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Research and Development in Forensic Toxicology

Prediction of drug interactions with methadone, buprenorphine and oxycodone from in vitro inhibition of metabolism

Final Technical Report

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Abstract

We are testing the hypothesis that inhibition of in vitro drug metabolism can predict potential drug interactions with the opioids buprenorphine, methadone and oxycodone. We have made substantial progress in meeting our specific aims.

Aim 1A) We have optimized incubation conditions for buprenorphine, methadone and oxycodone in human liver microsomes (HLM) and with the relevant DNA-expressed cytochrome P450s (rCYPs) (i.e., CYP3A4 for all three substrates, 2C8 for buprenorphine, 2B6 for methadone, and 2D6 and 2C18 for oxycodone). This was finally achieved with a uniform 20-µM concentration for all three substrates. We have also established respective positive controls for time-dependent inhibition (TDI) under these incubation conditions.

Aim 1B) A dual-incubation system measures the kinetics of an enzyme destructive form of TDI referred to as mechanism-based inhibition (MBI). We have established the following positive controls for the system: troleandomycin (TAO) with CYP3A4 metabolism of buprenorphine, oxycodone and methadone, and gemfibrozil glucuronide for CYP2C8 metabolism of buprenorphine. Work with thioTEPA as a MBI of CYP2B6 metabolism of methadone and paroxetine as a MBI of CYP2D6 metabolism of oxycodone continues.

Aim 2) We have screened the inhibitory potential of three drug classes in HLM using our ± 15-minute pre-incubation of inhibitor with HLM and source of NADPH protocol that indicates both in vitro inhibition and TDI. This has been done for four H_2-receptor antagonists, and five proton pump inhibitors (PPIs) with methadone and oxycodone at 2.0 µM substrate concentration, and for twelve antifungal azole compounds at 20 µM substrate for all three opioids. Subsequently cimetidine inhibition of methadone and oxycodone has also been performed using 20 µM substrate concentrations.

Aim 3) For the relevant compounds (most), we have determined IC_{50} values for the inhibition of relevant CYP450s, and have performed in vivo extrapolations to estimate in vivo inhibitory potential. The results of these studies have been published in two articles in the *Journal of Analytical Toxicology*, the "official" journal for the Society of Forensic Toxicologists.

Aim 4) HLM screens conducted at 20 µM provided evidence for TDI of buprenorphine, methadone and oxycodone metabolism by cimetidine, and several PPIs. Dual-incubation kinetic experiments run for cimetidine with: buprenorphine (CYP3A4 and 2C8), methadone (CYP3A4, 2B6 is yet to be done), and oxycodone (CYP3A4 and 2D6) were indicative of MBI. MBI by cimetidine has only previously been shown for a CYP2D6 pathway. Rabeprazole produced the pre-incubation time and inhibitor concentration dependent loss in dual-incubations of buprenorphine with CYP3A4 that is associated with MBI; other PPIs did not with CYP3A4, but it was seen with esomeprazole incubated with HLM. We hypothesized that PPI metabolites generated at another CYP contributed to inhibition of CYP3A4 metabolism. IC_{50} determinations with commercially available PPI metabolites revealed some are potent inhibitors. MBI of CYP3A4 by rabeprazole and the contribution of PPI metabolites to inhibition of CYP3A4 are novel findings. Protocols to confirm the MBI mechanism are under development, and initial studies have demonstrated the NADPH and CYP3A4 dependence of cimetidine and rabeprazole TDI of buprenorphine metabolism.

Following further mechanistic studies we intend to submit manuscripts on TDI by cimetidine and PPIs. While much remains to do in our studies on in vitro inhibition of opioid metabolism, significant in-roads have been achieved.

Executive Summary

ES 1.0 Introduction

We have now initiated a series of studies on the in vitro inhibition of buprenorphine, methadone and oxycodone metabolism in human liver microsomes (HLM) and cDNA-expressed cytochrome P450s[1] (CYP450). While the in vitro inhibitory action of many of the inhibitors we have already, as well as those we still plan to study, has been addressed previously in the literature, few previous studies compared large numbers of potential inhibitors. Further the pre-incubation time-dependence of many of these inhibitors has not been previously studied. Further, few studies have addressed the inhibition of specific drug pathways, preferring model substrates amenable to high throughput assays. Because drugs (substrates) have different K_m values, the modeling of in vitro inhibition cannot always be done with the model substrates. This can be particularly true for CYP3A4, and some other CYP, substrates, as the large substrate-binding domain of this enzyme allows various alignments, and thereby various potencies for inhibitor substrate interactions.

ES 2.0 Major Goals / Aims

The goals of this project are to measure the in vitro inhibition of metabolism of three opioids: methadone, buprenorphine and oxycodone. This includes studies to optimize incubation conditions, use a list of almost 100 potential inhibitors from several drug classes to provide direction of studies but to also take time to investigate findings indicative of time-dependent inhibition (TDI).

The aims of the grant are as follow:

1. Optimization of Assay Conditions

A. HLM Screen

- Incubation systems for HLM screens will be optimized such that substrate concentration is below K_m, but sufficient to prevent > 10% substrate loss over incubation period.
- Solvent controls (methanol, DMSO) will be included if 1% organic solvent is used in order to reach desired concentration of inhibitor.
- Positive controls will be included to demonstrate appropriate pre-incubation conditions.

B. TDI Kinetics

- Each CYP450 pathway will be tested with a TDI positive control to assure substrate concentration is sufficient to minimize reversible inhibition from inhibitor in primary incubation system (CYP2C18 excluded as no positive control has been described).

[1] The abbreviation CYP450 describes the cytochrome P450 enzyme family as a whole; for specific gene products CYP will be followed by the gene product name (e.g., CYP3A4). cDNA-expressed CYP450s will be referred to as rCYP.

2. HLM Screen

- The potential inhibitors list in Table 1 will continue as the directional menu for screening and follow-up studies of potential inhibitors.
- Screening procedures will test 3 concentrations of inhibitor ± 15 minute pre-incubation using optimized substrate concentrations, along with solvent (as needed) and positive controls (Aim 1A).

3. IC_{50} Determinations

- When sufficient inhibition is found in the HLM screen, IC_{50} experiments will be conducted using the CYP450 specific pathways. This will include compounds showing TDI in the HLM screen, as reversible inhibition may be potent, and the IC_{50} data will be needed to assess the results in the dual incubation systems.

4. TDI Kinetic Determinations and Follow-up Experiments

A. TDI Kinetic Determinations

- The TDI kinetic determinations will be conducted utilizing a dual incubation system. The specific rCYP will be incubated with 5-6 concentrations (4 at any one incubation) of inhibitor and the NADPH generating system. Aliquots are removed at approximately 5-minute intervals, and placed into a secondary incubation system to measure CYP activity for the specific opioid. Three-four repeat determinations will be needed to meet publishable criteria.

B. Follow-up Experiments

- Tests will be conducted using CYP specific pathways and dual incubation at a selected pre-incubation time and inhibitor concentration to determine the mechanistic dependence of TDI. This will include incubations ± NADPH, incubations with CYP450 specific inhibitor and excess model substrate, and incubations with catalase and superoxide dismutase to rule out impact of oxygen free radical mechanisms. Additional tests for irreversible nature of inhibition, or further studies for other mechanisms (e.g. inhibitory metabolites) will be performed as needed.

ES 3.0 Accomplishments Under Goals

ES 3.1 Background

The main premise of this research is that inhibition of the metabolism of opioids may contribute to their toxic effects. The laboratory has worked on the metabolism, pharmacokinetics and potential drug interactions of methadone and buprenorphine for several years. An early accomplishment funded in part by this grant was a review on the relationship between the metabolism and toxicity of methadone and buprenorphine.[2] The laboratory only more recently started studies on oxycodone. Another early accomplishment, also partially funded by this grant, was publication of a validated method for quantitation of oxycodone and metabolites

[2] Moody, D.E. Metabolic and toxicologic considerations of opioid replacement therapy and analgesic drugs: methadone and buprenorphine. *Exp. Opin. Drug Metab. Toxicol.* **9**: 675-697, 2013.

in biological fluids and HLM. Studies with HLM and rCYP confirmed the involvement of CYP3A4 and 2D6 in the metabolism, as well as demonstrated CYP2C18 involvement.[3]

This research focuses on in vitro inhibition of 5 metabolic pathways. Four of the pathways are mediated in part by CYP3A4, including N-dealkylation of buprenorphine to norbuprenorphine, the N-dealkylation-cyclization of R- and S-methadone to R- and S-EDDP, and the N-dealkylation of oxycodone to noroxycodone. Each has a respective co-CYP that also mediates the metabolism: these are CYP2C8, 2B6 and 2C18, respectively. One pathway, the O-demethylation of oxycodone to oxymorphone is mediated solely by CYP2D6. For drugs that show potential inhibition in the HLM screen, their effect on the pertinent 5 different CYP-mediated pathways will be determined.

Our screening incorporates a comparison of HLM that undergo no pre-incubation with those that are pre-incubated for 15 minutes with inhibitor. This is designed to differentiate inhibitors that display TDI, which require metabolism to produce the active inhibitor (also referred to as metabolism-dependent inhibition). With CYP450, TDIs are generally split into 3 different categories. 1) Mechanism-based inhibitors (MBI) require metabolism to a reactive metabolite that covalently binds to a portion of the enzyme (or the heme cofactor). 2) Metabolic intermediate complexes (MIC) form from a non-covalently tight binding with the CYP450 heme. MICs are considered quasi-irreversible, since under some in vitro conditions (e.g., incubation with ferricyanide) they can be disassociated from the heme with cessation of inhibition. 3) Metabolites can be formed that are potent reversible inhibitors.

The MBI and MIC inhibitors have potential for creating more insidious drug interactions. Their action requires synthesis of new protein to overcome the inhibition. Reported CYP450 turnover half-lives range from 25-100 hours. This could prove challenging for a forensic investigation as drug use could be halted and no longer detectable while the enzyme is still inhibited.

Our screen with HLM does not differentiate any of the TDIs mechanisms, as inhibitor is not separated from the target and reversible inhibition cannot be differentiated from irreversible inhibition. It does, however, determine if TDI has occurred. With suspected TDIs we can then study the effect of pre-incubation in a primary system that only contains the relevant CYP450(s), inhibitor and source of NADPH. At different time points, aliquots from this are then placed into a secondary incubation system containing substrate and additional source of NADPH, where the inhibitor is diluted 10-20 fold to minimize any reversible inhibitor effect. This provides a better estimate of MBI, but does not rule out MICs. Additional experiments are to establish MBI of CYP450.

[3] Fang, W.B., Lofwall, M.R., Walsh, S.L. and Moody, D.E. Determination of oxycodone, noroxycodone and oxymorphone by high performance liquid chromatography- electrospray ionization -tandem mass spectrometry in human matrices: In vivo and in vitro applications. *J. Anal. Toxicol.* **37**: 337-344, 2013.

ES 3.2 Accomplishments under Aim 1.A - Optimization of Incubation Conditions: HLM

Work under this aim can be summarized as follows:

- Incubations with oxycodone and methadone were first optimized at 2.0 µM substrate with 0.5 mg/mL HLM.
- Buprenorphine was metabolized too rapidly under these conditions. We had to increase substrate to 20 µM, and reduce HLM protein to 0.05 mg/mL.
- Oxycodone and methadone were then also optimized at 20 µM substrate with 0.3 and 0.2 mg/mL HLM protein, respectively.
- Under these conditions we established TDI positive controls ± pre-incubation screens, tested impact of solvents and pre-incubations on negative controls and determined the need for background samples to account for minor metabolite impurities that became significant with substrate at 20 µM.

ES 3.3 Accomplishments under Aims 2 and 3: HLM Screens, IC_{50} Determinations and Extrapolations to In Vivo Potency

Work under these aims can be summarized as follows:

- Four H_2-receptor antagonists and 5 proton pump inhibitors (PPIs) were ± pre-incubation screened in HLM for inhibitory action on methadone and oxycodone metabolism at 2.0 µM substrate concentration. No overt evidence of TDI was noticed.
- IC_{50} values were determined and in vitro to in vitro extrapolations of potency were determined. These findings were the basis for another publication.[4]
- Twelve azole antifungals and related compounds were ± pre-incubation screened in HLM for inhibitory action on buprenorphine, methadone and oxycodone metabolism at 20 µM substrate concentration. Many had significant inhibitory action towards multiple pathways. No overt evidence of TDI was noticed.
- While it was evident that CYP3A4 pathway would require IC_{50} determinations with most of these compounds, a further screen was conducted with other rCYPs to determine candidate pathways and compounds for IC_{50} determinations.
- IC_{50} values were determined and in vitro to in vitro extrapolations of potency were determined. These findings were the basis for another publication.[5]

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[4] Moody, D.E., Liu, F., and Fang, W.B. In vitro inhibition of methadone and oxycodone cytochrome P450-dependent metabolism: Reversible inhibition by H_2-receptor agonists and proton pump inhibitors. *J. Anal. Toxicol.* **37**: 476-485, 2013.

