The remaining eight drugs (26%) were classified as MBIs with an unknown mechanism of inactivation (Table 3), based on concentration- and time-dependent inactivation assays, but

lack of reports of a protein adduct, heme destruction, trapping of a reactive intermediate or detection of a spectral MI complex.

Overall, 54% of the inhibitors that displayed *in vitro* P450 inactivation kinetics also inhibited the same P450 enzyme *in vivo*, defined as at least a 1.25-fold increase in the AUC of the probe drug. Of these *in vivo* inhibitors, ten were potent (>5-fold increase in the substrate AUC), eight were moderate (2-5-fold AUC increase) and eight were weak (1.25-2-fold AUC increase) as classified based on the FDA recommendations[14]. The mechanism of P450 inactivation did not correlate with magnitude of clinical interactions. Six of the ten potent inhibitors, five of the seven moderate inhibitors, and three of the eight weak inhibitors were MI complex forming drugs. Interestingly, CYP3A4 appeared to be most susceptible to interactions via MBI. Eight (89%) out of the nine potent inhibitors displayed potent interactions with CYP3A4 probes *in vivo*. In fact, 22 of 31 (71%) MBIs were reported to inactivate CYP3A4, whereas CYP2C9 had only two reports.

All but five of the 31 irreversible inhibitors indentified have confirmed circulating metabolites (Tables 1-3). However, the role of the circulating metabolites in P450 inactivation is equivocal for many of these metabolites. Based on our current classification, inactivating metabolites were defined as such, if the metabolite was shown to inactivate the enzyme, and was on the metabolic route to the reactive intermediate (Figure 1, pathway a, b). Based on these criteria, nine (29%) of the inhibitors have circulating metabolites that have a confirmed role in the inactivation of the P450 enzymes. Of these circulating metabolites, six result in MI complex formation and three contribute to protein adduct formation. It is important to note that the reported inactivating metabolites (Tables 1 and 2) often undergo further metabolism before irreversible inhibition is sustained. As depicted in Figure 1, the parent drug has three possible routes of metabolism. The first is an off path metabolite, which results from metabolism removed from the inactivation site, however this metabolite may still undergo convergent secondary metabolism to P450 inactivation. The two other metabolic pathways (a, b) represent formation of metabolites that are precursors to the reactive species that leads to inactivation. These precursor metabolites can stay on path to the inactivation of the enzyme, or can be released into the media and circulate, and rebind to the P450 enzyme to cause inactivation

3. Role of metabolites in irreversible inhibition by protein adduct formation

The inhibitors that formed protein adducts (Table 1) were commonly oxidized to an electrophilic species that either binds to the P450 protein or is released from the active site and binds to other macromolecules. Hence, circulating metabolites associated with the reactive species were rarely observed. The reactive intermediates, usually quinone imine-derivatives, are commonly identified as glutathione adducts. For example, dasatinib, diclofenac, nefazodone, raloxifene, and zafirlukast have identified glutathione adducts but only one of these five drugs, nefazodone, displays *in vivo* inhibition of the inactivated enzyme[15-19]. Clopidogrel and ticlopidine, that both result in protein adduct formation, have similar pharmacological actions as well as similar inactivation mechanisms. The primary reactive metabolites identified for both clopidogrel and ticlopidine are further oxidized by CYP enzymes before forming a protein adduct, and in each case, there is controversy on the exact species responsible for the inactivation[20]. The remaining inhibitors in this group, efavirenz and gemfibrozil, have unique mechanism of inactivation and are discussed in detail as case studies. It is interesting to note that all of the protein adduct forming inhibitors that cause *in vivo* interactions, also have circulating metabolites that can contribute to the P450 inactivation.

4. Role of metabolites in irreversible inhibition by MI complex formation

Of the drugs characterized as MI complex forming inhibitors, 64% contained an alkyl amine moiety at the site of P450 metabolism. Alkyl amines are not only common moieties known to

cause P450 inactivation, but are also important entities to the pharmacological activity of the drugs. The formation of MI complexes from alkyl amine containing drugs is believed to occur via a multistep process, which results in the eventual inactivation of the P450 (Figure 2). As shown in Figure 2, there are multiple oxidative steps required to form an MI complex with the P450 enzyme. In addition, the intermediate metabolites, such as the primary amines often have high affinity for binding to the P450 enzymes. It is commonly believed that a primary amine is required for MI complex formation via hydroxylation of the amine nitrogen to form the primary hydroxylamine and further oxidation to a nitroso group that coordinates to the ferrous state of the heme iron[21]. It is important to note that the metabolite responsible for coordination to the heme iron has not been fully characterized and several different chemical pathways to inactive enzyme have been proposed[22,23]. For example, Lindeke et al. examined MI complex formation from the secondary amine, N-methylamphetamine, and proposed that the secondary amine is N-oxygenated to the secondary hydroxylamine, and then proceeds through further oxidative steps to coordination of the heme iron (Figure 2)[24]. Hence, the pathway to the formation of an MI complex from alkyl amine drugs is a complex process involving a variable number of metabolic steps.

