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Title page:

NADPH-independent Inactivation of CYP2B6 and NADPH-dependent Inactivation of CYP3A4/5 by Pyrrolobenzodiazepine Dimer (PBD): Potential Implication for Assessing Covalent Modulators for Time-dependent Inhibition

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Running Title:

NADPH-independent inactivation of CYP2B6 by PBD

Key Words: pyrrolobenzodiazepine, pyrrolobenzodiazepine dimer, cytochrome P450, CYPCYP4/5, CYP2B6, CYP inhibition, time-dependent inhibition, NADPH-independent time-dependent inhibition, covalent modulators, and reactive imines.

Abbreviations: CYP, cytochrome P450; TDI, time-dependent inhibition; GSH, gluthatione; NADPH, β-nicotinamide adenine dinucleotide phosphate; PBD, pyrrolobenzodiazepine dimer; KPi, potassium phosphate.

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Abstract

Pyrrolo[2,1-c][1,4]benzodiazepine dimer (PBD) has been shown broad antitumor properties and potential as a therapeutic agent for cancers. During a routine DDI assessment, it was found that PBD is a reversible inhibitor of CYP2C8 (IC_{50} = 1.1 μM) but not CYP1A2, 2B6, 2C9, 2C19, 2D6, or 3A4/5. Additionally, PBD is a classic TDI of CYP3A4/5, with >30-fold shift in IC_{50} following a pre-incubation with NADPH. All other CYPs tested did not show evidence for TDI, but potent inhibition of CYP2B6 (IC_{50} = 1.5 μM) was observed following a pre-incubation w/wo NADPH, an unexpected observation given the fact that no inhibition was observed in the direct inhibition assay. No other CYP isoforms were susceptible to this apparent non-NADPH dependent inhibition, suggesting that PBD may selectively inactivate CYP2B6 without metabolic activation. The washing of the HLM pellet after incubation with PBD did not fully recover CYP2B6 activity, indicating that PBD is covalently bound to CYP2B6 leading to inactivation of the enzyme. To further investigate the mechanism of NADPH independent inhibition, the IC_{50}-shift was determined for several PBD analogs, and it was found that the compounds without both reactive imines did not show NADPH-independent inhibition of CYP2B6, implying that NADPH-independent inactivation was likely caused by direct covalent binding of PBD to the enzyme in a highly structure specific manner. These data clearly highlight the need to assess direct and time-dependent inhibition w/wo NADPH to adequately characterize the in vitro CYP inhibitory properties of drug candidates with reactive moieties.

Significance Statement: We described a very unique in vitro CYP inhibition profile of PBD as a potent reversible CYP2C8 inhibitor, a NADPH dependent CYP3A4/5 TDI inhibitor and a NADPH independent CYP2B6 TDI inhibitor, and inhibition of CYPs occurs through three distinct mechanisms: reversible drug-enzyme binding, enzyme inactivation via bioactivation, and enzyme inactivation by covalent binding via chemical reactions. Our results suggest that, for compounds with reactive functional moieties, false positives can be reported when the conventional TDI assay is utilized.

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Introduction

The pyrrolo[2,1-c][1,4]benzodiazepines (PBDs) are an important class of DNA alkylators that bind to the DNA minor-groove in a sequence-selective manner to form a covalent aminal bond between their C_{11}-position and the C_2-NH_2 groups of guanine bases of the targeted double-stranded DNA region (Fig. 1A) (Hartley, 2011; Mantaj, et al. 2017). Compared to monomeric PBDs, PBD dimers contain two alkylating imines which, after binding to the DNA minor-groove, can form intra/inter-strand DNA cross-links leading to formation of a more stable PBD-DNA complex compared to mono-alkylated adducts (Fig. 1B). The PBD dimer molecular structure can influence DNA sequence recognition given that such compounds contain a chiral C_{11}a(S)-center that forms an appropriate conformation to fit in the minor groove of DNA, resulting in superior alkylating potency compared to PBD monomers.

Through covalent bonding to DNA, PBDs have been shown to exert a number of biological effects in cells including DNA strand breakage (Reid, et. al, 2011), inhibition of DNA processing enzymes such as endonuclease (Puvvada, et. al, 1993), RNA polymerase (Puvvada, et. al 1997) and ligase (Shameem, et al, 2015), and specific transcriptional factors (Prabhakar, et. al, 2011). Consequently, PBDs have been investigated for their broad anti-tumor activity and as potential therapeutic agents against a variety of oncology targets, and several PBD dimers such as SJG-136 and DSB-120 have been advanced to clinic for cancer treatments including leukemia and ovarian cancer (Cipolla et al. 2009; Hartley, 2011; Martin et al. 2005).

Apparently, cellular cytotoxicity of both PBD monomers and dimers is not highly target-selective, and their inherited off-target activity can potentially result in non-selective cytotoxicity which severely limits their therapeutic potential as anti-cancer agents (Mantaj, et al. 2017). To

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mitigate the non-selective cytotoxicity, PBD dimers have recently been attached as a cytotoxic warhead to tumor-specific antibodies via a self-immolative linker to the N_{10} nitrogen to generate antibody–drug conjugates (ADCs) (Zhang, et al. 2019). As a result, PBD dimers can be delivered by antigen-specific monoclonal antibodies specifically to the targeted tumor cells. Once bound to the tumor cells, ADCs can then be internalized via endocytosis and subsequently degraded to generate the C_{11}-N_{10} imine group upon linker cleavage, releasing PBD dimers to exert their cancer cell-killing activity (Gregson, et al, 2004; Hartley, et al. 2004). This immunological targeting strategy has resulted in several ADCs advancing to clinical development (Phillips T, et. al 2019; Zheng B, et. al 2019; Zammarchi, et. al, 2018).