[5] Moody, D.E., Liu, F., and Fang, W.B. In vitro inhibition of methadone and oxycodone cytochrome P450-dependent metabolism: Reversible inhibition by H_2-receptor agonists and proton pump inhibitors. *J. Anal. Toxicol.* **37**: 476-485, 2013.

ES 3.4 Accomplishments under Aims 1B and 4 - Studies on TDI Kinetic Determinations and Follow Up experiments

Work under these aims can be summarized as follows:

- Positive controls for TDI/MBI kinetics have been established for all of the CYP3A4 pathways (buprenorphine N-dealkylation, methadone N-demethylation and oxycodone N-demethylation) with TAO as the positive control and for CYP2C8 buprenorphine N-dealkylation with gemfibrozil glucuronide as the positive control.
- Similar studies with the CYP2B6 positive control thioTEPA have been initiated; similar experiments will be pursue with the 2D6 positive control paroxetine.
- A screen of inhibition of 20 µM buprenorphine, methadone and oxycodone metabolism in HLM by the H_2-receprtor antagonist cimetidine indicated TDI of this activity. Use of the higher substrate concentration probably uncovered the TDI for methadone and oxycodone metabolism.
- Dual-incubation experiments were performed to obtain MBI kinetics for cimetidine with buprenorphine and CYPs 3A4 and 2C8, oxycodone and CYPs 3A4 and 2D6, and methadone and CYP3A4. Experiments with methadone and CYP2B6 are pending.
- We have initiated the establishment of control experiments to confirm the MBI nature of inhibition and the first set of protocols have been applied to cimetidine and rabeprazole inhibition of buprenorphine metabolism.
- A screen of inhibition of 20 µM buprenorphine metabolism in HLM by the PPIs revealed that all 5 displayed TDI.
- Dual incubation experiments revealed MBI kinetics for rabeprazole and CYP3A4, but pre-incubation time and inhibitor concentration dependent loss of activity was not found when CYP3A4 was incubated with the other PPIs.
- As shown with esomeprazole, we could however, obtain MBI kinetics for buprenorphine metabolism if the PPI was incubated with HLM.
- This led to an alternative hypothesis that PPI metabolites generated at another enzyme may be causing reversible or irreversible inhibition of buprenorphine metabolism at CYP3A4. We have now shown that several commercially available PPI metabolites have fairly potent IC_{50} values for inhibition of CYP3A4 metabolism of buprenorphine.
- Studies continue to confirm this alternative hypothesis and to confirm the MBI of CYP3A4 by rabeprazole.

ES 4.0 Conclusions

ES 4.1 Summary of Accomplishments

We have made considerable progress on achieving our specific aims. We have completed optimization of the incubation conditions in HLM (Aim 1A), and have established positive TDI/MBI controls for about 2/3 of the metabolic pathways we are studying (Aim 1B). The HLM screen has now been performed for three classes of inhibitors, the H_2-receptor antagonists, the

PPIs and the azole antifungal agents (Aim 2). IC_{50} values have been determined for the respective pathways and those of significance have been extrapolated to estimate in vivo potency (Aim 3). We have found evidence for TDI inhibition with cimetidine and a number of PPIs, and are well underway in determining mechanisms of TDI, which include MBI for cimetidine and rabeprazole, and contribution of inhibitor metabolites for some of the PPIs. Five manuscripts related to these studies have already been published and our results have been presented in five platform and 4 poster presentations to forensic toxicologists and related disciplines (see section 7.0 for details).

ES 4.2 Implications for Policy and Practice

ES 4.2.1 What is the impact of the project on the criminal justice system?

The impact of drug interactions spreads beyond the field of forensic toxicology to encompass general medicine, pharmacy and other areas involved in drug use and dispensing. These can all directly and indirectly impact the criminal justice system, as many in these professions are called upon as consultants and expert witnesses for cases going through the criminal justice system. We anticipate that information we provide on potential drug interactions with methadone, buprenorphine and oxycodone is of benefit in these fields.

ES 4.2.2 How has it contributed to crime laboratories?

At this early stage of the project our impact has been modest. We would hope that our presentations to the forensic toxicology community, along with publications, have provided some thought among the audience of the potential impact drug interactions may have on the toxicity of other drugs. As crime laboratory personal often testify in regard to plasma concentrations of drugs, these findings will hopefully enhance the interpretation of these testimonies.

ES 4.3 Implications for Further Research

We will focus on further studies on TDI by cimetidine and some of the PPIs. This will include follow-up experiments listed in our revised aims. Experiments will be included with positive controls for TDI. The initial experiments for all three of these areas have been discussed above. With further 3-year funding now awarded from NIJ, we will continue these experiments. We will next screen another set of inhibitor class listed in Table 1. We will continue in this manner, either simply determining the IC_{50} values and extrapolating to in vivo potency if there is no evidence of TDI, or testing for mechanisms if evidence of TDI is found. We have now set in place most of the protocols to perform these studies, and look forward to continuing our progress in determining the potential for inhibition of the metabolism of the opioids, buprenorphine, methadone and oxycodone.

Technical Report

1.0 Introduction

Over the past decade and a half there has been a notable increase in mortalities arising from opioid use. This is related to the increased use of opioids in pain management and the increased abuse of these prescribed medications [1; 2; 3; 4]. The forensic toxicology community is tasked with assisting in the interpretation of the cause of these deaths, be they intentional or accidental, self-inflicted, a result of a medical misadventure or some other reason. A confounding factor is whether use of co-medications may have contributed to the resultant death [5; 6; 7; 8]. Such drug interactions may have an impact on causation. Drug interactions may change the pharmacokinetics of the drug. Three commonly used (and abused) opioids are buprenorphine, methadone and oxycodone [1; 2; 9]. A major site of drug interaction is the enzyme involved in the metabolism of the drug [10; 11]. While buprenorphine, methadone and oxycodone all share metabolism by cytochrome P450[6] (CYP) 3A4, they differ in the other enzymes involved in the metabolism and in the pharmacodynamic activity of their metabolites.

Buprenorphine is N-dealkylated to norbuprenorphine by CYP3A4 [12; 13] and CYP2C8 [14] Other pathways of ring and side chain hydroxylation have been identified; they are also catalyzed by CYP3A4 and 2C8 [15; 16], but appear to be of minor clinical relevance [17]. While norbuprenorphine has in vitro activity at the mu-opioid receptor, its central activity is limited due to efflux at the blood brain barrier by P-glycoprotein [3; 18; 19].

Methadone is a racemic drug. The R-enantiomer is the more potent mu opioid receptor agonist [20], while both R- and S-enantiomers are NMDA receptor antagonists [21]. S-Methadone is the more potent blocker (≈ 2.5-3.5x) of the human ether-a-go-go-related gene (hERG) K^+ channels that are associated with methadone-induced prolonged QT interval [22]. R- and S-methadone are N-demethylated, with an ensuing spontaneous cyclization, to R- and S-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), respectively. EDDP is further N-demethylated to 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP). We [23; 24], and others [25; 26; 27], have shown that CYP3A4 and 2B6 are the main enzymes involved in the in vitro N-demethylation of methadone. CYP2B6 has a higher affinity for S-methadone, while 3A4 is non-specific. CYP2C19 (R-methadone preferred) and 2D6 (non-specific) also carry out the reaction, but appear to have minor roles. EDDP and EMDP are essentially void of opioid activity [20].

Oxycodone is N-demethylated to noroxycodone and O-demethylated to oxymorphone; combined reactions form noroxymorphone. Lalovic et al. [28] have shown that the N-demethylation is carried out by CYP3A4 and the O-demethylation by CYP2D6. We recently found that CYP2C18 can also perform the N-demethylation [29]. Oxymorphone and noroxymorphone with the open 3-hydroxyl group are glucuronidated, while oxycodone and noroxycodone are either

[6] The abbreviation CYP450 describes the cytochrome P450 enzyme family as a whole; for specific gene products CYP will be followed by the gene product name (e.g., CYP3A4); rCYP refers to cDNA-expressed gene products.

not, or only slightly glucuronidated. Oxymorphone and noroxymorphone share mu opioid receptor activity with oxycodone, while noroxycodone does not. The impact of noroxymorphone is limited to the periphery as it poorly penetrates the blood brain barrier [28]. Oxymorphone is highly glucuronidated, and the mu opioid receptor activity of oxymorphone-glucuronide has not been determined, but this might limit its contribution to the overall mu opioid receptor activity.

Studies on drug interactions with buprenorphine have focused primarily on antiretrovirals. Some instances of inhibition and induction of metabolism have been described, but few have had adverse effects under the conditions of the studies [6; 30].

Drug interactions with methadone have been studied since Kreek's initial report in 1976 that rifampin induces methadone metabolism [31]. Subsequent studies were sporadic until the mid 90s, at which time a number of studies with selective serotonin receptor inhibitors (SSRIs) were published, and studies with antiretrovirals were just starting to appear [32]. Since that time a focus has been on the antiretrovirals with only a few other drug classes studied [6]. Most of these adverse effects, particularly opioid withdrawal, have arisen from CYP-related induction of methadone metabolism that is associated with withdrawal. Inhibition of metabolism was also seen, but under the controlled clinical conditions of the studies, was not associated with adverse effects. This, however, shows that many drugs can inhibit methadone metabolism and cause higher circulating concentrations that under conditions of undeveloped tolerance or ingestion of higher than intended doses could have severe adverse effects.

Drug interaction studies with oxycodone are limited, but have been published more often in recent years. Overholser et al., have reviewed many through 2010 [33]; a few examples are provided here. Many involve the testing of prototypical inhibitors of CYP2D6 and 3A4 [34] to confirm the involvement of these enzymes in the two main pathways of metabolism. A few other classic drug interactants are: rifampin [35], St. John's wort [36], and grapefruit juice [37]; and more recently the antiretrovirals ritonavir and lopinavir/ritonavir [38]. Inducers of metabolism decrease the effectiveness of oxycodone, inhibitors that increase systemic exposure to oxycodone can increase the effect; this is even more so if systemic exposure to oxymorphone is also increased. These studies show there is a danger from drug interactions increasing exposure to oxycodone, and thereby increasing risk of overdose.

We have now initiated a series of studies on the in vitro inhibition of buprenorphine, methadone and oxycodone metabolism in human liver microsomes (HLM) and cDNA-expressed (recombinant) CYP450s (rCYP). While the in vitro inhibitory action of many of the inhibitors we have already studied and still plan to study, has been addressed previously in the literature, few previous studies compared large numbers of potential inhibitors. Further, few studies have addressed the inhibition of specific drug pathways, preferring model substrates amenable to high throughput assays. Because drugs (substrates) have different K_m values, the modeling of in vitro inhibition cannot always be done with the model substrates. This can be particularly true for CYP3A4, and some other CYP, substrates, as the large substrate-binding domain of this enzyme allows various alignments, and thereby various potencies for inhibitor substrate interactions [39]. Also, many earlier studies did not address the impact or pre-incubation of the inhibitors.

2.0 Major Goals / Aims

The goals of this project are to measure the in vitro inhibition of metabolism of three opioids: methadone, buprenorphine and oxycodone. This includes studies to optimize incubation conditions, use a list of almost 100 potential inhibitors from several drug classes (Table 1) to provide direction of studies but to also take time to investigate findings indicative of time-dependent inhibition (TDI).

The aims of the grant are as follow:

1. Optimization of Assay Conditions

A. HLM Screen

- Incubation systems for HLM screens will be optimized such that substrate concentration is below K_m, but sufficient to prevent > 10% substrate loss over incubation period.
- Solvent controls (methanol, DMSO) will be included if 1% organic solvent is used in order to reach desired concentration of inhibitor.
- Positive controls will be included to demonstrate appropriate pre-incubation conditions.

B. TDI Kinetics

- Each CYP450 pathway will be tested with a TDI positive control to assure substrate concentration is sufficient to minimize reversible inhibition from inhibitor in primary incubation system (CYP2C18 excluded as no positive control has been described).

2. HLM Screen

- The potential inhibitors list in Table 1 will be the directional menu for screening and follow-up studies of potential inhibitors.
- Screening procedures will test 3 concentrations of inhibitor ± 15 minute pre-incubation using optimized substrate concentrations, along with solvent (as needed) and positive controls (Aim 1A).

3. IC_{50} Determinations

- When sufficient inhibition is found in the HLM screen, IC_{50} experiments will be conducted using the CYP450 specific pathways. This will include compounds showing TDI in the HLM screen, as reversible inhibition may be potent, and the IC_{50} data will be needed to assess the results in the dual incubation systems.