The most remarkable feature of the alkylamine drugs is that with the exception of troleandomycin and erythromycin, they all have characterized circulating metabolites. Most commonly, the N-dealkylated metabolites circulate (Table 2) and their plasma concentrations are higher than the parent drug. Based on current mechanistic understanding, it is assumed that these N-dealkylated metabolites also contribute to P450 inactivation, although very little evidence is available of inactivation of P450 enzymes by the primary amine metabolites of the drugs listed in Table 2. It is interesting that despite the fact that the primary amines are believed to be the direct precursors of MI complexes, there is no direct correlation between formation of the primary amine by a given P450 enzyme and inactivation of that same P450 enzyme by MI complex formation. For example, CYP2D6 has the lowest reported K_m (2.1μM) [Cl_{int} = 2.9 $\mu M^{-1}min^{-1}$] with regards to the N-demethylation of fluoxetine to norfluoxetine, but this CYP does not form an MI complex with fluoxetine; whereas, CYP2C19 which has a 82-fold higher K_m (172μM) [Cl_{int}= 0.23 $\mu M^{-1}min^{-1}$] for norfluoxetine formation undergoes rapid inactivation by fluoxetine[25,26]. At present, it is not known why some P450 enzymes are capable of oxidizing the parent alkyl amine drug to inhibitory metabolites but are incapable of forming an MI complex. A plausible explanation may be the structure of the P450 active site, given the prevalence of CYP3A4 to form MI complexes (100%), and the absence of an MI complex formation with CYP2C9 (0%). Given CYP3A4 promiscuity to metabolize a large number of drugs and its large active site, it may be more prone to forming and binding reactive metabolites. However, the prevalence of reports of MI complexes with CYP3A4 may be a result of more studies conducted with this enzyme. There may also be a concentration dependence to the inactivation of P450 enzymes as with CYP3A4 and troleandomyocin[27]. At concentrations higher than 10μM, troleandomycin no longer displays inactivation kinetics due to the amount of competitive primary and secondary metabolites formed that are able to compete for the active site of CYP3A4.

Due to the complex, and potentially multiple, parallel pathways to MI complex formation with P450's, it is unlikely that the inactivation kinetics of the P450 by a tertiary amine or secondary amine (parent drug) will correctly represent the cellular or *in vivo* system, in which multiple inactivating metabolites are present and may dominate the overall *in vivo* inactivation. In addition, the metabolites may be potent reversible inhibitors of the relevant P450 adding further complexity into the characterization of inhibition. Hall et al. recently proposed the need to add both the competitive and irreversible inhibition, when determining the extent of inhibition *in vivo* with the alkyl amine drugs, erythromycin and diltiazem[28]. A semiphysiological model was presented, which suggested that both diltiazem and its metabolite N-desmethyldiltiazem contributed to the overall inhibitory effect after diltiazem administration *in vivo*[29].

Unfortunately, sufficient data *in vivo* and *in vitro* for the metabolites is lacking to develop a more detailed model of *in vivo* MI complex formation kinetics.

Four of the MI complex forming drugs are anti-HIV protease inhibitors (amprenavir, indinavir, nelfinavir and ritonavir). Figure 3 shows the structures of these four protease inhibitors and their primary metabolic sites. No apparent reactive metabolite(s) or intermediates that would lead to MI complex formation have been identified *in vitro* for any of these drugs. As is evident from the structures, no obvious mechanism for the MI complex formation that would agree with the pathway in Figure 2 can be readily proposed. However, a spectroscopic detection of a peak at ~455 nm after incubation of HLM's and recombinant CYP3A4 with these compounds suggested MI complex formation, and warrants further mechanistic studies with these compounds with focus on metabolites[30].

Finally, paroxetine forms a distinctive type of MI complex with CYP2D6. Paroxetine contains a methylenedioxyphenyl moiety, a structural alert known to exhibit MBI of P450 enzyme [31]. P450-catalyzed metabolism of the methylenedioxyphenyl substituent results in initial hydroxylation at the methylene carbon forming an unstable intermediate. This unstable intermediate can partition between demethylenation yielding a cathecol metabolite or dehydration to a carbene, which forms an MI complex with the P450 enzyme[32]. Based on the mechanism it is unlikely that circulating metabolites play a role in CYP2D6 inactivation by paroxetine.