Clinical interest in developing ADCs as potential cancer therapeutics led us to investigate PBD dimer (referred as PBD below) for its potential to inhibit common human CYPs in vitro, in order to pursue extrapolation of such findings to clinical DDI potential. As a routine experimental assessment, we evaluated pyrrolo[2,1-c][1,4]benzodiazepine, a commonly used PBD warhead, for inhibition of seven common cytochrome P450 enzymes including CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and CYP4/5 in human liver microsomes (HLM). Also, IC_{50} shift assay was conducted to determine whether PBD is a time-dependent inactivator (TDI) of those common CYP enzymes. Additionally, several PBD analogs were studied in order to investigate the effect of the reactive moieties on the time dependent inhibition (TDI) of CYP2B6. Since PBDs represent a novel class of drug molecules with chemically reactive moieties, the outcomes of the present investigation would not only help understand the inhibitory properties of this unique compound class against human CYP enzymes but also advance our strategy in assessing DDI potential for other chemically reactive drug molecules such as various covalent modulators, another novel chemical class of increasing interest in drug discovery and development.

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Materials and Methods

Chemicals and reagents.

All reagents were in the highest grade commercially available. Bupropion, phenacetin, paclitaxel, *(S)*-(-)-warfarin, dextromethorphan, *(S)*-(+)-mephenytoin, ticlopidine, and β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich (St. Louis, MO). Midazolam, 1-hydroxy midazolam–[^{13}C3], human liver microsomes (mixed gender, 150 donors pooled) and *Supersomes* containing rCYP2B6 (rCYP2B6) were all obtained from Corning (Corning, New York). Acetaminophen-D_4, phenyl-D_5-7-hydroxywarfarin, hydroxybupropion-D_6, 4'-hydroxymephenytoin-D_3, 6α–hydroxypaclitaxel-D_5 and dextrorphan-D_3, were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Acetonitrile and formic acid were purchased from EMD Millipore (Hayward, CA). Glacial acetic acid was purchased from BDH Chemicals (London, England). Potassium phosphate, pyrrolobenzodiazepine dimer (PBD) and the PBD analogs were obtained internally from Genentech compound management.

Reversible CYP inhibition.

CYP inhibition assay was performed in triplicate on a Tecan Freedom Evo (Morrisville, NC). Briefly, PBD dimer, PBD analogs or positive controls in DMSO stock was serially diluted in phosphate buffer (KPi 10 mM, pH 7.4) containing human liver microsomes (HLM) and individual CYP isoform specific substrates. After brief warm-up at 37°C, enzymatic reaction was initiated by adding 1 mM NADPH to HLM-substrate mixture. The final reaction mixtures contain 0.03 mg/mL HLM, a test compound (PBD or analog) at various concentrations of (0, 0.0032, 0.016, 0.08, 0.4, 2, and 10 μM) and individual CYP specific substrates including

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phenacetin (50 µM, 1A2), bupropion (80 µM, 2B6), paclitaxel (4 µM, 2C8), *S*-(-)-warfarin (2 µM, 2C9), *S*-(+)-mephenytoin (60 µM, 2C19), dextromethorphan (5 µM, 2D6), and midazolam (2 µM, CYP4/5). After incubation at 37°C for 10-30 min depending on the CYP specific substrate, reaction was terminated with acetonitrile (ACN) containing 6% acetic acid and internal standards. The samples were centrifuged at 2000 x g for 10 min to remove precipitated protein; the resulting supernatants were transferred to a fresh plate and diluted with 0.1% formic acid water, and subsequently were subjected to LC-MS/MS analysis for CYP specific metabolites including acetaminophen (1A2), hydroxybupropion (2B6), 6-α-hydroxypaclitaxel (2C8), 7-hydroxywarfarin (2C9), *(S)*-(+)-4'-hydroxymephenytoin (2C19), dextrorphan (2D6), and 1-hydroxymidazolam (CYP4/5). Stable isotope labelled CYP specific metabolites were used as internal standards. CYP activity was expressed as the peak area ratio of a CYP specific metabolite relative to its internal standard, and percent inhibition was calculated from CYP activity of samples containing PBD relative to the solvent control (no PBD). Inhibition data were processed using Prism to calculate IC_{50} values for individual CYP enzymes.

Time-dependent CYP inhibition (TDI).

The diluted method was used as previously described in literature (Parkinson et al., 2011) for TDI assessment using the same incubation condition of the reversible inhibition assay. First, PBD dimer was serially diluted to 0, 0.032, 0.16, 0.8, 4, 20, and 100 µM in phosphate buffer (pH 7.4) containing 0.3 mg/mL HLM, and two separate sets of pre-incubation were performed at 37°C for 30 min in the presence and absence of 1 mM NADPH. After pre-incubation, aliquots of reaction mixture were transferred to a fresh plate and mixed with 9-volume of KPi buffer containing individual CYP specific substrates and 1 mM NADPH, and incubation continued for additional 10-30 min depending on the CYP specific substrate before quenching the incubation

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with ACN (6% acetic acid) containing internal standards. Samples were processed and then analyzed as described in the reversible CYP inhibition assay. For each CYP enzyme, a pair of sigmoidal dose–response curves was generated to calculate two IC_{50} values respectively for both in the presence and absence of NADPH in the pre-incubation.

Inactivation rate of CYP2B6 by PBD.

PBD dimer (2 μM) was initially incubated with 0.3 mg/mL HLM in 100 mM phosphate buffer (pH 7.4) in the absence and presence of 1 mM NADPH. At different time intervals, aliquots of reaction mixture were transferred and immediately mixed with 9-volume of KPi buffer containing both 1 mM NADPH and bupropion (80 μM), and then incubation continued for 15 min before quenching the incubation with ACN (6% acetic acid) containing hydroxybupropion-D_6 (IS). Resulting samples were subjected to LC-MS/MS analysis for CYP2B6 activity as described above.

Irreversibility of CYP2B6 inhibition by PBD.

HLM was first diluted to 0.5 mg/mL in 100 mM KPi buffer, and was divided into three portions (A, B and C, 0.5 mL each) in triplicate. After spiking 10 μM PBD in portion C, all three HLM samples (A, B and C) were incubated for 60 min at 37°C. At the end of the incubation, portion B was supplied with PBD dimer (10 μM). Subsequently, all three samples were centrifuged at 10,000 RPM for 5 min using an Effendorf Microfuge 5424 to remove supernatants, and the resulting HLM pellets were then washed with 1 mL PBS and followed by centrifugation to remove residual PDB dimer in supernatants. Finally, the treated HLM pellets were re-suspended with 100 mM KPi with final HLM concentration of 0.05 mg/mL to measure CYP2B6 activity in A, B and C by adding bupropion (80 μM) and NADPH (1 mM) as described

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in the above section. CYP2B6 activity in both B and C was expressed in relative to that in A (the control, 100%).