4. TDI Kinetic Determinations and Follow-up Experiments

A. TDI Kinetic Determinations

- The TDI kinetic determinations will be conducted utilizing a dual incubation system. The specific rCYP will be incubated with 5-6 concentrations (4 at any one incubation) of inhibitor and the NADPH generating system. Aliquots are removed at approximately 5-minute intervals, and placed into a secondary incubation system to measure CYP activity for the specific opioid. Three-four repeat determinations will be needed to meet publishable criteria.

B. Follow-up Experiments

- Tests will be conducted using CYP specific pathways and dual incubation at a selected pre-incubation time and inhibitor concentration to determine the mechanistic dependence of TDI. This will include incubations ± NADPH, incubations with CYP450 specific inhibitor and excess model substrate, and incubations with catalase and superoxide dismutase to rule out impact of oxygen free radical mechanisms.
- Inhibitor will be incubated with HLM and NADPH and then scanned in dual-beam spectrophotometer to determine if evidence exists for metabolic intermediate complex (MIC) formation.
- If evidence of MIC formation is noted, incubations with potassium ferricyanide would be included.
- Further studies of a different nature may be required based on preliminary findings. For example, our experience with the proton pump inhibitors (PPIs) and CYP3A4 metabolism of buprenorphine suggest inhibition has arisen from generation of a (ir)reversible inhibitor at another enzymatic site. Suspected metabolite inhibitors, if commercially available, will then be tested for inhibition.

3.0 Accomplishments Under Goals

3.1 Background

The main premise of this research is that inhibition of the metabolism of opioids may contribute to their toxic effects. The laboratory has worked on the metabolism, pharmacokinetics and potential drug interactions of methadone and buprenorphine for several years. An early accomplishment funded in part by this grant was a review on the relationship between the metabolism and toxicity of methadone and buprenorphine [3]. The laboratory only more recently started studies on oxycodone. Another early accomplishment, also partially funded by this grant, was publication of a validated method for quantitation of oxycodone and metabolites in biological fluids and HLM. Studies with HLM and rCYP confirmed the involvement of CYP3A4 and 2D6 in the metabolism, as well as demonstrated CYP2C18 involvement [29].

This research focuses on in vitro inhibition of 5 metabolic pathways (Figure 1). Four of the pathways are mediated in part by CYP3A4, including N-dealkylation of buprenorphine to norbuprenorphine, the N-dealkylation-cyclization of R- and S-methadone to R- and S-EDDP, and the N-dealkylation of oxycodone to noroxycodone. Each has a respective co-CYP450 that also mediates the metabolism: these are CYP2C8, 2B6 and 2C18, respectively. One pathway, the O-demethylation of oxycodone to oxymorphone is mediated solely by CYP2D6. For drugs that show potential inhibition in the HLM screen, their effect on the 5 different CYP-mediated pathways will be determined.

Table 1. Drugs to test for inhibition potential

H_2-Receptor Antagonists	ß-Blockers	Atypical Antipsychotics
Cimetidine	Acebutolol	Aripiprazole
Famotidine	Alprenolol	Asenapine
Nizatidine	Atenolol	Clozapine
Ranitidine	Betaxolol	Iloperidol
Proton Pump Inhibitors	Bisoprolol	Lurasidone
Deslansoprazole	Carteolol	Olanzapine
Esomeprazole	Carvedilol	Paliperidone
Lansoprazole	Celiprolol	Quetiapine
Omeprazole	Esmolol	Risperidone
Pantoprozole	Labetalol	Ziprasidone
Rabeprazole	Metoprolol	Other Antipsychotics
Imidazole Antifungal Agents	Nadolol	Chlorpromazine
Albendazole	Nebivolol	Fluphenazine
Clotrimazole	Penbutolol	Haloperidol
Econazole	Pindolol	Perphenazine
Fluconazole	Propranolol	Thioridazine
Itraconazole	Sotalol	Benzodiazepines
Ketoconazole	Timolol	Alprazolam
Metronidazole	Tricyclic Antidepressants	Norchlordiazepoxide
Miconazole	Amitryptyline	Clonazepam
Terconazole	Clomipramine	Demoxepam
Voriconazole	Desipramine	Diazepam
Macrolid Antibiotics	Doxepin	Estazolam
Azithromycin	Imipramine	Flunitazepam
Clarithromycin	Marprotiline	Flurazepam
Erythromycin	Nortryptyline	Lorazepam
Fidaxomicin	Protriptyline	Midazolam
Telithromycin	SSRIs	Nitrazepam
Ca-Channel Blockers: Dihydros	Citalopram	Nordiazepam
Amlodipine	Escitalopram	Oxazepam
Clevidipine	Fluoxetine	Temazepam
Felodipine	Fluvoxamine	Triazolam
Isradipine	Paroxetine	
Nicardipine	Sertraline	
Nifedipine	SNRIs	
Nilvadipine	Desvenlafaxine	
Nimodipine	Duloxetine	
Nisoldipine	Mirtazapine	
Ca-Channel Blockers: Others	Nefazodone	
Diltiazem	Venlafaxine	
Verapamil	Vilazodone	

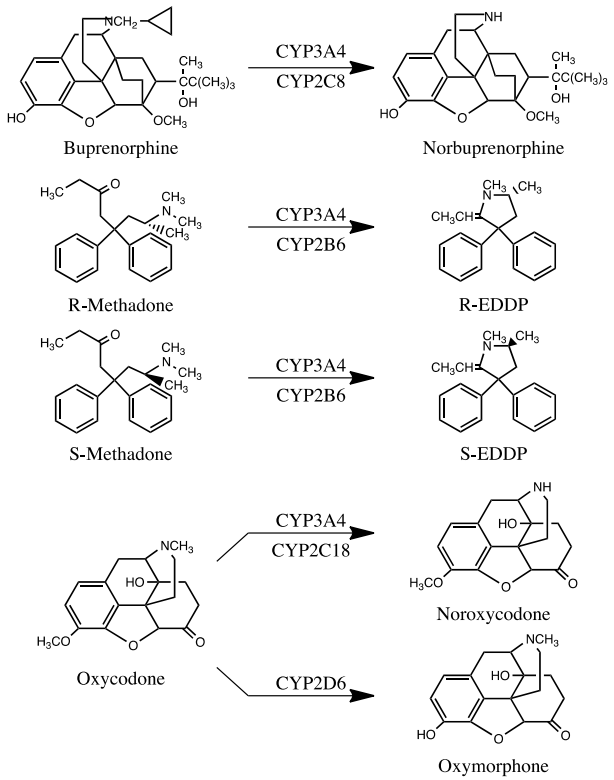

Figure 1. Metabolic routes under study with primary cytochrome P450 (CYP450) involved in metabolism indicated.

Our screening incorporates a comparison of HLM that undergo no pre-incubation with those that are pre-incubated for 15 minutes with inhibitor and a source of NADPH. This is designed to identify inhibitors that display TDI, which require metabolism to produce the active inhibitor (also referred to as metabolism-dependent inhibition). With CYP450, TDIs are generally split into 3 different categories. 1) Mechanism-based inhibitors (MBI) require metabolism to a reactive metabolite that covalently binds to a portion of the enzyme (or the heme cofactor). 2) MICs form from a non-covalently tight binding with the CYP450 heme. MICs are considered quasi-irreversible, since under some in vitro conditions (e.g., incubation with ferricyanide) they can be disassociated from the heme with cessation of inhibition. 3) Metabolites can be formed that are potent reversible inhibitors [40; 41].

The MBI and MIC inhibitors have potential for creating more insidious drug interactions. Their action requires synthesis of new protein to overcome the inhibition. Reported CYP450 turnover half-lives range from 25-100 hours [42]. This could prove challenging for a forensic investigation as drug use could be halted and no longer detectable while the enzyme is still inhibited. A hypothetical scheme of how blood concentrations of a drug may change following reversible and irreversible inhibition is presented in Figure 2.

Our screen with HLM does not differentiate any of the TDIs mechanisms, as inhibitor is not separated from the target and reversible inhibition cannot be differentiated from

irreversible inhibition. It does, however, determine if TDI has occurred. With suspected TDIs we can then study the effect of pre-incubation in a primary system that only contains the relevant CYP450(s), inhibitor and source of NADPH. At different time points, aliquots from this are then placed into a secondary incubation system containing substrate and additional source of NADPH, where the inhibitor is diluted 10-20 fold to minimize any reversible inhibitor effect. This provides a better estimate of MBI, but does not rule out MICs. Additional experiments would be performed to establish MBI of CYP450 [40; 41].

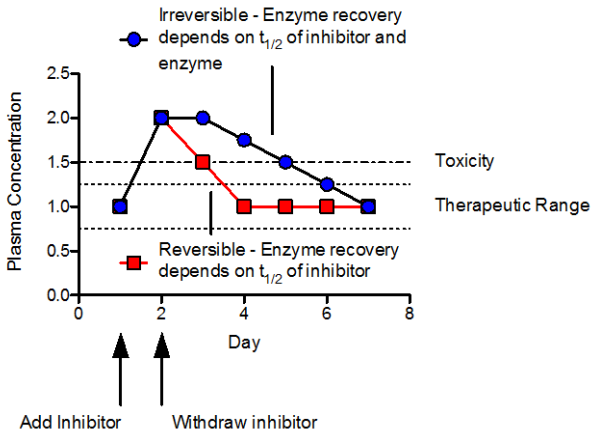

Figure 2. Hypothetical effect of reversible versus irreversible inhibitors on the plasma concentration of a target drug during a pharmacokineitic drug interaction. Note the main difference is that irreversible inhibition can last longer since it requires rejuvenation of the enzyme. Potentially, the irreversible inhibitor may already have diminished in the blood while its inhibitory effect continues at the enzyme.

3.2 Accomplishments under Aim 1.A - Optimization of Incubation Conditions: HLM

3.2.1 Initial Studies with Oxycodone and Methadone

Studies on in vitro inhibition of CYP450 metabolism are a routine part of drug-discovery research; as such the US Food & Drug Administration (FDA) has published guidance principals for such tests. A major recommendation for in vitro inhibition experiments is to set incubation conditions such that substrate utilization does not exceed 10% [43]. We initially optimized incubation conditions for methadone and oxycodone at a substrate concentration of 2 µM with 0.5 mg protein/mL in the HLM incubations, and appropriate pmoles/mL in the CYP-specific pathways. Representative time-course plots are shown for oxycodone metabolism in Figure 3, further details, including methadone metabolism optimization, are available in the published study [44].

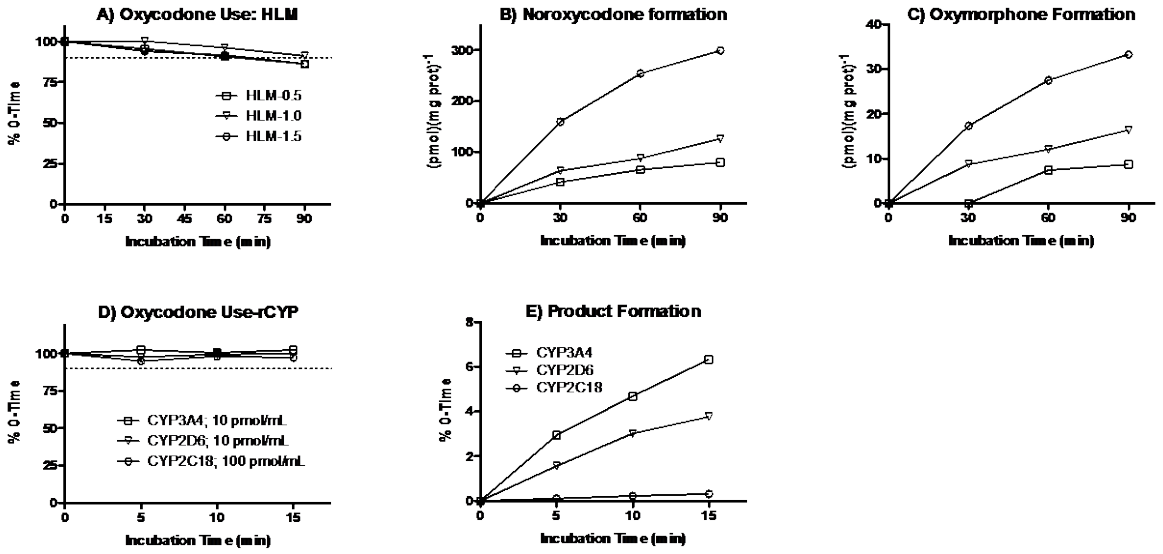

Figure 3. Initial optimization of oxycodone metabolism in HLM (0.5 mg protein/mL) (A-C). A) Oxycodone utilization, B) noroxycodone formation and C) oxymorphone formation in HLM at 0.5, 1.0 and 1.5 µM. With use of a shorter time-point, use of 2.0 µM oxycodone was selected. Optimization in rCYP using 2 µM oxycodone; D) oxycodone utilization and E) noroxycodone (CYP3A4 and 2C18) and oxymorphone production.