5. MBIs of unknown mechanisms of inhibition

Eight (26%) of the inhibitors identified have an unknown mechanism of inactivation based on lack of any described mechanism in the literature. These inhibitors were characterized as MBIs based on reports of concentration- and time-dependent inactivation assays, which are not necessarily conclusive in characterizing a drug as an MBI. Six of the eight (75%) drugs indentified have *in vivo* interactions with probe drug substrates. Based on the structures of these eight drugs, it could be hypothesized that MI complex is unlikely for all of the drugs, with the exception of delavirdine, which contains an alkyl amine moiety.

6. Case Studies

6.1 Irreversible Inhibition by P450 protein adduct

6.1A—Efavirenz (Figure 4) is metabolized mainly by CYP2B6 to two metabolites: 8-hydroxyefavirenz (major) and 8,14-dihydroxyefavirenz (minor)[33], and is an example of a drug that has a circulating metabolite that appears to be responsible for protein adduct formation. Efavirenz and both metabolites contain an ethynyl group, which is a known alert for P450 mechanism-based inactivation, particularly with the CYP2B family of enzymes[34]. Both efavirenz and its major metabolite 8-hydroxyefavirenz inhibit CYP2B6 but the inactivation of CYP2B6 by 8- hydroxyefavirenz was markedly different from the inactivation by efavirenz[35]. A comparison of the k_{inact}/K_I-ratio (efavirenz =0.0013; 8-hydroxyefavirenz =0.0094) shows that the metabolite is a more efficient inactivator than the parent compound. Interestingly, the metabolism of efavirenz and 8-hydroxylefavirenz by CYP2B6 leads to inhibition by two distinct mechanism although the reactive species responsible for the inactivation are not yet known. Efavirenz was a potent apparent reversible inhibitor (time- and concentration-dependent inactivation yet reversible by dialysis), whereas the 8-hydroxylefavirenz was an irreversible inhibitor (not reversible by dialysis). Based on this in vitro data, it appears that for *in vitro* to *in vivo* extrapolation of CYP2B6 inactivation after efavirenz administration, 8-hydroxyefavirenz rather than efavirenz should be modeled.

6.1B—Gemfibrozil (Figure 4) is known to be a more potent *in vitro* inhibitor of CYP2C9 than CYP2C8[36-38]. However, *in vivo*, gemfibrozil is an inhibitor of CYP2C8 but not of CYP2C9 [39]. An important step in providing a potential explanation of this *in vitro* to *in vivo* discrepancy was provided by demonstrating that gemfibrozil 1-*O*-β-glucuronide, the circulating metabolite of gemfibrozil, is a more potent inhibitor than gemfibrozil of CYP2C8 [40]. The mechanism of inactivation was recently characterized to occur via formation of a heme adduct[41]. It is important to note that the MBI of CYP2C8 was not observed with the parent drug in microsomes and phase II metabolites are not routinely tested for MBI, suggesting that testing for MBI in hepatocytes may be advantageous[42]. Oxidation of phase II metabolites by P450 enzymes appears rare; however, some examples include the oxidation of sulfate conjugates of testosterone, dehydroepiandrosterone, and estrogens[43-45] and oxidation of the acyl glucuronide of diclofenac by CYP2C8[46]. Gemfibrozil 1-*O*-β-glucuronide is the first report of a phase II metabolite that irreversibly inhibits CYP2C8 but this type of inhibition may be more widespread than currently acknowledged and true incidence may not be appreciated, especially given that phase II metabolites are rarely evaluated.

6.1C—Nefazodone (Figure 4) is known to have incidences of idiosyncratic hepatotoxicity, and the reactive intermediates may be responsible for liver injury[47-49]. Nefazodone has both on path and off path metabolites that circulate and two circulating metabolites are known to inactivate CYP3A4[50,51]. Nefazodone is oxidized by P450 enzymes to the major circulating metabolite, hydroxynefazodone, which is then further oxidized by CYP3A4 to an electrophilic quinonoid intermediate. The structure of this reactive intermediate was inferred through the characterization of the corresponding glutathione conjugate. A second circulating metabolite, para-hydroxyl-m-CPP (mCPP), which is formed mainly by CYP2D6, can also be activated to quinone-imine by CYP3A4 and may play a role in the inactivation of CYP3A4[17]. It is interesting that P450 inactivation by nefazodone may involve different pathways in single enzyme systems and in more complex matrices due to the involvement of different P450's in the formation of the intermediates. Due to the multi-enzyme involvement in inactivation and the presence of two distinct inactivating metabolites, *in vitro* to *in vivo* extrapolation of CYP3A4 inactivation by nefazodone is expected to require complex models.