Inhibition of rCYP2B6.

PBD or its analogs were serially diluted in PBS buffer in triplicates, and briefly warmed at 37°C after mixing with NADPH and bupropion. The reactions were initiated by the addition of rCYP2B6 in PBS. The reaction mixtures contain PBD (0.003-10 µM), 1 mM NADPH, 80 µM bupropion and 1.2 pmol/mL rCYP2B6 in a final volume of 100 µL each. After 15 min incubation, the reaction mixtures were quenched with a final volume of 200 µL of acetonitrile containing hydroxybupropion – D_6 as the internal standard. The samples were centrifuge at 2000 x g for 10 minutes; the resulting supernatants were transferred to a 96-well sample plate and diluted with 0.1% formic acid water, and then subjected to LC-MS/MS analysis for CYP2B6 activity as described previously.

Time-dependent inhibition of rCYP2B6.

Time-dependent inhibition of rCYP2B was performed similarly as the regular CYP TDI assay using HLM as described above. The only difference is replacing HLM with *Supersomes* containing rCYP2B6 (1.3 pmol/mL).

LC-MS/MS method

The samples were analyzed using LC 1200SL Series System from Agilent (Santa Clara, CA) and a HTS PAL auto sampler from CTC Analytics (Carrboro, NC) coupled with a 5500 QTRAP® MS equipped with an electronspray ionization (ESI) source. The separation of the analytes was performed using Luna Omega C18 columns (1.6 µm, 30 x 2.1 mm) from

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Phenomenex (Torrance, CA), and analytical methods used are summarized in the Supplementary Material.

Results

Reversible inhibition of CYP activities by PBD in human liver microsomes.

The inhibitory effect of PBD on the activities of seven common CYP isozymes (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYPCYP4/5) was assessed at microsomal protein concentration of 0.03 mg/ml. As summarized in Table 1, PBD showed little or no inhibition for CYP1A2, 2B6, 2C9, 2C19, 2D6, and CYPCYP4/5 with IC_{50} greater than 10 μM for all those isoforms. The only exception is inhibition of CYP2C8 with an IC_{50} value of 1.1 ± 0.3 μM.

Time-dependent inhibition of CYP activities by PBD in human liver microsomes.

The IC_{50} shift method was used to investigate the time-dependent inhibition properties of PBD in HLM. Similar to what was observed in the reversible inhibition assay, PBD showed little or no inhibition for CYP1A2, 2C9, 2C19 and 2D6 in the TDI assay, and no marked change in inhibition potency was observed between two pre-incubations with and without NADPH. For CYP3A4/5, 2C8 and 2B6, different inhibition profiles and were observed (Fig. 2A-C) and their IC_{50} shift patterns are summarized in Table 2. For CYP3A4/5 (Fig. 2A), PBD showed a significant potency shift, and its IC_{50} value decreased from 7.50 μM without NADPH to 0.20 μM with NADPH in the preincubation (Table 2); a 37-fold shift in IC_{50} indicates that PBD is a potent time dependent CYP3A inhibitor. For CYP2C8, PBD showed nearly two identical inhibition profiles with and without NADPH (Fig. 2B), and two corresponding IC_{50} values, 0.6 μM and 0.9 μM (Table 2), are comparable to that (1.1 μM) obtained in the reversible inhibition assay (Table 1), which suggest that PBD is not a time dependent inhibitor of CYP2C8. Similar to that of

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CYP2C8, PBD exhibited two nearly identical inhibition profiles against CYP2B6 (Fig. 2C), and two comparable IC_{50} values (1.50 μM vs 1.8 μM) (Table 2), suggesting that PBD is not a time dependent inhibitor for CYP2B6. However, both values are markedly lower than the IC_{50} (> 10 μM) obtained in the direct reversible inhibition assay.

NADPH-independent CYP2B6 inactivation.

To further confirm NADPH-independent inactivation of CYP2B6, formation of hydroxybupropion was monitored at different incubation times in PBD-HLM mixtures in the absence and presence of NADPH. As shown in Fig.3, remaining CYP2B6 activity in HLM decreased with incubation time and the slope of activity loss over the time course is almost identical between two preincubations with and without NADPH.

Time-dependent inhibition of rCYP2B6 by PBD and analogs.

PBD and five different analogs (PBD1-5) were further tested for both reversible and time dependent inhibition in *Supersomes* expressed CYP2B6. As shown in Table 3, PBD showed weak inhibition against rCYP2B6 in the direct reversible inhibition (IC_{50} > 10 μM), but potent inhibition was observed in the TDI assay in both in the absence and presence of NADPH with IC_{50} of 1.5 μM and 0.2 μM, respectively. In contrast, none of those five analogs showed potent inhibition (> 10 μM) against CYP2B6 in both reversible inhibition and TDI assay.

Irreversibility of CYP2B6 inhibition by PBD.

To determine the reversibility of the inactivation of CYP2B6 by PBD, three different HLM incubations were performed separately: HLM only (A), HLM spiked PBD after incubation (B), and HLM incubated with PBD (C), which was followed by centrifugation and washing to remove remaining PBD in HLM pellets (B and C) as described in the **Materials and Methods**. As shown in Fig 4, HLM spiked with PBD after incubation (B) showed nearly the same level of

CYP2B6 activity compared to that in HLM without PBD (control A), indicating that PBD was completely removed from the samples by centrifugation. In contrast, incubation of HLM with PBD (C) resulted in a loss of more than 30% CYP2B6 activity compared to either one without PBD in the incubation (A) or the other spiked with PBD after incubation (B), suggesting that removal of unbound PBD by centrifugation and washing did not lead to complete recovery of hydroxylase activity of PBD-treated samples.