3.2.2 (Re)Optimization of Assays with 20-µM substrate

Ensuing experiments with buprenorphine demonstrated that by reducing the amount of HLM protein used and using buprenorphine at 20 µM, substrate loss could be kept at ≤ 10% over an incubation time sufficient to generate measureable norbuprenorphine (Figure 4). Such high concentrations of buprenorphine greatly exceeded the upper limit of quantitation (ULOQ); we routinely monitored buprenorphine peak area counts to assure minimal substrate loss. At the time of optimization, we performed a more detailed experiment where incubates were diluted 200-fold to bring them within the analytical range and assure substrate loss remained ≤ 10% (Figure 4A). This was possible when incubations were limited to 10 min. We then went on to optimize the amount of CYP3A4 and 2C8 to use with 20-µM buprenorphine (Figure 4B).

To permit comparison across substrates we then conducted similar experiments with methadone and oxycodone to optimize incubation conditions for these substrates at 20 µM. The final incubation conditions are shown in Table 2. Further details are available in published form [45]. For the dual-incubations used to determine MBI kinetics (see below), we have adjusted the procedure so the second incubation uses the CYP concentrations optimal for 20 µM of substrate. We are currently evaluating the positive controls for these incubations.

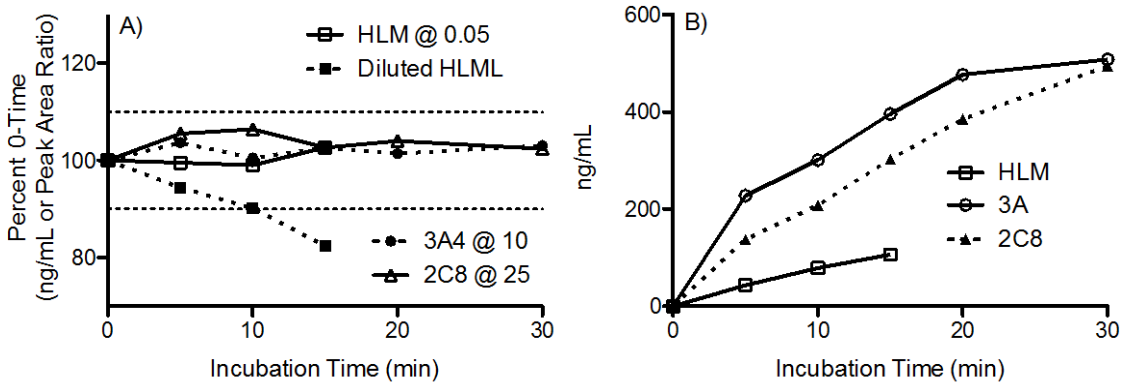

Figure 4. A) Buprenorphine utilization and B) norbuprenorphine formation in HLM, CYP3A4 and CYP2C8 incubated with 20-µM buprenorphine. Note: Due to the high concentration of buprenorphine it was generally monitored as peak area ratio. In one experiment (diluted HLM), the incubates were diluted 200-fold to allow ng/mL quantitation of buprenorphine).

Table 2. Optimized Incubation Conditions for Buprenorphine, Methadone and Oxycodone Metabolism Assays

Substrate	Substrate Concentration	Enzyme Source	Enzyme Amount	Incubation Time (min)
Buprenorphine	20 µM	HLM	0.05 mg/mL	10
		CYP3A4	2.5 pmol/mL	10
		CYP2C8	10 pmol/mL	10
Methadone	20 µM	HLM	0.2 mg/mL	15
		CYP3A4	5 pmol/mL	15
		CYP2B6	10 pmol/mL	15
Oxycodone	20 µM	HLM	0.3 mg/mL	60
		CYP3A4	5 pmol/mL	30
		CYP2D6	5 pmol/mL	30
		CYP2C18	25 pmol/mL	30

3.2.3 Background Formation With Use of 20 µM Substrates

With increase of all three substrate concentrations to 20 µM, background contamination was a consideration. As shown in Figure 5, the presence of minor amounts of impurity in substrate can create background amounts of metabolite that are detectable within the analytical range of the assays. While minimal for R- and S-EDDP and oxymorphone, the background is substantial for norbuprenorphine and noroxycodone. Background controls are now routinely included in all incubation batches.

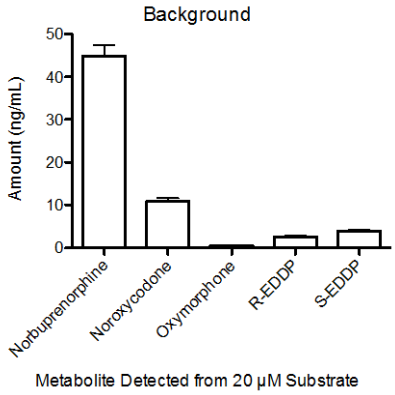

Figure 5. Amount of metabolite detected in HLM that were incubated for 0-time with no inhibitors added (i.e., background samples). Note: 20 µM is 9360, 6300 and 6200 ng/mL for buprenorphine, oxycodone and methadone respectively. Respective percent impurities are 0.48, 0.17, 0.006, 0.042 and 0.064%.

3.2.4 Negative Controls ± Solvent

A mitigating factor in the concentration of inhibitor that can be tested is its solubility in aqueous media. Organic compounds often need to be brought into solution in an organic solvent, but if the organic solvents concentration is too high it may inhibit the metabolizing enzyme. A rule of thumb, as recommended by the FDA [43], is to keep final organic solvent concentrations < 1%. We have now taken the precaution of including a solvent control in methanol or DMSO, as needed, with each run were solvent approaches 1%. Figure 6 shows the negative controls (no inhibitor) with or without solvent (DMSO in this case). In many, but not all of the assays, the addition of the solvent has a significant effect on the activity. In most cases it causes a decrease in activity, however, for oxymorphone production from oxycodone, DMSO at 1% actually increased activity. An important finding from these comparisons is that the activity after pre-incubation is typically decreased. This is understandable as the enzyme source is exposed to a warm environment for the 15-minute pre-incubation period, and the reaction with NADPH can be uncoupled producing oxygen radicals. While this difference from pre-incubation is not always significant, we routinely do not pool the data from the two groups.

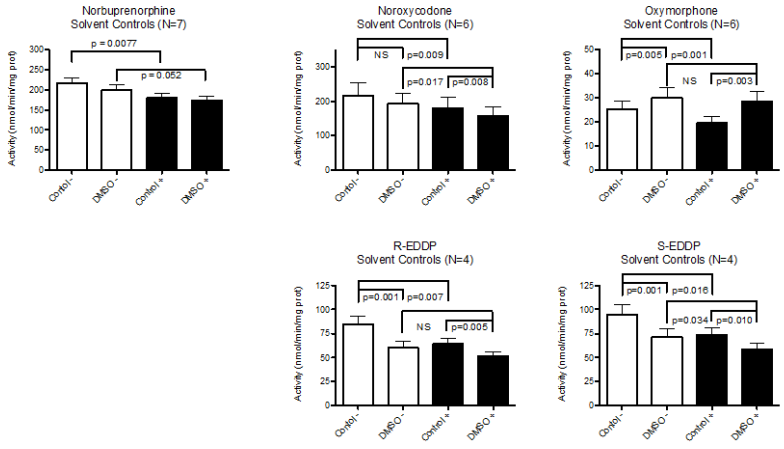

Figure 6. Effect of addition of 1% DMSO and pre-incubation on the activity in negative controls (i.e., no inhibitor added). Differences between conditions were first tested using 1-way ANOVA ($p < 0.05$). If significance was found individual groups were tested with the Tukey posthoc test ($p < 0.05$).

3.2.5 Positive Controls for TDI

Another addition to our assays is the use of positive controls. With the start of our studies on the azole antifungal agents (see below), we have instituted inclusion of positive controls with the HLM screens (Figure 7). Troleandomycin at 5 µM was used with all 3 substrates as the CYP3A4 positive control, 10 µM thioTEPA, 0.5 µM paroxetine and 5 µM gemfibrozil glucuronide was included as the CYP2B6 (methadone), CYP2D6 (oxycodone) and CYP2C8 (buprenorphine) positive controls, respectively [46; 47]. The effect of pre-incubation of 5 µM troleandromycin with CYP3A4 pathways, norbuprenorphine, noroxycodone, R- and S-EDDP production; 0.5 µM paroxetine with the CYP2D6 mediated formation of oxymorphone; and 10 µM thioTEPA with CYP2B6 mediated formation of S-EDDP are readily evident. The CYP2C8 TDI control, gemfibrozil glucuronide, did not show any effect on norbuprenorphine formation at 5 µM, probably due to the relatively lower content of CYP2C8 compared to 3A4 in HLM. A CYP2C18 specific TDI has not yet been identified.

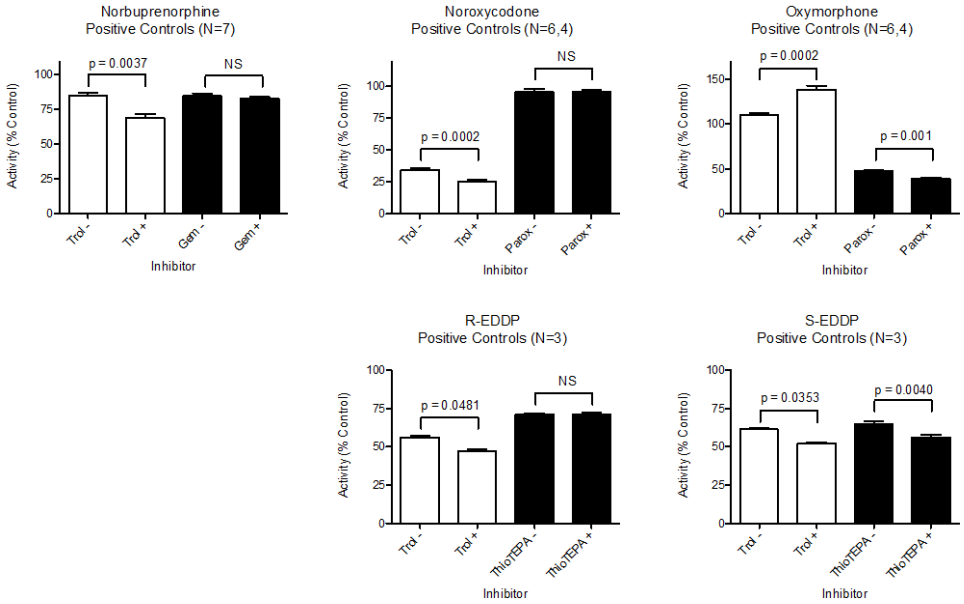

Figure 7. Effect of pre-incubation on inhibition by the positive controls 5 µM troleandromycin (Trol), 5 µM gemfibrozil glucuronide (Gem), 0.5 µM paroxetine (Parox), and 10 µM ThioTEPA. (see Figure 6 for approach to statistics)

3.3 Accomplishments under Aims 2 and 3: HLM Screens, IC_{50} Determinations and Extrapolations to In Vivo Potency

3.3.1 Methadone and Oxycodone Inhibition by H_2-Receptor Antagonists and Proton Pump Inhibitors with Substrate at 2.0-µM

While trouble-shooting the buprenorphine assay, we proceeded with the screen of the H_2-receptor antagonists and proton pump inhibitors (PPI) for inhibition of methadone and oxycodone metabolism in HLM, and conducted follow up IC_{50} determinations with specific CYP450s using the 2.0 µM substrate conditions [44]. The upper concentration of inhibitor

tested was usually limited by solubility in aqueous solutions. For the H_2-receptor antagonists this was 1000 μM; for the PPIs it was 200 μM (100 μM for lansoprazole). Under these conditions both cimetidine and famotidine caused > 50% inhibition in all four pathways; inhibition by nizatidine and ramotidine did not exceed 50%.

Pre-incubation appears to have enhanced the inhibition of noroxycodone formation and perhaps R-EDDP formation by cimetidine (Table 3). With the PPIs, excepting oxymorphone formation with omeprazole and pantoprazole, all exhibited at least 50% inhibition. This was most noticeable for noroxycodone formation, which was substantially inhibited by all 5 PPIs. Only rabeprazole exhibited greater inhibition with pre-incubation and this was seen with all four pathways.