6.2 Irreversible Inhibition of P450 by MI complex formation

6.2A—Verapamil (Figure 5) has two major metabolites formed by CYP3A4 via N-dealkylation: N-desalkylverapamil (D-617) and norverapamil[52,53]. Verapamil and these two major metabolites form MI complexes with CYP3A4[54]. Based on the ratio of k_{inact} to K_I, the inactivation potency was norverapamil > verapamil > D-617. Although the plasma concentration of D-617 is comparable to verapamil and norverapamil, and D-617 is a secondary amine, the potency of inactivation is weak and D-617 probably does not contribute to the *in vivo* inactivation. However, the steady-state levels of norverapamil reach that of verapamil and incorporating the inactivation kinetics of the secondary amine metabolite improved the *in vivo* predictions[55].

6.2B—Fluoxetine (Figure 5), is a substrate and inhibitor of multiple CYP enzymes. The major route of fluoxetine metabolism is by N-demethylation to norfluoxetine by CYP2D6 and other P450 isoforms, including CYP3A4 and CYP2C9[56,57]. Fluoxetine also undergoes CYP2C19-mediated O-dealkylation to a p-trifluoromethylphenol metabolite[58]. Fluoxetine and norfluoxetine have been shown to be reversible inhibitors of CYP2D6[59,60], CYP2C19 [61,62], CYP3A4[63] and CYP2C9[64]. In all cases of CYP inhibition, norfluoxetine was a more potent competitive inhibitor than the parent compound fluoxetine. However, fewer studies have been conducted on the MBI potential of fluoxetine and norfluoxetine. Mayhew et al.[65] showed fluoxetine to be an MBI of CYP3A4 and McGinnity et al.[26] demonstrated time- and concentration-dependent inhibition of CYP3A4 and CYP2C19 in multiple *in vitro*

systems, including hepatocytes. Recently, Stresser et al.[66] reported that norfluoxetine exhibited an 11-fold shift in IC_{50} value when tested in human liver microsomes with the CYP2C19 probe (S)-mephenytoin, suggesting that conversion of fluoxetine to norfluoxetine represents a metabolic pathway leading to time-dependent inhibition. As mentioned previously, it is important to point out that not all the enzymes that form norfluoxetine are inactivated by fluoxetine. CYP2D6 and CYP2C9 both form norfluoxetine efficiently, but do not undergo MBI by fluoxetine. More so, fluoxetine is an MBI of CYP2C19 but CYP2C19 has a relatively high K_m (172 ± 25 μM) for the formation of norfluoxetine[25]. It is possible that norfluoxetine formed by CYP2D6 and CYP2C9 is released and then inactivates other enzymes such as CYP3A4 and CYP2C19, again emphasizing the importance of testing for MBI in multiple CYP systems. Given that the route to inactivation is a multi-step process and metabolites are released into circulation, it is not clear which alkyl amine metabolites are important in making accurate predictions of *in vivo* inhibition. For example, CYP3A4 is clearly inactivated *in vitro* by fluoxetine but there is no *in vivo* interaction with CYP3A4 probes[67, 68]. In contrast, *in vitro* inactivation of CYP2C19 is associated with moderate inhibition of CYP2C19 *in vivo*[69].

6.2C—Amiodarone (Figure 5) is a tertiary amine that interacts *in vivo* with a number of drugs metabolized by CYP1A2, CYP2C9, CYP2D6 and CYP3A4 but the *in vitro* inactivation profile for amiodarone is not fully established[70-72]. Amiodarone is known to form an MI complex in rodents, which is consistent with tertiary amine metabolism to the nitrosoalkane reactive intermediate (Figure 2, [73]). The identity CYP isozymes inactivated by MI complex formation is unclear as the major circulating metabolite of amiodarone, N-desethylamiodarone, inactivates different isozymes than the parent drug. Ohyama, K et al.[74] report a k_{inact} value of 0.06 min^{-1} and a K_I value of 13.4μM for CYP3A4 and amiodarone but no inactivation of CYP3A4 by N-desethylamiodarone. In contrast, both N-desethylamiodarone and amiodarone were recently shown to be time- and concentration- dependent inactivators of CYP3A4[75]. N-desethylamiodarone, but not amiodarone, is consistently reported as an MBI of CYP2D6 highlighting the importance of studying metabolites separately for MBI[74,75]. Interestingly, CYP2D6 is capable of N-dealkylation reaction with an *in vitro* intrinsic clearance to the secondary amine of 106.8 μM/min/nmol CYP[74]. Although, CYP2D6 is very efficient in forming N-desethylamiodarone, the inhibitory metabolite, MBI of CYP2D6 by the parent drug is not observed.