Discussions

CYP inhibition is regarded as one of the most common mechanisms leading to clinical drug-drug interactions (DDI). CYP inhibition is classified mechanistically as reversible and irreversible inhibition, and irreversible inhibition is usually caused by bioactivation catalyzed by a CYP enzyme, resulting in a reactive intermediate that tightly binds to the enzyme (Hollenberg, et al. 2008). Thus, irreversible CYP inhibition is NADPH dependent. Our results showed that PBD is not an inhibitor for CYP1A2, 2C9, 2C19 and 2D6 up to 10 μM, but a potent reversible inhibitor for CYP2C8 (IC_{50} 1.1 μM). Additionally, PBD is a time dependent inhibitor for CYPCYP4/5, as evidenced by a more than 30-fold shift in IC_{50} observed in the TDI assay (Table 2). PBD is predominately metabolized by CYP3A4/5 in HLM (internal data). When incubated in HLM in the presence of GSH and NADPH, two glutathione conjugates were detected by as a direct conjugate (PBD-GSH, m/z 891.3475) and an oxidative conjugate (PBD-O-GS, m/z 907.3425) respectively (internal data), suggesting that reactive intermediates were formed in HLM incubation. Therefore, it is reasonable to speculate that those reactive intermediates likely bind and inactivate CYP3A4/5, although involvement of other CYPs is also possible.

Interestingly, PBD did not inhibit CYP2B6 in the reversible inhibition assay, but showed potent inhibition of CYP2B6 in the TDI assay w/wo NADPH (IC_{50} 1.5 μM vs 1.8 μM).

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Although no marked shift in IC_{50} was observed in TDI assay, a more than 5-fold decrease in IC_{50} was observed between TDI and reversible inhibition assay, and the shift in potency is unlikely due to the difference in $f_{u,mic}$ since HLM concentration was the same in both assays; additionally, highly comparable IC_{50} values were obtained for CYP2C8 between the same two assays (1.1+/-0.3 uM vs 0.9+/-0.03 uM), suggesting that an decrease in IC_{50} of CYP2B6 was not caused by different incubation conditions. Thus, we concluded that inactivation of CYP2B6 by PBD in HLM is NADPH independent. This conclusion is supported by the observation that the rate of CYP2B6 inactivation in HLM-PBD mixtures is almost identical w/wo NADPH in pre-incubation (Fig. 3). Additionally, NADPH-independent inhibition of CYP2B6 by PBD seems irreversible since removal of PBD from HLM mixtures did not fully restore enzymatic activity (Fig. 4).

NADPH-independent inactivation of CYP2B6 by PBD was further confirmed in *Supersomes* containing rCYP2B6, which showed a more than 5-fold decrease in IC_{50} in the absence of NADPH compared to that measured in the reversible inhibition assay. Interestingly, PBD is more potent in the presence of NADPH (IC_{50} 0.2 μM) compared to that in the absence of NADPH (IC_{50} 1.5 μM), a 7-fold shift in IC_{50} raising the possibility that, in addition to NADPH independent inactivation, CYP2B6 was also inactivated by PBD via NADPH-dependent bioactivation. Apparently, NADPH-dependent inactivation of CYP2B6 seems unnoticeable in HLM, as indicated by two comparable IC_{50} values (1.8 μM vs 1.5 μM) measured in TDI assay w/wo NADPH respectively; additionally, in TDI assay, PBD appeared to be more potent against CYP2B6 in *Supersomes* (IC_{50} 0.2 μM) than HLM (IC_{50} 1.8 μM) when NADPH was present in preincubation. Both differences in inhibition potency observed between *Supersomes* and HLM might be explained by potential metabolism of PBD by CYP3A4/5, leading lower PBD concentrations in HLM than in *Supersomes*.

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Approximately, CYP2B6 accounts for 2-10% of total hepatic P450 content, and it metabolizes 3-12% of all drugs (Wang et. al, 2008; Zanger et al., 2007). So far, several CYP2B6 TDI inhibitors have been reported including isopsoralen (Lu, et al. 2016), isoimperatorin (Cao, et al 2015), selegiline (Sridar, et al. 2012), ticlopidine and clopidogrel (Richter, et. al 2004). Compared to those known TDI inhibitors, PBD is highly unusual given that inactivation of CYP2B6 does not require NADPH-dependent bioactivation. Presumably, this uncharacteristic TDI inhibition is attributed to the fact that PBD contains two imines that can chemically react with both thiol and amines of different amino acid residues, resulting in covalent binding and subsequent inactivation of the enzyme without CYP2B6-mediated bioactivation of PBD. This assumption is quite reasonable since it has been well established that two imines of PBD can chemically react with the C_2-NH_2 groups of guanine bases of the targeted DNA to form a covalent aminal bond (Hartley, 2011; Mantaj, et al. 2017). Consistently, PBD-1, a highly similar analog containing two amines instead of imines, did not show any TDI under the same assay conditions.

It is apparent that inactivation of CYP2B6 is not simply due to random chemical reactions of PBD with the enzyme, since PBD-4, a monomer analog containing one reactive imine group, did not inactivate CYP2B6. Additionally, it is important to note that, PBD-2 and PBD-3, two structurally similar analogs containing only one imine at different locations, did not exhibit TDI against CYP2B6, suggesting that formation of two distinct covalent bonds is likely required to stabilize the PBD-enzyme complex, which is consistent with the fact that PBD forms a covalent aminal bond only after the molecule is secured within the minor groove even though the major DNA major-groove also contains guanine bases (Mantaj, et al. 2017). Therefore, we hypothesized that PBD must first associate with CYP2B6 reversibly to bring two reactive imine

groups within close proximity of their corresponding amino acid residues of the enzyme in order to form two covalent bonds, resulting in a stable and inactive PBD-enzyme complex.

CYP2B6 contains multiple cysteine residues and a number of arginine and lysine residues with amine groups (Mo, et al. 2005). It is difficult to postulate how exactly PBD binds to the enzyme. Treatments of recombinant CYP2B6 with PBD followed by gel electrophoresis/trypsin digestion and subsequent LC-MS/MS analysis achieved more than 85% sequence coverage but failed to locate the binding sites, despite repeated attempts. One possible explanation for the failure is the reversible nature of aminal bonding between PBD and CYP2B6 which may require the intact PBD-protein complex to be stabilized, and such postulation is substantiated by our finding that no putative adducts were detected by LC-MS after PBD was incubated with free guanine or lysine. Alternatively, it is possible that PBD-peptide complexes derived from trypsin digestion are not chemically stable under experimental conditions.