We then proceded to determine IC_{50} values with the specific rCYPs. We then used scaling models to predict potential for in vivo inhibition (Table 4). One of the simpler equations to extrapolate in vitro to in vivo inhibition is:

Table 3. Approximate IC_{50} Determinations for H_2-Receptor Antagonists and Proton Pump Inhibitors Incubated with HLM ± Pre-Incubation

	R-EDDP Formation		S-EDDP Formation		Noroxycodone Formation		Oxymorphone Formation	
	Approximate IC_{50} (μM) ± Pre-incubation							
Inhibitor	-	+	-	+	-	+	-	+
H2-Receptor Antagonists								
Cimetidine	395	305	620	610	201	101	90	90
Famotidine	405	490	460	550	315	310	505	505
Nizatidine	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Ranitidine	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
Proton Pump Inhibitors (PPIs)								
Omeprazole	21	22	102	105	1.8	1.2	> 200	> 200
Esomeprazole	80	81	105	108	1.6	2.9	> 200	200
Lansoprazole	70	50	> 100	> 100	11	6.2	50	80
Rabeprazole	70	50	78	51	5.3	4.0	170	105
Pantoprazole	103	104	105	102	19	16	> 200	> 200

Note: Incubations with HLM were performed at 10, 300 and 1000 μM with H_2-receptor antagonists and at 1, 30 and 200 (100 for lansoprazole) μM with PPIs ± a 15 minute pre-incubation of HLM, NADPH source and inhibitor. IC_{50} were approximated from these 3-concentrations.

$$AUC_i / AUC_n = 1 + [I] / K_i \qquad (1)$$

Where AUC_i and AUC_n are the area under the time versus plasma concentration curve in presence of inhibitor and absence of inhibitor, [I] is the in vivo concentration of inhibitor, and K_i is the inhibition constant. Ratios of $AUC_i / AUC_n \geq 2$ are considered to be potentially significant inhibitions. This equation is discussed in numerous articles, Zvyaga et al. [48] is a recent example that looks at PPI inhibitors. When one assumes competitive inhibition the K_i can be estimated from the IC_{50} using the Cheng-Prusoff equation [49]:

$$K_i = IC_{50} / (1 + S/K_m) \qquad (2)$$

Where S is the substrate concentration used in vitro and K_m is the Michaelis-Menton constant for the reaction being studied. When equation 2 is applied to the IC_{50} values we calculated, we see the estimated K_i is quite similar. As the substrate concentration used of 2.0 μM is so much smaller than the K_m values of our reactions there is little adjustment (Table 4). When equation 1 is then used, we find a number of estimated ratios of AUC_i / AUC_n exceed 2. Most notable are the inhibition of rCYP3A4-mediated oxycodone formation by pantoprazole, rCYP2D6-mediated oxymorphone formation by lansoprazole, rCYP3A4-mediated S-EDDP formation by pantoprazole and rCYP3A4-mediated R-EDDP formation by omeprazole (Table 4). Changes in the ratios are not directly proportional to [I], as is shown if we assume a 10-fold increase in [I], that may be found in a toxic dose of the inhibitor (Table 4).

Table 4. IC_{50}s and Estimates of K_i and In Vivo Inhibitory Potential at Average and 10X Average Plasma Concentrations of Inhibitor

Inhibitor	[I]pl (μM)[a]	IC_{50} (μM)	K_i[b] (μM)	AUC_i[c] /AUC_n	AUC_{ix10} /AUC_n[c]	IC_{50} (μM)	K_i[b] (μM)	AUC_i[c] /AUC_n	AUC_{ix10} /AUC_n[c]
		Oxycodone by CYP3A4				Oxymophone by CYP2D6			
Cimetidine	6.42	78.0	77.8	1.08	1.83	110	105	1.06	1.61
Famotidine	0.33	190	189	1.00	1.02	300	286	1.00	1.01
Esomeprazole	0.90	0.450	0.449	3.01	21.1	14.0	13.3	1.07	1.68
Omeprazole	1.80	0.800	0.798	3.26	23.6	15.0	14.3	1.13	2.26
Lansoprazole	2.70	2.90	2.89	1.93	10.3	0.65	0.62	5.36	44.6
Pantoprazole	12.0	1.0	1.0	13.0	121	15.0	14.3	1.84	9.40
Rabeprazole	1.57	4.90	4.89	1.32	4.22	14.0	13.3	1.12	2.18
		R-Methadone by CYP3A4				S-Methadone by CYP3A4			
Cimetidine		450	444	1.01	1.14	405	400	1.02	1.16
Famotidine		370	365	1.00	1.01	390	385	1.00	1.01
Esomeprazole		0.90	0.89	2.01	11.2	0.80	0.79	2.14	12.4
Omeprazole		0.72	0.71	3.54	26.4	0.69	0.68	3.64	27.4
Lansoprazole		3.50	3.45	1.78	8.83	3.10	3.06	1.88	9.83
Pantoprazole		7.00	6.90	2.74	18.4	3.20	3.16	4.80	39.0
Rabeprazole		17.0	16.8	1.09	1.94	14.0	13.8	1.11	2.14
		R-Methadone by CYP2B6				S-Methadone by CYP2B6			
Cimetidine		>2000				>2000			
Famotidine		420	406	1.00	1.01	420	373	1.00	1.01
Esomeprazole		92.0	89.0	1.01	1.10	92.0	81.8	1.01	1.11
Omeprazole		120	116	1.02	1.16	120	107	1.02	1.17
Lansoprazole		>100				>100			
Pantoprazole		200	194	1.06	1.62	200	178	1.07	1.67
Rabeprazole		62.0	60.0	1.03	1.26	62.0	55.1	1.03	1.29

a – Plasma (pl) concentrations are from Schulz & Schmoldt [50] and Suzuki et al. [51]

b – K_i was estimated from IC_{50} using the Cheng-Prusoff equation where $K_i = IC_{50}/(1 + S/K_m)$ [49], this assumes competitive inhibition. K_m values (μM) were from the literature as follows: Oxycodone by CYP3A4 (600) and 2D6 (39.8) [28], R-methadone by CYP3A4 (112) and 2B6 (13.6) and S-methadone by CYP3A4 (136) and 2B6 (12.4) [52].

c – Inhibitory potential $AUC_i/AUC_n = 1 + [I]/K_i$, where AUC = ratio of area under the concentration curve in presence of inhibitor (i), 10-times inhibitor (ix10) or no inhibitor (n)

Several modifications can be made to equation 1 as more and more complex models are derived for in vitro to in vivo extrapolations. This too is discussed in numerous articles; again see Zvyaga et al. [48] for a recent example that looks at PPI inhibitors. One of the main modifications is use of free (unbound) fraction of inhibitor in plasma ($f_{u,pl}$) and in enzyme source ($f_{u,HLM}$ or $f_{u,rCYP}$). These were not calculated for this study. Plasma protein binding is available in the literature. We have used these along with some hypothetical rCYP binding to show how protein binding effects the ratios of AUC_i / AUC_n (Table 5). When $f_{u,pl}$ is small and $f_{u,rCYP}$ is negligible, the ratio decreases dramatically. When $f_{u,pl} \approx f_{u,rCYP}$ the ratio is essentially the same as when binding was not considered. As non-specific binding to membranes is often less than to plasma protein (e.g., we found $f_{u,HLM}$ for buprenorphine was 0.42, while $f_{u,pl}$ is 0.01 [53]), we provide an intermediate hypothetical $f_{u,rCYP}$ for omeprazole (Table 5). These may be closer to reality; similar adjustments can be made for the other drugs.

Table 5. Examples of the Impact of Plasma and Microsome Drug Binding on Calculation of Inhibitory Potential: Oxycodone Metabolism by CYP3A4

	f_u [a] plasma	f_u [b] rCYP	[I]pl, u (μM)	IC_{50}, u (μM)	K_i, u (μM)	AUC_i [c] /AUC_n	AUC_{ix10} /AUC_n [c]
Cimetidine	1.00	1.00	6.42	78.0	77.8	1.08	1.83
	0.80	1.00	5.14	78.0	77.8	1.07	1.66
	0.80	0.80	5.14	62.0	62.2	1.08	1.83
Omeprazole	1.00	1.00	1.80	0.800	0.798	3.26	23.6
	0.03	1.00	0.054	0.800	0.798	1.07	1.66
	0.03	0.03	0.054	0.024	0.024	3.25	23.5
	0.03	0.50	0.054	0.400	0.399	1.14	2.35

a – Plasma protein binding from Somogyi & Gugler [54] for cimetidine and Andersson & Weidoff [55] for omeprazole.
b – Hypothetical non-specific binding for rCYP.

CYP2C18 is an extrahepatic enzyme not suitable for scaling. Its IC_{50} values for oxycodone N-demethylation are summarized separately in the published account [45].

We found that the H_2-receptor antagonists were generally weak reversible inhibitors of the four pathways studied, N-demethylation of oxycodone to noroxycodone, O-demethylation of oxycodone to oxymorphone, and N-demethylation (with cyclization) of R- and S-methadone to R- and S-EDDP. Ranitidine and nizatidine did not exceed 50% inhibition in HLM at up to 1000 μM and were not further studied. Ranitidine has continuously been found to be a weak inhibitor of CYP-dependent metabolism, particularly in comparison to cimetidine [56; 57; 58]. Nizatidine was also a weaker inhibitor of CYP-dependent metabolism in comparison to cimetidine, except for its effect on CYP2C19 [59], which is not a factor in the metabolic routes studied. The in vitro effects of famotidine (1-100 μM) in human tissue have only been studied against phenacetin O-demethylation, which it was found not to inhibit [57]. We did find famotidine inhibited the four pathways we studied with reversible IC_{50}s of 190-390 μM, which were only slightly weaker or comparable to the reversible inhibition exhibited by cimetidine. The extrapolation of in vitro to in vivo inhibition suggests that reversible inhibition will not produce significant in vivo inhibition unless concentrations exceed 10-fold of those associated with therapeutic use. Cimetidine does, however, produce in vivo inhibition of the metabolism of some drugs [60].

In contrast to the H_2-receptor antagonists, many of the PPIs demonstrated at least moderate reversible inhibition of the pathways studied and could be predicted as having the potential to produce in vivo drug interactions at therapeutic concentrations. For the pathways studied, we found the CYP3A4-mediated reactions were more susceptible to omeprazole, esomeprazole and pantoprazole. The CYP2D6-mediated pathway was almost selectively susceptible to lansoprazole. The CYP2B6-mediated pathways were only weakly inhibited by the PPIs. In general, the PPIs are known to most potently inhibit CYP2C19 [61; 62; 63; 64]. CYP2C19 inhibition by omeprazole and esomeprazole has recently been found to be TDI or MBI [48; 65]. The sub-micromolar IC_{50} values we found for omeprazole and esomeprazole with CYP3A4-mediated metabolism and for lansoprazole and CYP2D6 mediated metabolism are lower than those reported for non-CYP2C19 mediated metabolism. Substrate differences in susceptibility to PPI inhibition may be one explanation. This has been seen with CYP2C19 [48]. Rabeprazole was a less potent reversible inhibitor of the pathways with IC_{50} values that ranged from 4.9 to 17 μM. Rabeprazole, however, showed potential TDI in our screen for a number of the pathways and has also shown TDI with CYP1A2, 2C8 and 2D6-mediated reactions [48]. Further studies on the TDI/MBI of the PPIs are certainly warranted.

The H_2-receptor antagonists, cimetidine and famotidine showed weak in vivo reversible inhibition of the opioid pathways studied, but appear unlikely to be significant reversible inhibitors in vivo unless concentrations exceed 10X therapeutic. The PPIs showed CYP-selective moderate reversible inhibition with omeprazole, esomeprazole and pantoprazole having potential for in vivo reversible inhibition of CYP3A4-mediated pathways, while lansoprazole has potential for reversible inhibition of CYP2D6-mediated pathways. The potential for in vivo MBI cannot, however, be ruled out for either class of drugs and will be the subject of further study.

3.3.2 Buprenorphine, Methadone and Oxycodone Inhibition by Azole Antifungal Agents with Substrate at 20 μM

When proceeding with the third group of inhibitors, the azole antifungal agents, we initiated the use of 20 μM substrate concentrations for all three opioids. Background on these agents is provided in the published account [45]. The azole compounds have a wide range of polarity/aqueous solubility, which limited the final concentrations that could be tested for some compounds (e.g., itraconazole) as low as 5 μM. A correlation ($R^2 = 0.473$, $p = 0.0194$) was found between the partition coefficient (XLogP3) and the highest final concentration tested (Figure 8).

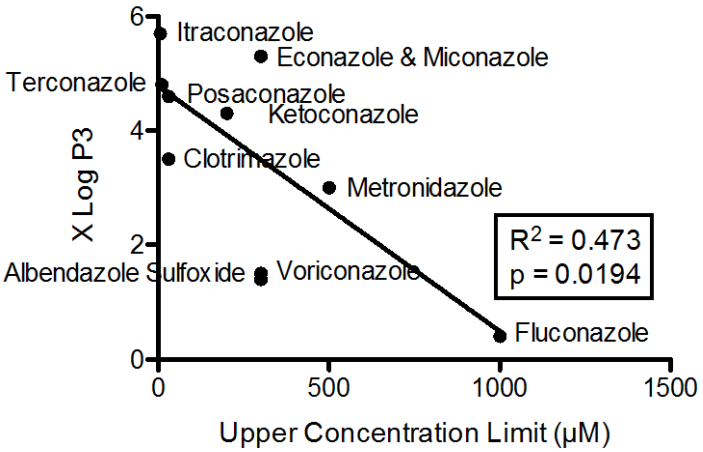

Figure 8. Plot of upper concentration of inhibitor used to not exceed 1% DMSO in incubation versus log of the octanol-water partition coefficient (X Log P3); X Log P3 values are from PubChem.