Amiodarone also inactivates CYP2C8 and CYP2C9 (Polasek, PM et al. 2004, Mori, K. et al.) and CYP2C8 plays a significant role in amiodarone deethylation[70,75,76]. However, spectral studies did not detect MI complex formation or heme loss with CYP2C8 and the exact mechanism by which amiodarone inactivates CYP2C8 remains unclear[76]. N-desethylamiodarone may play a role in CYP2C8 inactivation, as the inhibition by N-desethylamiodarone increased by 42% between co- and pre-incubated samples[76]. CYP2C9 has similar time- and concentration-dependent inactivation by both amiodarone and N-desethylamiodarone. It is likely that N-desethlyamiodarone is essential for CYP2C9 inactivation as a correlation was found between the ΔINR/ warfarin dose and plasma concentration of N-desethylamiodarone but not with amiodarone concentration[77]. This again emphasizes the critical role of the metabolites in CYP inactivation and suggests that accounting of the metabolites is important for quantitative understanding and predictions of in vivo CYP inactivation.

7. Conclusions

Given the limited number of predictive models for complex DDIs involving parent drugs and their metabolites, it is difficult to fully evaluate the importance of inhibitory metabolites. Many drugs that display *in vitro* MBI kinetics, do not display significant *in vivo* DDIs. In fact only

9 of the 31 inhibitors (29%) are potent inhibitors *in vivo*. On the other hand, drugs such as mibefradil, a potent MBI of CYP3A4, was withdrawn from the market as a result of unpredicted CYP inhibition, most likely due to interactions caused by a metabolite[78]. From the available data for the compounds reviewed here, it appears that circulating metabolites are much more important in *in vivo* CYP inactivation than is currently acknowledged. The data suggests that CYP inactivation by circulating primary and secondary metabolites needs to be characterized for accurate predictions as well as for better mechanistic understanding of *in vivo* MBI. Due to the multi-enzyme involvement of CYP inactivation, testing for MBI in complex enzyme systems such as hepatocytes, may help in the overall understanding of the inhibition and significantly improve *in vitro* to *in vivo* predictions.

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Abbreviations

CYP	cytochrome P450 enzyme
DDI	drug-drug interaction
HLMs	human liver microsomes
k_{inact}	maximum inactivation rate constant
K_I	inactivation binding constant
K_i	reversible binding constant
MBI	mechanism-based inactivation (inhibitor)
MI complex	metabolic-intermediate complex

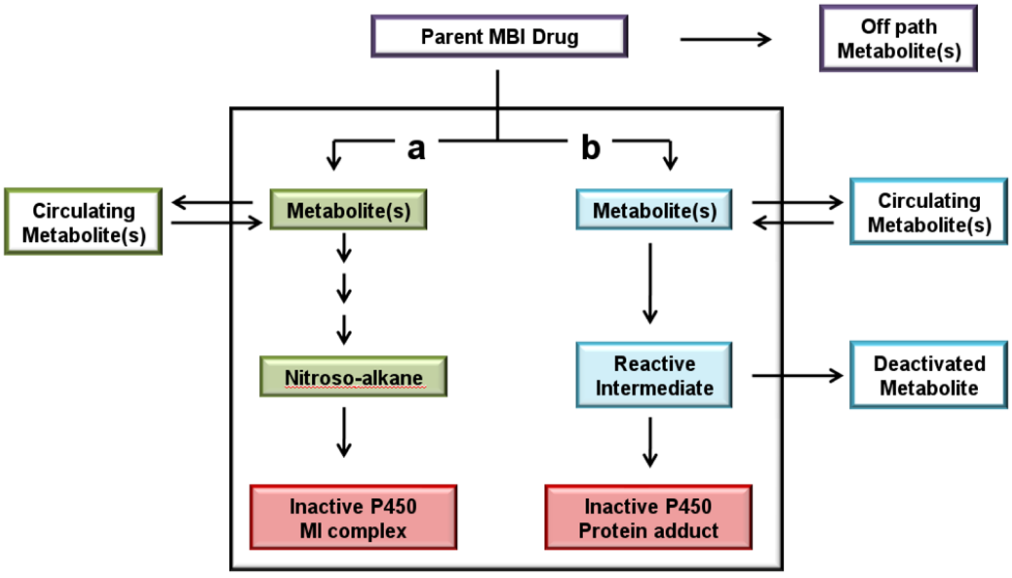

Figure 1.