PBD dimer was designed to be a DNA minor groove covalent binder (Hartley et al., 2004; Zhang et al., 2016) and no protein covalent binding is known so far. In fact, PBD dimer was nearly recovered completely (>95%) after 24-hour incubations with human and mouse plasma (internal data), indicating its low potential for protein covalent binding. Inactivation of CYP2B6 through protein covalent binding is a new finding although the chemical nature of the covalent binding is not known between PBD and the enzyme. The adduct formation of PBD dimer with GSH in buffer suggested addition of thiol to an imine. There are limited examples that demonstrated the chemical binding properties of reactive intermediates or drugs to endogenous proteins. Covalent binding of acetaminophen to proteins was through cysteine thiol group connecting to quinone imine intermediate (Leeming et al. 2015). MaxiPost formed an irreversible covalent bond with the ε-amino group of a lysine residue of albumins (Zhang et al.,

2004). Acrylamide-containing covalent inhibitors, such as neratinib, osimertinib and ibrutinib, are intrinsically reactive to endogenous proteins. For example, neratinib was found to form covalent adducts on Lys-190 of human serum albumin (Wang et al. 2010).

Screening for TDI is commonly achieved by comparing two IC_{50} values determined w/wo NADPH (Riley et al, 2007). As shown in this study, drug candidates with reactive moieties can directly inactivate CYPs in a NADPH independent manner resulting in two comparable IC_{50} values and false positives in the TDI assay. Therefore, a revised TDI assay is proposed specifically for compounds with reactive groups. As shown in Fig. 5, in addition to two pre-incubations w/wo NADPH, a third incubation without the drug is included; after preincubation, the drug is added to the third preincubation, resulting in three IC_{50} values respectively: $IC_{50\text{-}N}$ (without NADPH), IC_{50+N} (with NADPH) and $IC_{50\text{-}D}$ (without drug). For NADPH-dependent TDI, $IC_{50\text{-}N}$ and $IC_{50\text{-}D}$ will be comparable and both values should be higher than IC_{50+N} due to inactivation of CYP in preincubation; for NADPH-independent TDI, both $IC_{50\text{-}N}$ and IC_{50+N} are lower than $IC_{50\text{-}D}$ due to direct inactivation of CYP enzyme by the reactive drug. If the CYP enzyme is inactivated by both reaction intermediates and the reactive moiety of the drug, one will expect that IC_{50+N} will be lower than $IC_{50\text{-}N}$ and both values will be lower than $IC_{50\text{-}D}$. NADPH-independent and NADPH-dependent TDI is ruled out if three IC_{50} values are in agreement.

In conclusion, we have shown a unique CYP inhibition profile of PBD: a potent reversible CYP2C8 inhibitor, a NADPH dependent CYP3A4/5 TDI inhibitor and a NADPH independent CYP2B6 TDI inhibitor. To the best of our knowledge, the present study is the first example showing three types of CYP inhibition through different mechanisms: reversible drug-enzyme binding, enzyme inactivation via bioactivation, and enzyme inactivation by covalent

binding via chemical reactions. Because of the unique nature of covalent modulators, a revised TDI assay is advised to avoid false positives.

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Authorship Contributions.

Participated in research design: Mika Kosaka, Donglu Zhang, Simon Wong, Zhengyin Yan

Conducted experiments: Mika Kosaka

Performed data analysis: Mika Kosaka, Donglu Zhang, Simon Wong, Zhengyin Yan

Contributed to writing of the manuscript: Mika Kosaka, Donglu Zhang, Zhengyin Yan

Conflict of Interest.

Authors have no conflict of interest to declare.

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Legends for figures

Figure 1. Formation of the aminal bond between guanine and PBD monomer (top) and dimer (bottom), respectively.

Figure 2. Time dependent inhibition of CYP2C8, 3A and 2B6 by PBD. The concentrations of PBD were 0, 0.032, 0.16, 0.8, 4, 20, and 100 μM in HLM mixtures preincubated w/wo NADPH for 30-min, and dilution was performed before CYP specific substrates were added to determine relative enzyme activity as previously described under the *Materials and Methods*. A pair of sigmoidal dose–response curves were fitted using PRISM to determine two IC_{50} values respectively. The assay was performed in triplicates

Figure 3. Inactivation of CYP2B6 in HLM incubated with PBD in the absence of NADPH (○) *and presence of NADPH* (Δ). PBD (2 μM) was first incubated in triplicates with 0.3 mg/mL HLM w/wo NADPH. Aliquots of reaction mixtures were transferred at different time intervals, and immediately mixed with 9-volume of KPi buffer containing both NADPH and bupropion to determine CYP2B6 activity. CYP2B6 activity at different time points was expressed relative to that at T0 (100%). CYP2B6 activity loss caused by thermo-inactivation was 16% approximately after 60 min incubation at 37 ^{o}C.

Figure 4. Recovery of CYP2B6 activity in HLM incubated with PBD after washing. Three incubations were performed for 60 min at 37°C in triplicate: HLM alone (left bar), HLM + 10 μM PBD (right bar) and HLM spiked with PBD after incubation (middle bar). All samples were then centrifuged to pellet HLM followed by washing with 1 mL PBS to remove residual PDB. HLM pellets were re-suspended with 100 mM KPi to a final HLM concentration of 0.05 mg/mL to measure CYP2B6 activity as described under the *Materials and Methods.* CYP2B6 activity loss in HLM incubated with PBD (right bar) suggests that removal of unbound PBD by centrifugation and washing did not lead to complete recovery of hydroxylase activity of PBD-treated samples.

Figure 5. A revised TDI assay setup for reactive modulators. Three pre-incubations are performed: HLM + drug (a, left), HLM + drug + NADPH (b, middle), and HLM alone (c, right). After preincubation, the drug is spiked to incubation sample c. Subsequently, incubation mixtures are diluted and then assayed for CYP-specific activity to obtain three IC_{50} values respectively: $IC_{50\text{-}N}$ (a, without NADPH), IC_{50+N} (b, with NADPH) and $IC_{50\text{-}D}$ (c, without drug in preincubation).

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Table 1. Reversible inhibition of common CYPs in HLM by PBD.