The results of the initial screen in HLM ± pre-incubation are summarized in Table 6. Albendazole and metronidazole did not exceed 50% inhibition and are not shown in the table. Most azole compounds caused >50% inhibition for norbuprenorphine, R- and S-EDDP, and noroxycodone formation. Metranidazole and albendazole (data not shown) were notable exceptions. Only econazole and miconazole produced > 50% inhibition of oxymorphone formation in HLM. Only posaconazole incubated with methadone demonstrated TDI (Table 6). We have not further explored mechanisms for this TDI.

Table 6. Approximate IC_{50} Determinations for Azole Antifungals Incubated with HLM ± Pre-Incubation

Inhibitor (highest concentration tested, µM)	Norbup-renorphine Formation		R-EDDP Formation		S-EDDP Formation		Nor-oxycodone Formation		Oxy-morphone Formation	
	Approximate IC_{50} (µM) ± Pre-incubation									
	-	+	-	+	-	+	-	+	-	+
Albendazole Sulfate (300)	>300	>300	≈300	≈310	≈310	≈310	>300	>300	>300	>300
Clotrimazole (5)	0.19	0.19	0.55	0.71	0.71	1.10	0.27	0.33	> 5	> 5
Econazole (5)	0.49	0.33	1.0	1.8	1.0	1.8	1.1	1.7	3.0	3.9
Fluconazole (300)	19	31	9.9	12	9.9	10	4.3	4.0	>300	>300
Itraconazole (5)	4.9	4.1	>5	>5	>5	>5	5.0	3.0	>5	>5
Ketoconazole (5)	<0.1	<0.1	<0.1	0.15	0.13	0.21	0.10	0.25	>5	>5
Miconazole (5)	1.2	2.1	2.0	1.8	2.0	1.7	2.0	1.4	5.0	≈7
Posaconazole (30)	6.8	10	211	4.2	30	6.0	2.0	2.0	>30	>30
Terconazole (10)	5.0	3.4	1.2	2.1	1.1	2.1	0.59	0.55	>10	>10
Voriconazole (300)	8.0	11	5.0	10	2.3	4.0	5.1	4.2	>300	>300
Note: Incubations with HLM were performed ± a 15 minute pre-incubation of HLM, NADPH source and inhibitor. IC_{50} were approximated from 3-concentrations.										

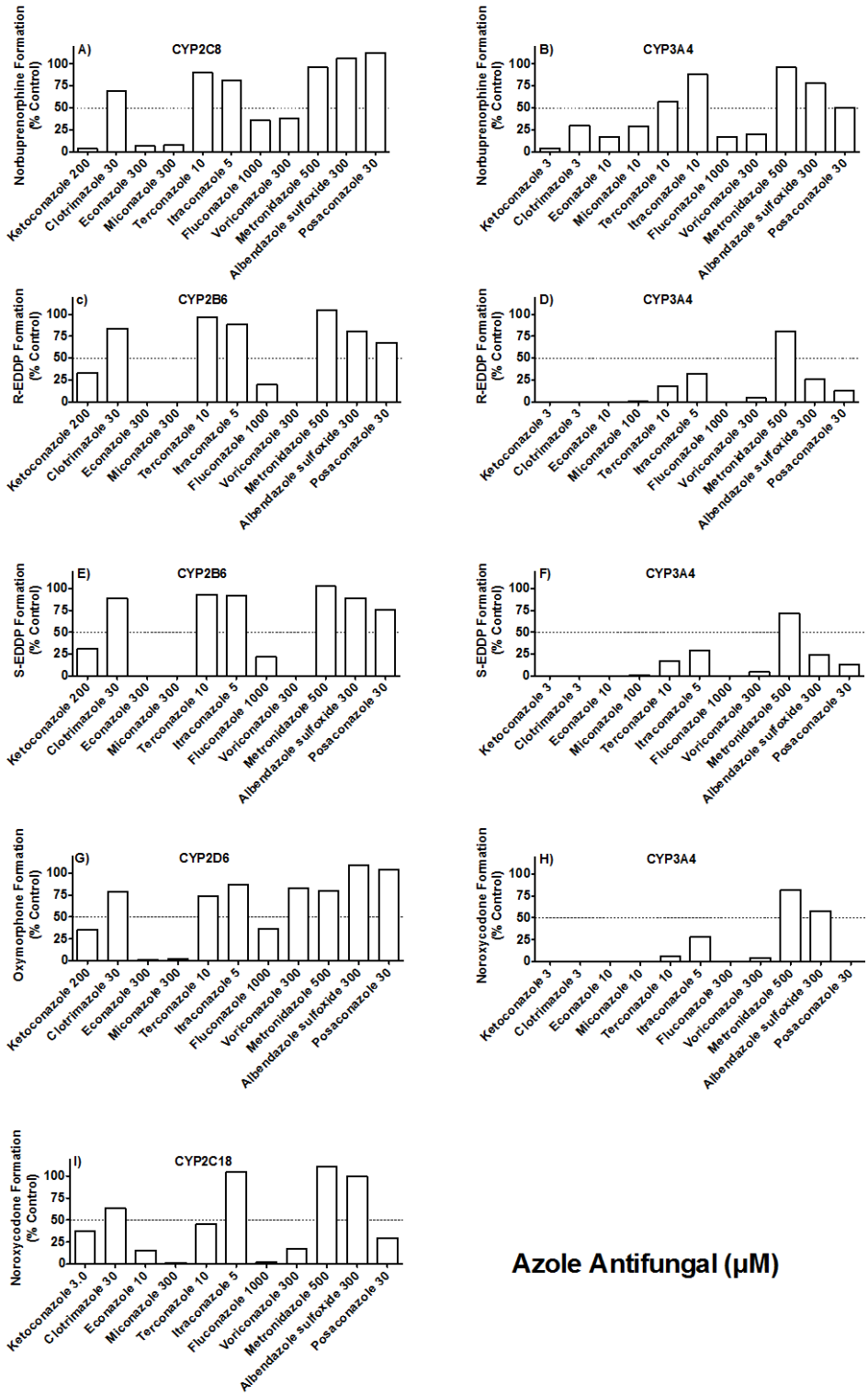

Figure 9. Inhibition of the rCYP450 mediated metabolism using the highest achievable concentration for non-CYP3A4 pathways, and highest concentration used in IC_{50} determinations for CYP3A4 pathways.

CYP3A4 is the major CYP present in HLM, and the magnitude of inhibition found in HLM is a fairly good indicator of inhibition of CYP3A4. All azoles were selected for IC_{50} determinations with CYP3A4 pathways according to the findings in Table 6, except that albendazole sulfoxide and metronidazole were screened against CYP3A4 pathways to assure inhibitory activity greater than 50% was not missed. As the magnitude of inhibition of CYP2B6, 2C8 and 2D6 may not be accurately expressed in HLM due to their lesser percentage of total CYP protein, inhibition of these pathways was first tested at maximum concentration of azole to determine which would require IC_{50} determinations (Figure 9). Based on these studies, CYP3A4 IC_{50} determinations would include albendazole sulfoxide with methadone; CYP2B6, 2C8, 2C18 and 2D6 IC_{50} determinations would include: econazole, fluconazole, ketoconazole, miconazole, and voriconazole (except 2D6); and CYP2C18 IC_{50} determinations would also include terconazole and posaconazole (Figure 9).

IC_{50} determinations for selected azoles were carried out as described above. rCYPs were incubated with the selected substrates and azoles at 6 concentrations (each in duplicate) based on the findings with the screen and the upper limit of solubility of the compound. For most compounds, the IC_{50} was determined using non-linear regression. For a few azoles, extrapolation from where the curve intercepted the 50% inhibition point was used. A summary of the IC_{50} determinations for each CYP-dependent pathway is presented in Table 7. Comparison of the IC_{50} determinations for the CYP3A4 pathways is shown in Figure 10. In general there was a fair agreement in the rankings. In about one-half of the comparisons, the IC_{50} determinations are inversely proportional to the reported K_m for the respective pathways, with oxycodone highest, methadone intermediate and buprenorphine lowest [15; 28; 52], as expected for competitive inhibition.

Table 7. In Vitro Reversible Inhibition of CYP-Mediated Metabolism of Methadone, Buprenorphine and Oxycodone by Azole Antifungal Agents

Antifungal Azole	CYP Pathways and Products [a]								
	3A4				2B6		2C8	2C18	2D6
	R-ED	S-ED	Norbu	Norox	R-ED	S-ED	Norbu	Norox	Oxym
	IC_{50} (μM)								
Alendazole sulfoxide	224*	224*	> 300	> 300	> 300	> 300	> 300	> 300	> 300
Clotrimazole	0.348	0.350	0.354	0.303	> 30	> 30	> 30	> 30	> 30
Econazole	2.38	2.18	4.94	2.58	9.46	6.75	6.04	1.04	1.22
Fluconazole	16.1	16.3	65.8	7.69	313	361	1240	17.2	1000
Itraconazole	2.48*	2.45*	> 5	3.16*	> 5	> 5	> 5	> 5	> 5
Ketoconazole	0.0793	0.0853	0.0935	0.0320	11.7	31.2	77.6*	0.976	182*
Metronidazole	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
Miconazole	2.34	2.28	7.59*	3.73	2.76	2.78	5.34	3.10	5.89
Posaconazole	3.44	3.41	19.8	3.82	> 30	> 30	> 30	3.82	> 30
Terconazole	1.70	1.53	> 10	0.481	> 10	> 10	> 10	8.13*	> 10
Voriconazole	2.28	2.89	14.6	0.397	2.40	2.53	170	12.7	> 300

* - Determined by extrapolation

a - Abbreviations: Pathways studied: CYP3A4 and 2B6 - methadone conversion to R-EDDP (R-ED) and S-EDDP (S-ED); CYP3A4 and 2C8 – buprenorphine conversion to norbuprenorphine (Norbu); CYP3A4 and 2C18 – oxycodone conversion to noroxycodone (Norox); and CYP2D6 – oxycodone conversion to oxymorphone (Oxym).

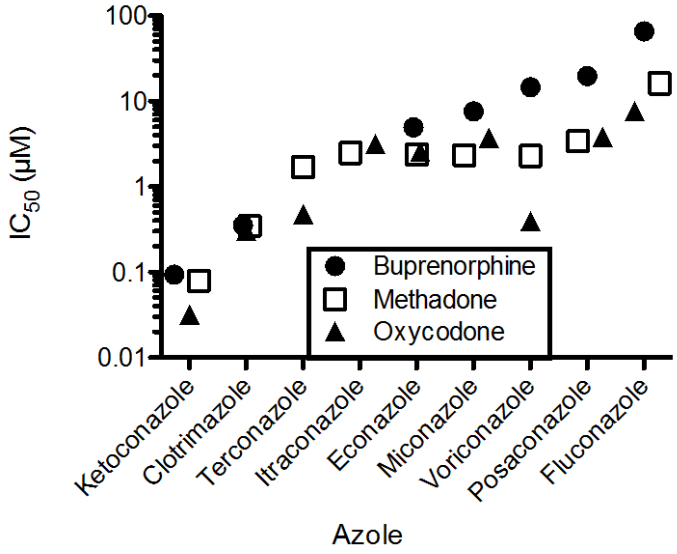

Figure 10. Ranking of IC_{50} determinations for CYP3A4 mediated pathways.

Estimates of in vivo potency were performed using equations 1 and 2 described above. When equation 2 is applied to the calculated IC_{50} values (Table 8), the largest differences between K_i and K_m were found for the pathways where the K_m approximates the 20 μM substrate concentration, as is the case for CYP3A4 and 2C8 metabolism of buprenorphine and CYP2B6 metabolism of methadone. The difference between K_i and K_m is less so as the K_m increases for CYP2D6 metabolism of oxycodone and CYP3A4 metabolism of methadone, and almost nonexistent for CYP3A4 metabolism of oxycodone (Table 8). When equation 1 is then used, the estimated ratios of AUC_i / AUC_n exceed 2 for ketoconazole and voriconazole on CYP3A4 pathways and for voriconazole on the CYP2B6 pathway. If the level of concern is lowered to a factor of 1.5, the impact of itraconazole, fluconazole and posaconazole are also seen with a number of the CYP3A4 pathways, as well as ketoconazole and CYP2B6 metabolism (Table 8). This modeling system would not predict even 1.5-fold factor changes in CYP2D6 or by plasma concentrations published for the mucosal and topical formulations. The topical formulation did not exceed this factor even when the plasma concentrations were increased ten-fold to mimic a serious overdose situation (Table 8). In the discussion of the published report, we have compared the findings in Table 8 with those in the literature for azole antifungal inhibition of the relevant CYP450 pathway [45].