Pathways to irreversible inhibition of P450 enzymes. Pathways a and b indicate the two metabolic routes to inactivate P450 (red boxes) by MI complex formation (green boxes) or by protein alkylation (blue boxes). The off path metabolites (purple box) are not directly involved in the inactivation of the P450 enzymes. Circulating metabolites (pathway a and b) are able to rebind to the P450 enzymes and are further metabolized to inactivate the P450 enzymes. The deactivated metabolites (pathway b) are dead end products and often detected *in vitro*.

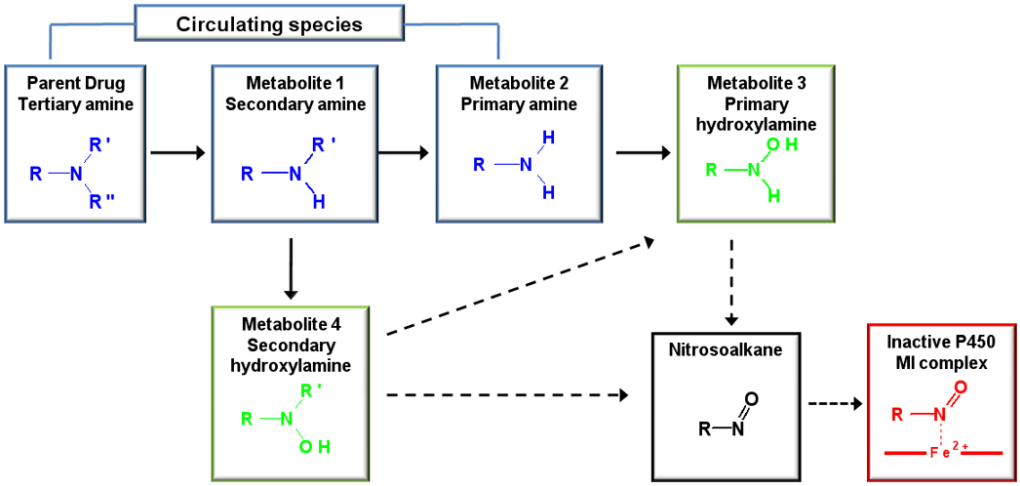

Figure 2.

Proposed route of alkyl amine metabolism to MI complex. Solid arrows indicate P450 metabolic reactions indentified in HLMs, dashed arrows are proposed metabolic reactions on route to MI complex formation but have not been verified. Blue compounds are known circulating metabolites, green compounds have been shown to form MI complexes but are not known circulating metabolites, the black colored compound is the proposed proximal species to MI complex formation, the red colored compound is the inactivated P450 enzyme. The pathways were adapted from references [21,22,24]