CYP inhibition assay was performed in triplicate as described under *Materials and Methods*. Individual CYP activity was expressed as the peak area ratio of a CYP specific metabolite relative to its internal standard, and percent inhibition was calculated from CYP activity of samples containing PBD relative to the solvent control (no PBD). IC_{50} values of individual CYP enzymes were determined using Prism.

CYP	**Reversible CYP inhibition** IC_{50} (μM)
1A2	>10
2B6	>10
2C8	1.1 ± 0.3
2C9	>10
2C19	>10
2D6	>10
3A4/5	>10

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Table 2. TDI inhibition of common CYPs in HLM by PBD.

The concentrations of PBD were 0, 0.032, 0.16, 0.8, 4, 20, and 100 μM in HLM mixtures preincubated w/wo NADPH for 30-min, and a 10-folds dilution was performed before individual CYP specific substrates were added to determine relative enzyme activity as previously described under the *Materials and Methods*. The assay was performed in triplicates.

CYP	**TDI inhibition: IC_{50} (μM)**		
	- NADPH	+ NADPH	IC_{50} ratio
1A2	>10	>10	NA
2B6	1.5 ± 0.07	1.8 ± 011	0.8
2C8	0.90 ± 0.03	0.6 ± 0.02	1.5
2C9	>10	>10	NA
2C19	>10	>10	NA
2D6	>10	>10	NA
3A4/5	7.5 ± 0.18	0.20 ± 0.06	>35

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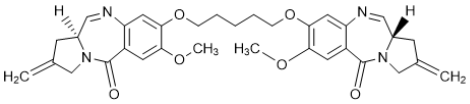
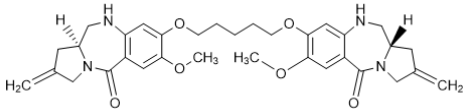
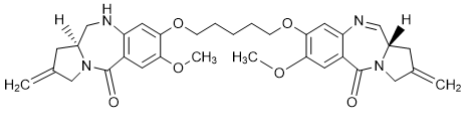
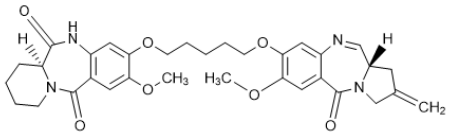
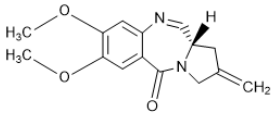
Table 3. TDI inhibition of common CYP2B6 in HLM by PBD analogs.

The concentrations of PBD and its analogs were 0, 0.032, 0.16, 0.8, 4, 20, and 100 µM in HLM mixtures preincubated w/wo NADPH for 30-min, and then dilution was performed before individual CYP specific substrates were added to determine relative enzyme activity as previously described under the *Materials and Methods*.

Recombinant CYP2B6				
Name	**Structure**	**Direct Inhibition IC_{50} (µM)**	**- NADPH IC_{50} (µM)**	**+ NADPH IC_{50} (µM)**
PBD		> 10	1.5	0.2
PBD - 1		> 10	> 10	> 10
PBD – 2		> 10	> 10	> 10
PBD - 3		> 10	> 10	> 10
PBD – 4		> 10	> 10	> 10

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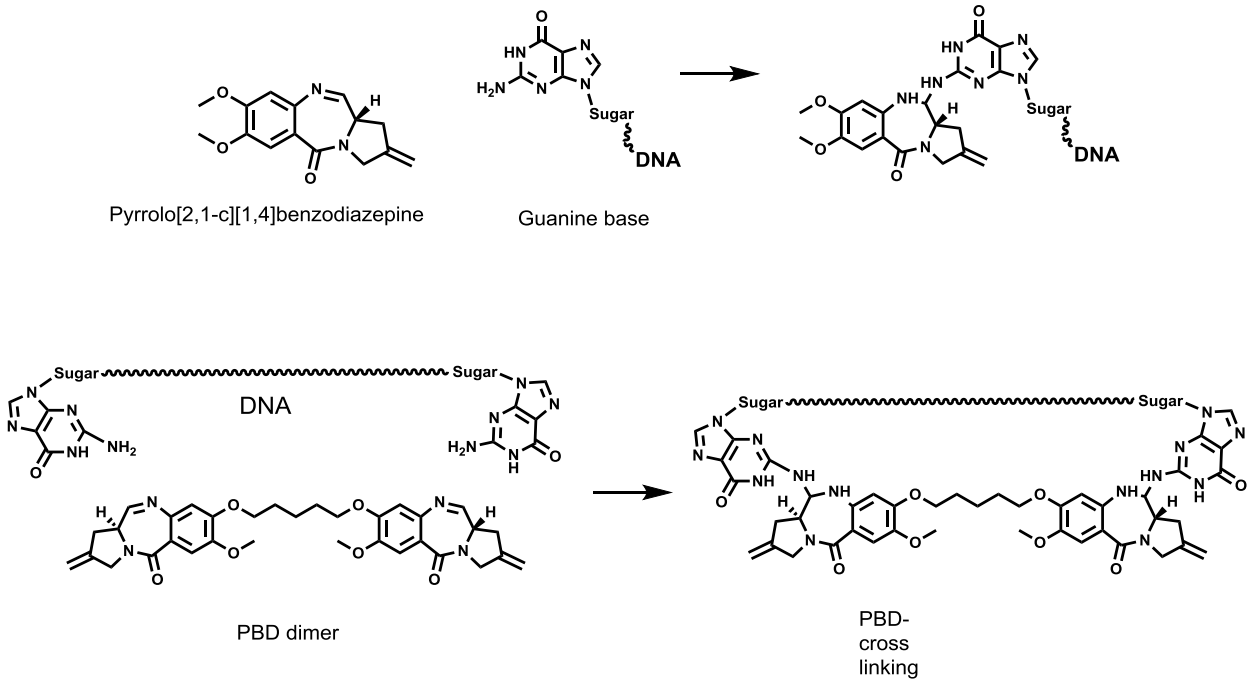

Figure 1.