Many of the azole agents inhibited the in vitro metabolism of oxycodone, methadone and buprenorphine. Ketoconazole and clotrimazole had sub-micromolar IC_{50} values for the CYP3A4 mediated pathways, as did terconazole and voriconazole for oxycodone metabolism by CYP3A4. Several azoles had IC_{50} values between 1-10 μM for the CYP3A4 pathways. Two to three azoles also had IC_{50} values in this range for the CYP2B6, 2C8, 2C18 and 2D6 pathways. When unbound plasma concentrations of the azoles were used there was fair agreement between extrapolation predicted increases in AUC and those seen in the literature for clinical studies. Noted exceptions were with itraconazole, which has

Table 8. Extrapolation of IC_{50} Determinations to K_i and Inhibitory Potential at Average and 10 x Average Plasma Concentration of Azole Inhibitors.

Inhibitor	[I]pl (μM) [a]	IC_{50} (μM)	K_i (μM) [b]	AUC_i /AUC_n[c]	AUC_{i10} /AUC_n	IC_{50} (μM)	K_i (μM)	AUC_i /AUC_n	AUC_{i10} /AUC_n
		Oxycodone by CYP3A4				Oxycodone by CYP2D6			
Fluconazole	4.24	7.69	7.44	1.57	6.70	1000	666	1.01	1.06
Itraconazole	1.98	3.16 *	3.06	1.65	7.47	---	---	---	---
Ketoconazole	7.53	0.0320	0.0310	244	2433	182 *	121	1.06	1.62
Posaconazole	1.86	3.82	3.70	1.50	6.03	---	---	---	---
Voriconazole	10.9	0.397	0.384	29.4	285	---	---	---	---
Clotrimazole	0.029	0.303	0.293	1.10	1.99	---	---	---	---
Miconazole	0.024	3.73	3.61	1.01	1.07	5.89	3.92	1.01	1.06
Terconazole	0.019	0.481	0.465	1.04	1.41	---	---	---	---
Econazole	0.0029	2.58	2.50	1.00	1.01	1.22	0.812	1.00	1.04
		R-Methadone by CYP3A4				S-Methadone by CYP3A4			
Albendazole Sulfoxide	4.90	224 *	190	1.03	1.26	224 *	195	1.03	1.25
Fluconazole		16.1	13.7	1.31	4.10	16.3	14.2	1.30	3.98
Itraconazole		2.48 *	2.10	1.94	10.4	2.45 *	2.14	1.93	10.3
Ketoconazole		0.0793	0.0673	113	1120	0.0853	0.0744	102	1014
Posaconazole		3.44	2.92	1.64	7.37	3.41	2.97	1.63	7.26
Voriconazole		2.28	1.93	6.63	57.3	2.89	2.52	5.33	44.3
Clotrimazole		0.348	0.295	1.10	1.98	0.350	0.305	1.10	1.95
Miconazole		2.34	1.99	1.01	1.12	2.28	1.99	1.01	1.12
Terconazole		1.70	1.44	1.01	1.13	1.53	1.33	1.01	1.14
Econazole		2.38	2.02	1.00	1.01	2.18	1.90	1.00	1.02
		R-Methadone by CYP2B6				R-Methadone by CYP2B6			
Fluconazole		313	210	1.02	1.20	361	185	1.02	1.23
Ketoconazole		11.7	7.86	1.96	10.6	31.2	16.0	1.47	5.71
Voriconazole		2.40	1.61	7.76	68.6	2.53	1.30	9.41	85.1
Miconazole		2.76	1.86	1.01	1.13	2.78	1.42	1.02	1.17
Econazole		9.46	6.36	1.00	1.00	6.75	3.46	1.00	1.01
		Buprenorphine by CYP3A4				Buprenorphine by CYP2C8			
Fluconazole		65.8	26.6	1.16	2.59	1240	475	1.01	1.09
Ketoconazole		0.0935	0.0378	200	1991	77.6 *	29.7	1.25	3.54
Posaconazole		19.8	8.01	1.23	3.32	---	---	---	---
Voriconazole		14.6	5.91	2.84	19.4	170	65.1	1.17	2.68
Clotrimazole		0.354	0.143	1.20	3.02	---	---	---	---
Miconazole		7.59 *	3.07	1.01	1.08	5.34	2.04	1.01	1.12
Econazole		4.94	2.00	1.00	1.01	6.04	2.31	1.00	1.01

a – Plasma concentrations for the oral azoles were taken from literature C_{max} values as follows: albendazole sulfoxide [66], fluconazole, itraconazole and voriconazole [67], ketoconazole [68], and posaconazole [69]; the mucosal formulations clotrimazole, miconazole and terconazole were calculated from an estimated C_{max} of 10 ng/mL and the topical econazole from an estimated C_{max} of 1.0 ng/mL.

b – K_i was estimated from the IC_{50} using the Cheng-Prusoff, equation, where $K_i = IC_{50}/(1 + S/K_m)$. K_m values (μM) were from the literature as follows: Oxycodone by CYP3A4 (600) and 2D6 (39.8), [28] R-methadone by CYP3A4 (112) and 2B6 (13.6) and S-methadone by CYP3A4 (136) and 2B6 (12.4), [52] and buprenorphine by CYP3A4 (13.6 and by 2C8 (12.4). [15]

c – Inhibitory potential $AUC_i/AUC_n = 1 + [I]/K_i$, where AUC = area under the concentration curve in the presence of inhibitor (i), 10-times inhibitor (i10) or no inhibitor (n).

* - Insufficient points were available for calculation of IC_{50} by nonlinear regression, value was determined by extrapolation from curve.

contributing inhibitory metabolites, and with oral gel use of miconazole. The data provided by this study should assist in interpretation of clinical and forensic findings when pharmacokinetic drug interactions may have occurred because of co-administration of these opioids and azoles.

3.4 Accomplishments under Aims 1B and 4 - Studies on TDI Kinetic Determinations and Follow Up experiments

This section focuses on four primary areas: establishing positive controls for the dual-incubation MBI experiments, evidence for MBI with cimetidine, different mechanisms for TDI for PPIs, and establishing protocols to establish these mechanisms.

3.4.1 TDI Positive Controls

The dual-incubation system to determine TDI kinetics is a labor-intensive procedure, with timing of the initial incubations to permit withdrawal of aliquots for 5 test concentrations (including the zero), and then 10-20 minute secondary incubations. This limits the number of samples that can be conveniently handled in an analytical batch. Our normal batch will comprise 2-4 background samples, 5 concentrations of inhibitor (the zero being control for all batches along with 4 experimental concentrations), and 4 time points all in duplicate. This results in an initial plot of "ln % remaining activity" versus pre-incubation time (see Figure 11A-D and G for examples). The slope of these lines equals the negative Kobs. An eventual plot of Kobs versus inhibitor concentration is used to calculate the TDI kinetic parameters K_{inact} and K_i. A half-life ($t_{1/2}$) for enzyme degradation is then calculated as $t_{1/2} = 0.693/K_{inact}$.

To achieve publishable data, we aimed for 5 experimental concentrations with a negative slope (i.e., positive Kobs), and ≥ 3 sets of incubations per concentration. Under optimal conditions this can be achieved with 4 batches. If however, the initial range selection of experimental conditions includes a concentration too low to achieve a negative slope over time, or too high to capture inhibition within the time frame studied, up to 6 batches may be required. This took 2-4 weeks per positive control.

In Figure 11, we show how these data accumulate for the study of TAO as a positive MBI control of CYP3A4 mediated metabolism of buprenorphine. The top four graphs (Figures 11A-D) show individual batch results where we have collected data for TAO used at 0.25-10 µM. The 0.25 µM concentration used in the 1st batch was inadequate to measure TDI. Final results were based on 0.5, 1.0, 2.5, 5 and 10 µM experiments. Once the data were collected, we compared two approaches to summarizing the results. In one approach, we determined the mean Kobs determined for individual batches and plotted average Kobs versus the inhibitor concentration (Figure 11E). Alternatively, we can calculate the mean ln % remaining and then plots these versus pre-incubation time (Figure 11F). The negative slopes of these lines are then plotted as Kobs versus inhibitor concentration (Figure 11G). The results of the two approaches are nearly identical. The presentations shown in Figures 11F and G will be used as they allow presentation of the mean ln % remaining versus pre-incubation time and the mean Kobs versus mean inhibitor concentration.

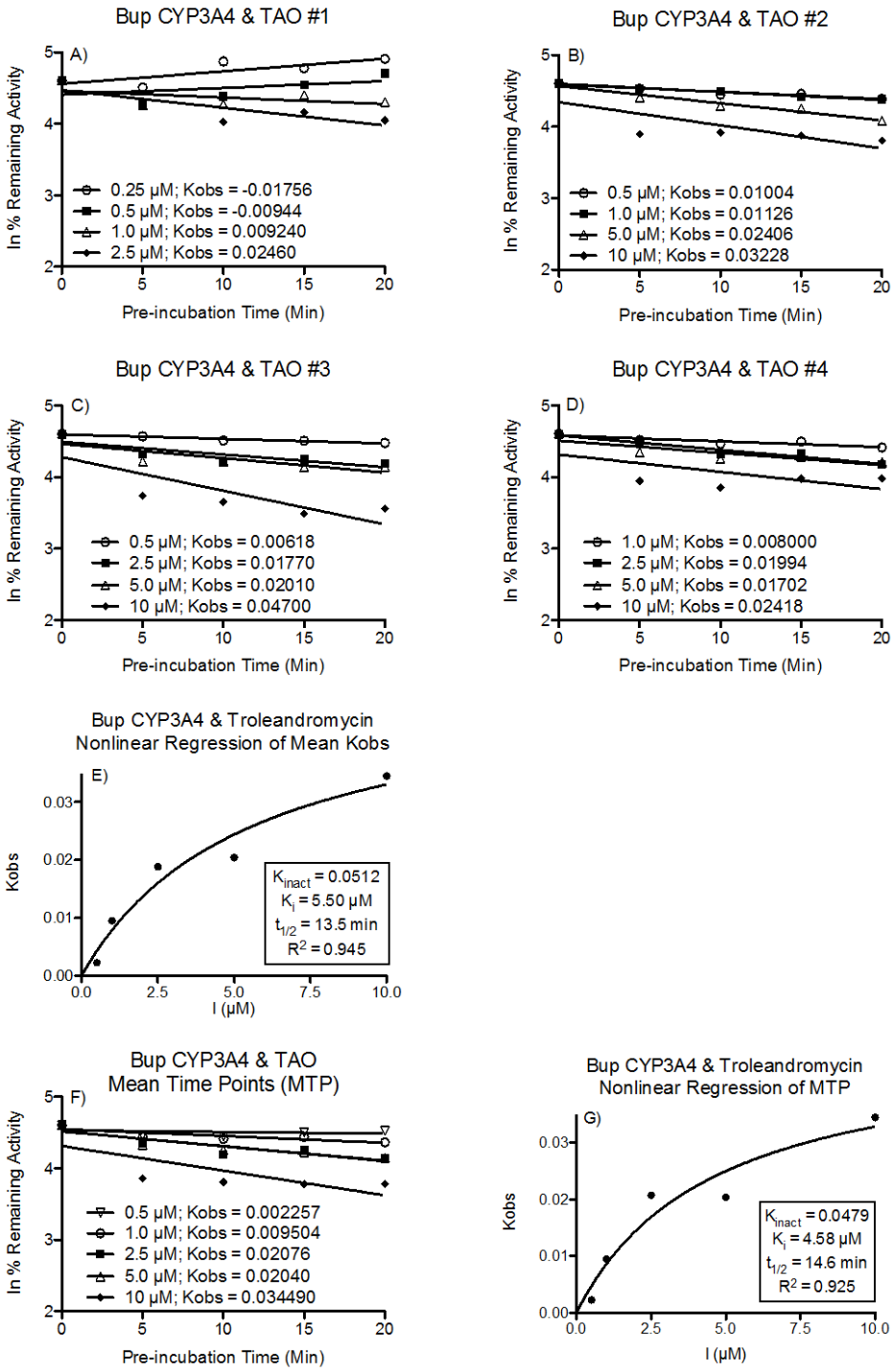

Figure 11. Determination of kinetics for MBI of CYP3A4 metabolism of buprenorphine by troleandomycin (TAO); A-D Individual batch results, E nonlinear regression of mean Kobs, F plot of mean ln % remaining versus pre-incubation time, and G nonlinear regression of mean time point Kobs versus inhibitor concentration.

We have also finalized studies on use of TAO as a positive control for CYP3A4 metabolism of oxycodone and methadone (Figure 12), and for gemfibrozil glucuronide on buprenorphine metabolism by CYP2C8 (Figure 13). Similar studies with thioTEPA as a positive control for CYP2B6 metabolism of methadone have been initiated; we have completed 2 incubations with this control (data not shown). We must still determine the effect of paroxetine on oxycodone metabolism by CYP2D6.