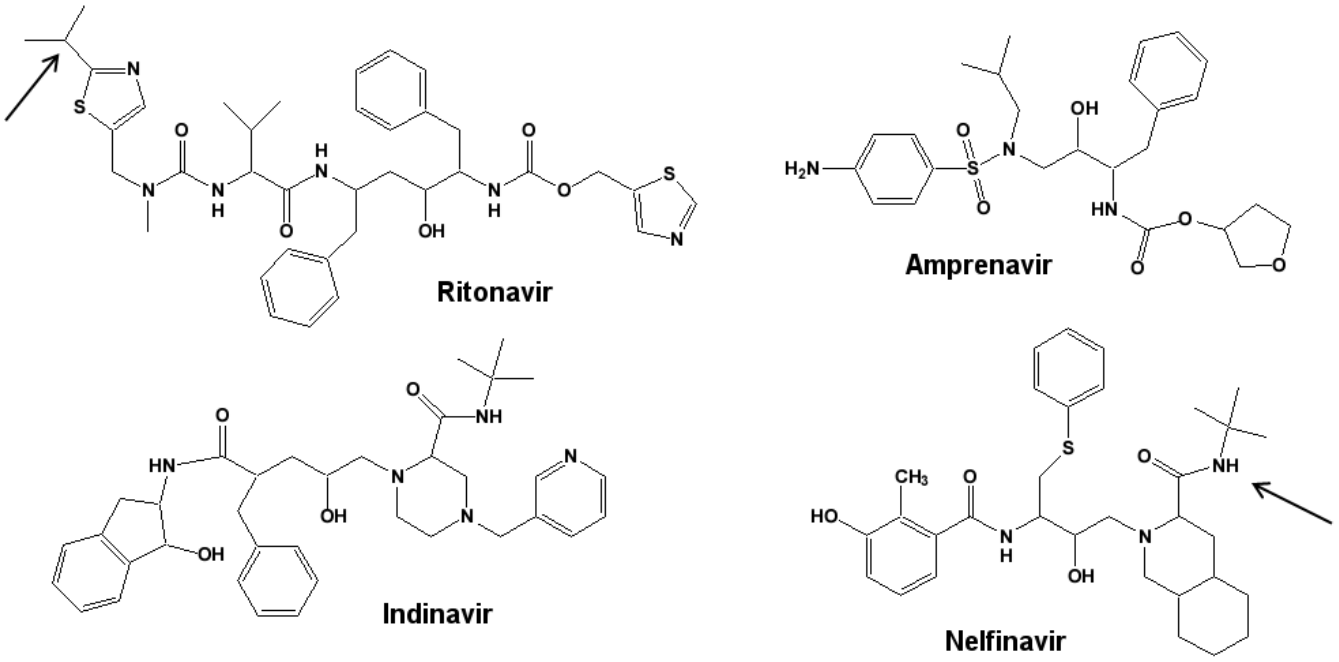

Figure 3.
Structures of anti-HIV protease inhibitors that irreversibly inhibit CYP3A4 via an MI complex formation. The arrows indicate sites of metabolism for known circulating metabolites (Table 2).

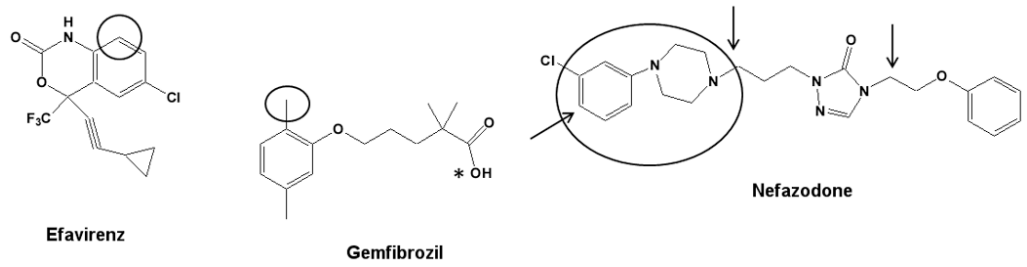

Figure 4.
Structures of three drugs that form protein adducts with P450 enzymes. Circles indicate the site of metabolism that leads to inactivation, the arrows indicate site of metabolism for off path circulating metabolites (Table 1). *indicates the site of glucuronidation to form the inactivating glucoronide metabolite.

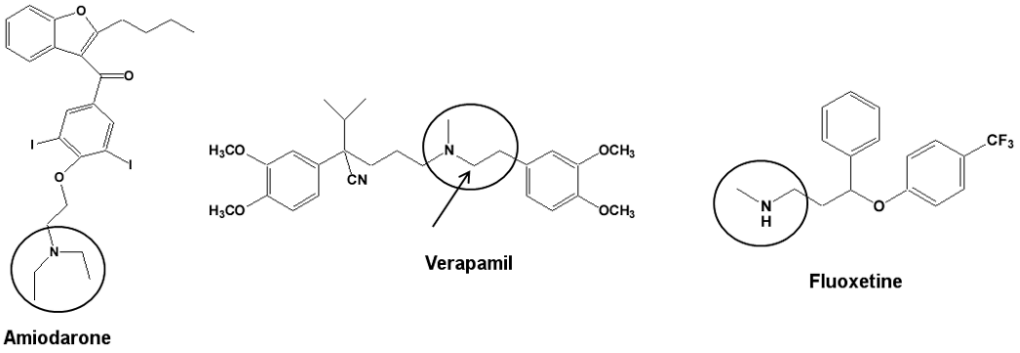

Figure 5.
Structures of three drugs that result in MI complex formation with P450 enzymes. Circles indicate the site of metabolism that leads to inactivation, the arrows indicate site of metabolism for off path circulating metabolites (Table 2).

Table 1

Inhibitors that inactivate CYP enzymes by protein adduct formation. The inactivating metabolites were classified by determining the proximal reactive species reported on the pathway to the irreversible inhibition. $^{\Pi}$ circulating metabolite, * indicated DDI in vivo (w= weak, m=moderate, p=potent as indicated in text), ‡ inhibition by metabolite only, ^{N} negative control study of inhibition reported (< 20% inhibition of an FDA approved probe drug).