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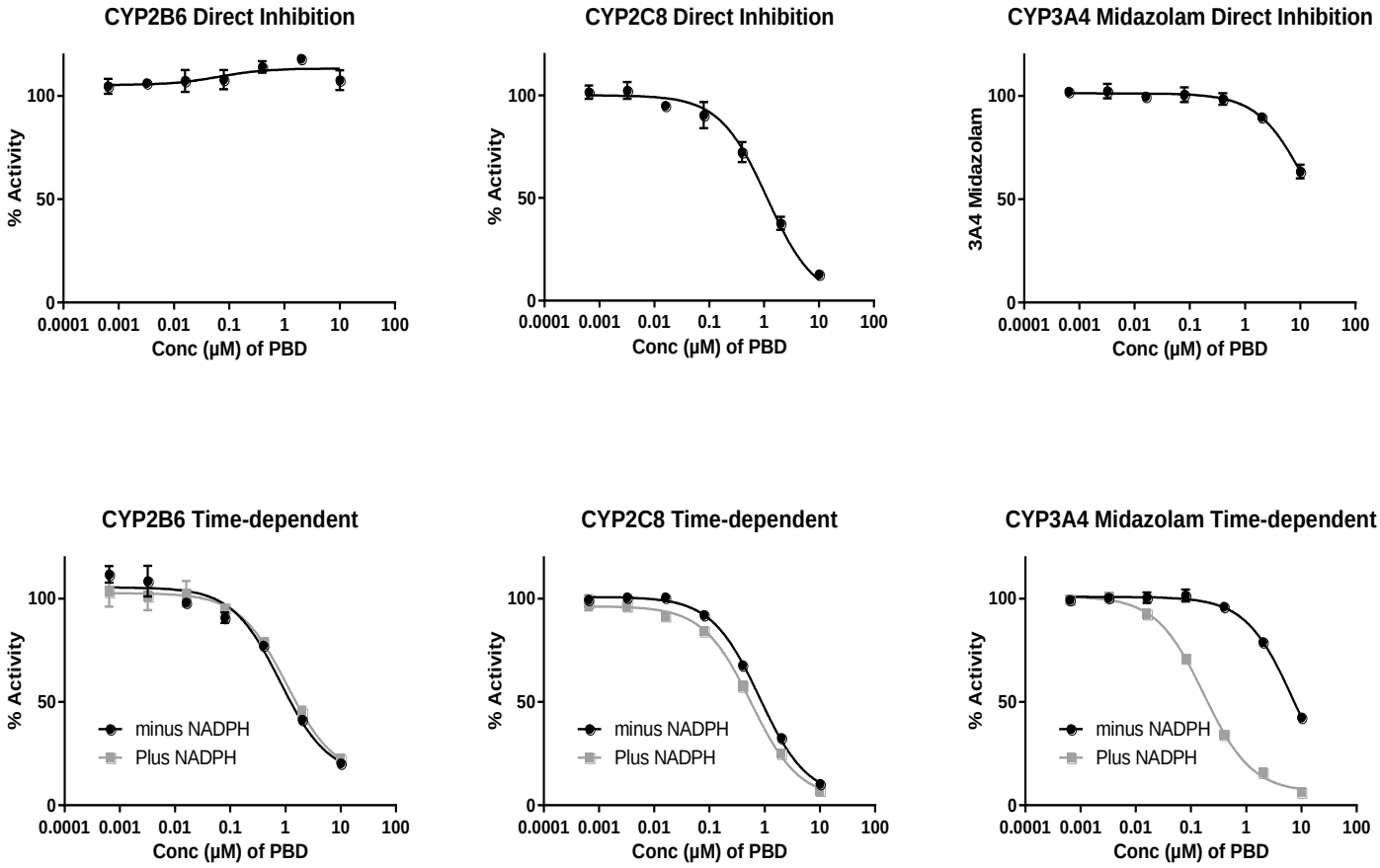
Figure 2.



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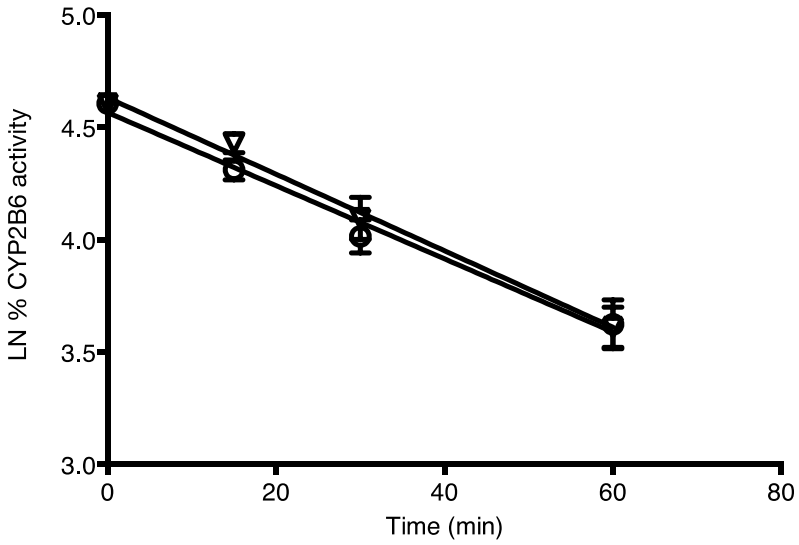

Figure 3.

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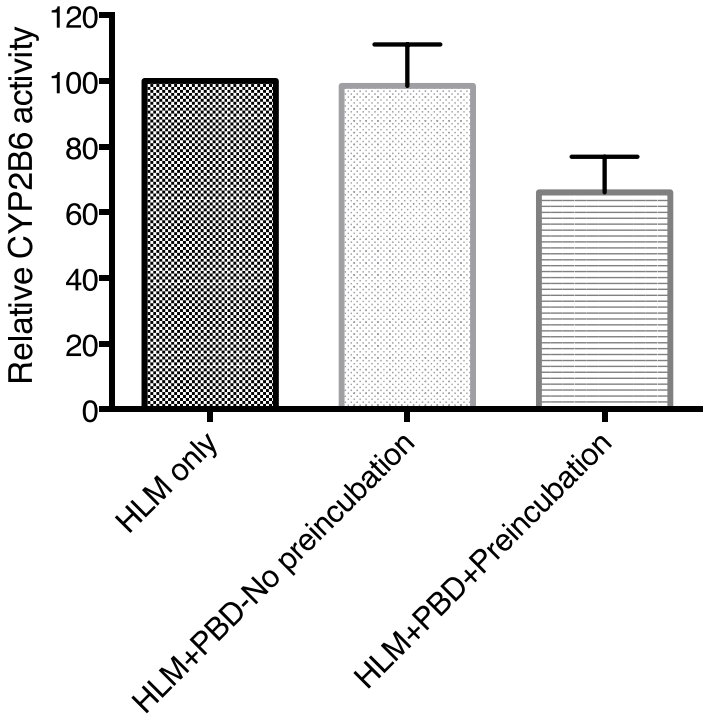

Figure 4.

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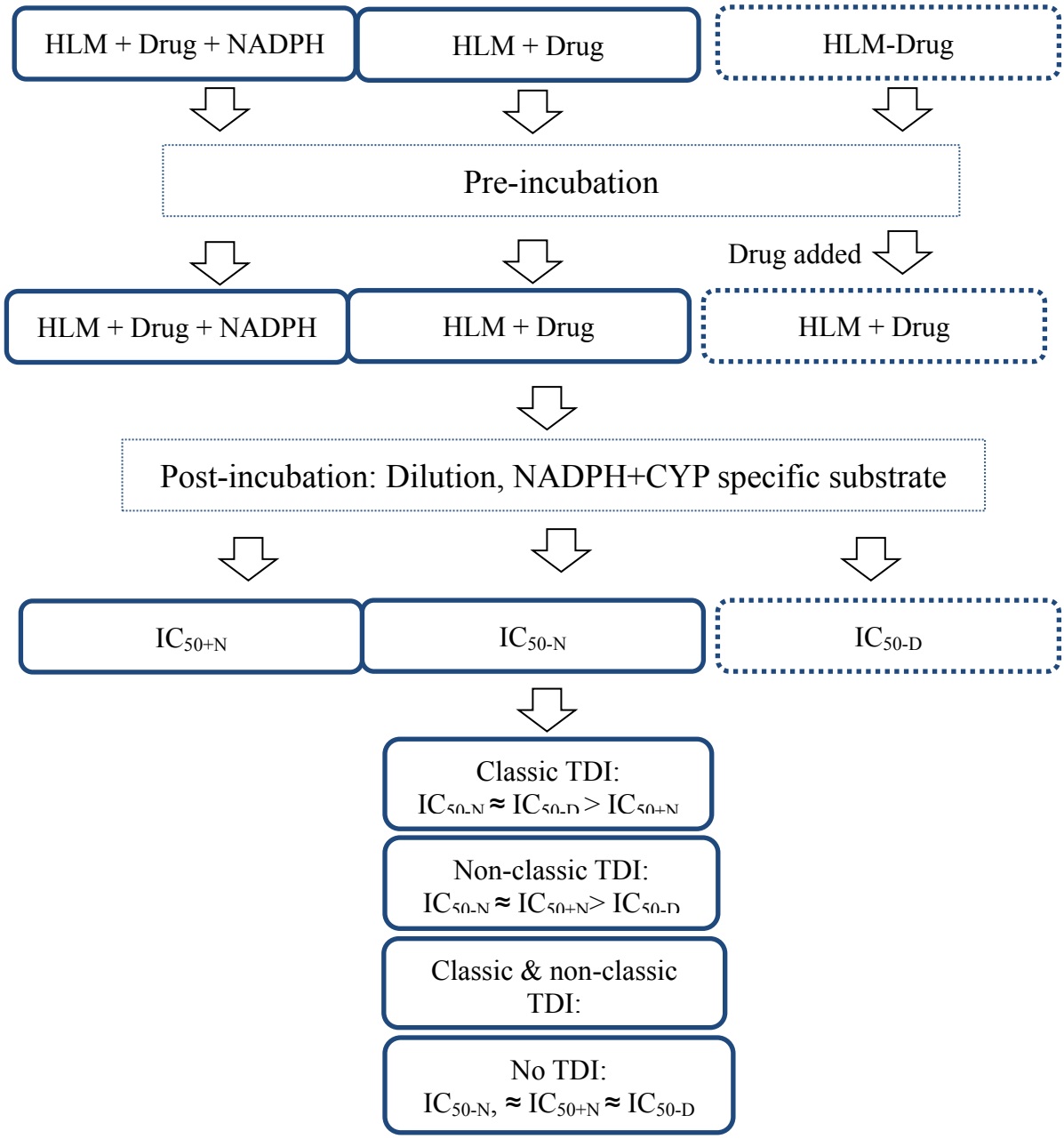
Figure 5.



Supplemental Material:

LC method: The mobile phases consist of water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The flow rate was 0.5 mL/min and the sample injection volume was 20 µL. The gradient was as follows: 1% mobile phase B for 0.5 min, 25% mobile phase B for 0.8 min, 60% mobile phase B for 2 min, 95% mobile phase B for 0.25 min and finally 1% mobile phase B for 1 min. The total LC run time was 4.55 min.

MRM transitions of CYP specific metabolites and internal standards

CYP	CYP Probe Substrate	Metabolite (MRM transition)	Internal Standard (MRM transition)
1A2	Phenacetin	Acetaminophen (152.0→110.1)	Acetaminophen-D_4 (156.0→114.1)
2B6	Bupropion	Hydroxybupropion (256.0→238)	Hydroxybupropion-D_6 (262.0→244.0)
2C9	*(S)*-(-)-Warfarin	7-Hydroxywarfarin (322.9→177.0)	Phenyl-D_5-7-hydroxywarfarin (328.0→177.0)
2C19	*(S)*-(+)-Mephenytoin	(±)-4'-Hydroxymephenytoin (232.9→190.0)	4'-Hydroxymephenytoin-D_3 (236.0→193.0)
2D6	Dextromethorphan	Dextrorphan (258.2→157.0)	Dextrorphan-D_3 (261.1→156.7)
3A4	Midazolam	1'-Hydroxymidazolam (305.0→269.0)	1'-Hydroxymidazolam-C_3 (308.0→272.0)
2C8	paclitaxel	6-α-hydroxy paclitaxel (870.0→286.0)	6-α-hydroxy paclitaxel-D_5 (875.0.→291.0.)