Parent	Inactivating metabolites	1A2	2B6	2C8	2C9	2C19	2D6	3A4	Off path circulating metabolites	Ref
clopidogrel	2-oxo-clopidogrel (thiolactone)$^{\Pi}$		**X*^{w}**						SR26334 (clopidogrel acid)	[79,80]
dasatinib	para-quinone-imine intermediate							**X,^{N}**	M4, M5, M6, M20, and M24	[15,81]
diclofenac	benzoquinone imine intermediate							**X**	3-OH,4methoxy-diclofenac, 4-OH-diclofenac	[46,82]
efavirenz	8-hydroxyefavirenz$^{\Pi}$		**X*^{m}** ‡							[33.35]
gemfibrozil	gemfibrozil 1-*O*-β-glucuronide$^{\Pi}$			**X*^{m}**						[41,42]
nefazodone	para-quinone-imine intermediate							**X*^{p}**	mCPP,hydroxynefazodone,desethylhydroxy	[17,51]
raloxifene	diquinone methide intermediate							**X**		[83]
ticlopidine	S-oxide ticlopidine$^{\Pi}$ (2C19), thiolactone (2B6)		**X*^{w}**			**X*^{p}**			metabolites 85-95% of plasma radioactivity	[79,84]
zafirlukast	α,β-unsaturated iminium ion intermediate							**X**	hydroxylated metabolites	[19]

Table 2

Inhibitors that inactivate CYP enzymes by MI complex formation. [Π] circulating metabolite, * indicated DDI in vivo (w= weak, m=moderate, p=potent as indicated in the text), [‡] inhibition by metabolite only, [#] inhibition by both parent and metabolites, [N] negative control study of inhibition reported (< 20% inhibition of an FDA approved probe drug), [o] parent did not form MI complex but was an MBI of unknown mechanism, [1] metabolite may be on path to inactivation

Parent	Inactivating metabolites	1A2	2B6	2C8	2C9	2C19	2D6	3A4	Off path circulating metabolites	Ref
Alkyl amines										
amiodarone	N-desethylamiodarone[Π]	X[‡]	X[‡]	X[o]	X[o]		X*[w] [‡]	X*[w]		[70,75]
amitriptyline	nortriptylline[Π]	X[#]		X[‡]		X[#]	X[‡]	X[‡]		[76,85]
clarithromycin	N-desmethylclarithromycin							X*[p]	14-hydroxyclarithromycin	[86]
diltiazem	N-demethyldiltiazem[Π]							X*[m] [#]	N-deacetyldiltiazem	[65,87]
erythromycin	N-desmethylerythromycin							X*[m]		[28,88]
fluoxetine	norfluoxetine[Π]			X		X*[m] [#]		X*[w],[N]		[26,66,76]
tamoxifen	N-desmethyltamoxifen[Π]		X					X[#]	4OH-tamoxifen, N-didesmethyltamoxifen[1]	[89,90]
troleandomycin	N-desmethyltroleandomycin							X*[p]		[12,26]
verapamil	N-desalkylverapamil, norverapamil[Π]			X				X*[m]	D-617, D-620	[54,76]
Protease inhibitors										
amprenavir	unknown							X*[m]		[30]
indinavir	unknown							X*[p]		[30]
nelfinavir	unknown							X*[p]	Hydroxy-t-butylamide nelfinavir	[30]
ritonavir	unknown							X*[p]	M-2 (Hydroxy-isopropyl ritonavir)	[30]
Other										
paroxetine	methylenedioxy carbene intermediate						X*[p]			[32]

Table 3

Inhibitors that inactivate CYP enzymes by unknown mechanism. * indicated DDI in vivo (w= weak, m=moderate, p=potent as indicated in the text), [1] P450 heme-destruction implied based on mechanism of action.

Parent	Inactivating metabolites	1A2	2B6	2C8	2C9	2C19	2D6	3A4	circulating metabolites	Ref
cimetidine	unknown						X*w		sulfoxide, hydroxymethyl cimetidine	[91,92]
delavirdine	unknown							X*w	N-desisopropyl, N-desakyl delavirdine	[93]
lopinavir	unknown							X*p		[30]
phenelzine	unknown[1]	X		X	X			X		[76,94]
saquinavir	unknown							X*p	mono- and di-hydroxylated saquinavir	[30]
thiotepa	unknown		X						tepa	[95-97]
zileuton	unknown	X*m							N-dehydroxylated	[98]
zolpidem	unknown							X*w		[99]

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