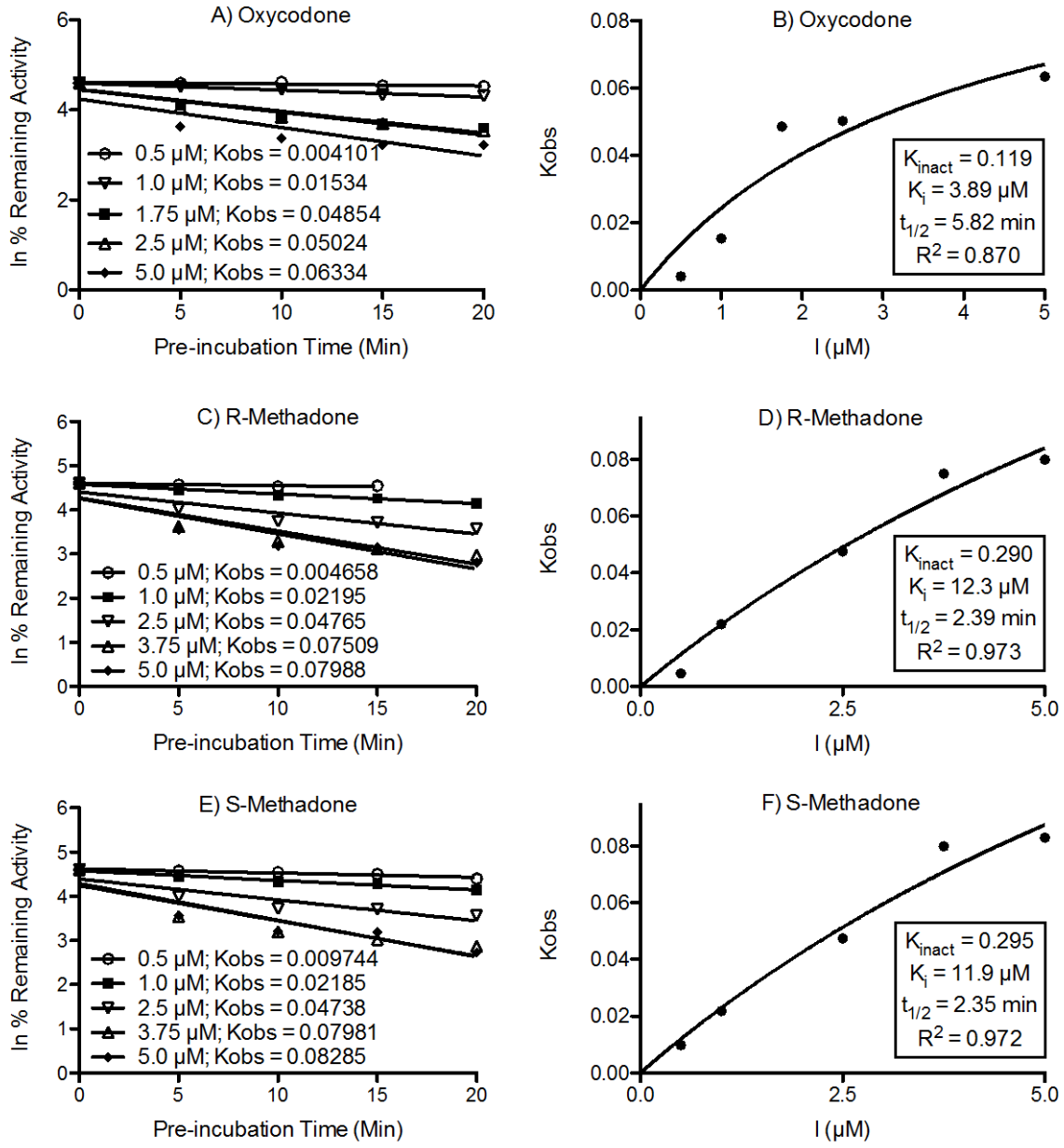

Figure 12. Determination of kinetics for MBI by TAO of CYP3A4 metabolism of A & B) oxycodone to noroxycodone, C & D) racemic methadone to R-EDDP, and E & F) racemic methadone to S-EDDP. (Left - mean "ln % remaining activity" versus "pre-incubation time" where slope = 1Kobs; Right - nonlinear regression of "Kobs" versus "inhibitor concentration").

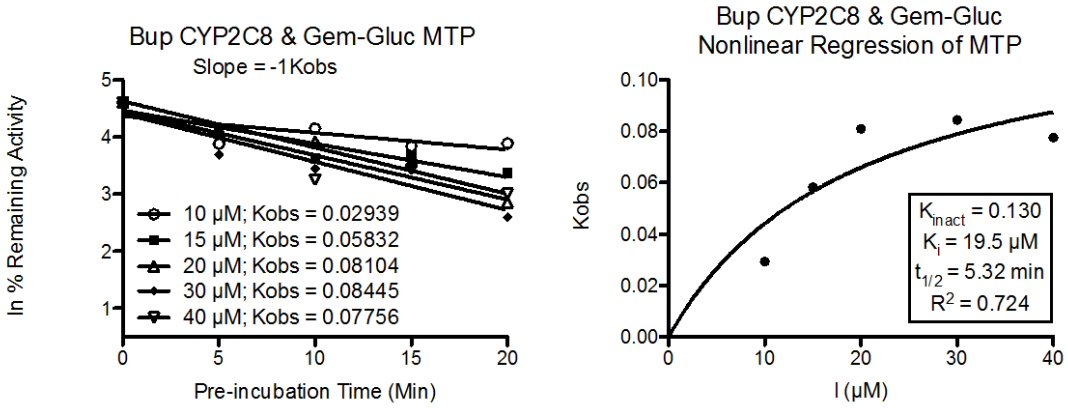

Figure 13. Determination of kinetics for MBI by gemfibrozil glucuronide (Gem-Gluc) of CYP2C8 metabolism of buprenorphine. (see Figure 12 for Left and Right chart definition)

3.4.2 Determining TDI kinetics for Cimetidine and Different CYP-mediated Pathways

With buprenorphine assays now optimized at 20 µM substrate concentration, the effect of the H_2-receptor antagonists on buprenorphine metabolism in HLM was now determined (Figure 14). Significant inhibition was found with cimetidine and famotidine, with little or none seen with nizatidine and ranitidine, as previously seen for oxycodone and methadone metabolism with substrate at 2.0 µM [44]. In this experiment, we found evidence for TDI by cimetidine. The inhibition of oxycodone and methadone metabolism by cimetidine in HLM was then repeated but with substrate at 20 µM (Figure 15). Evidence for TDI was seen for all pathways, except oxymorphone formation from oxycodone.

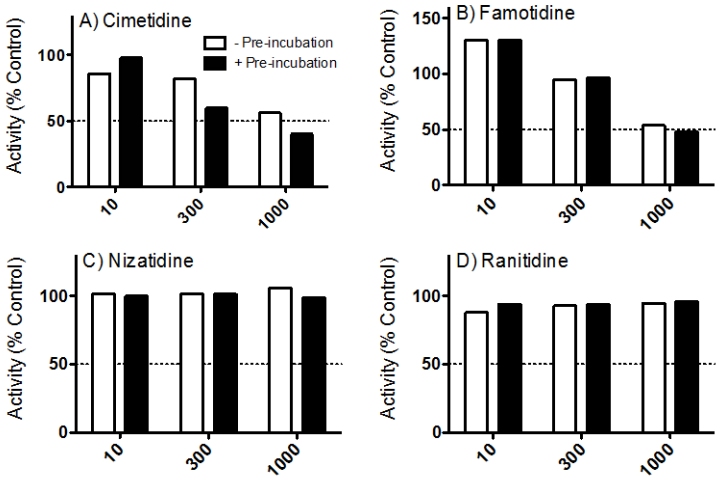

Figure 14. Inhibition of buprenorphine (20-µM) metabolism in HLM by the H_2-receptor antagonists ± 15 minute pre-incubation with A) cimetidine, B) famotidine, C) nizatidine and D) ranitidine.

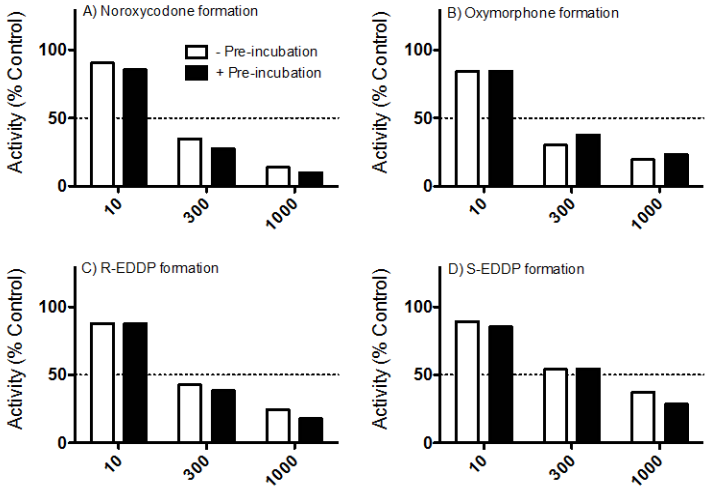

Figure 15. Inhibition of 20-µM oxycodone metabolism to A) noroxycodone and B) oxymorphone, and 20-µM methadone metabolism to C) R-EDDP and D) S-EDDP in HLM ± 15 minute pre-incubation with cimetidine.

Based on these indications of TDI with most pathways, we have used the dual incubation system to study TDI by cimetidine (Figure 16). Studies have been completed for buprenorphine metabolism by CYP3A4 and 2C8, methadone metabolism by CYP3A4, and oxycodone metabolism by CYP3A4 and 2D6. Forthcoming studies will determine the kinetics for CYP2B6 metabolism of methadone.

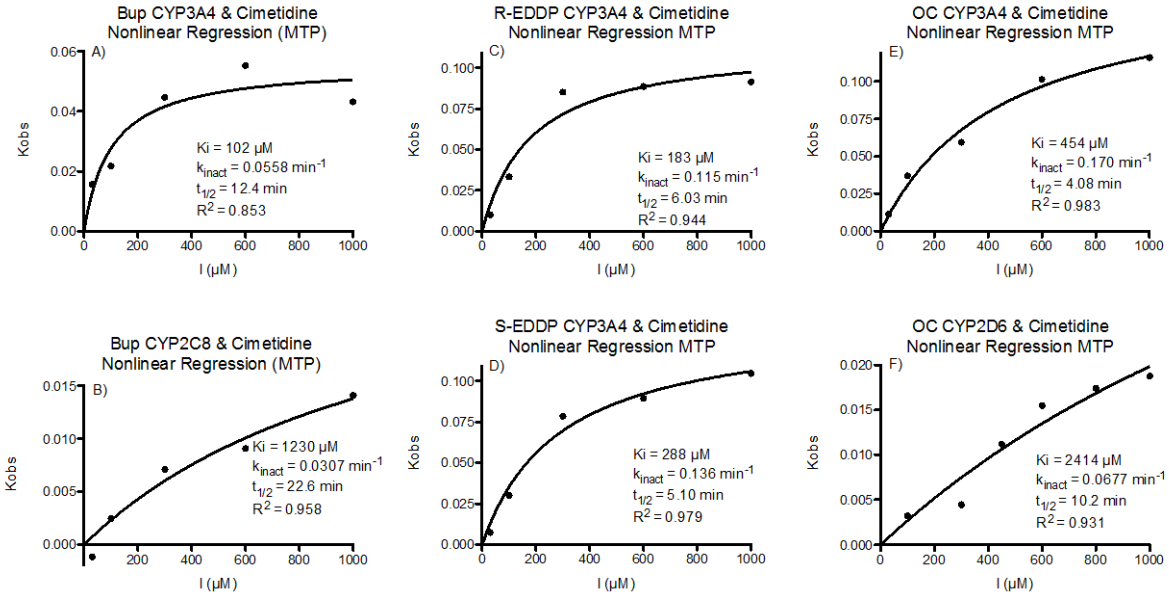

Figure 16. Kobs versus inhibitor concentration nonlinear regression plots for TDI by cimetidine on A) CYP3A4 metabolism of buprenorphine, B) CYP2C8 metabolism of buprenorphine, C) CYP3A4 metabolism of methadone to R-EDDP and D) CYP3A4 metabolism of methadone to S-EDDP, E) CYP3A4 metabolism of oxycodone and F) CYP2D6 metabolism of oxycodone (N ≥ 3).

3.4.3 TDI and PPIs

Our research with PPIs and buprenorphine metabolism unveiled some previously unseen potential for TDI of CYP3A4 mediated metabolism (Figure 17). This was not seen in our earlier work with the effect of PPIs on methadone and oxycodone metabolism possibly because these studies were done at a much lower substrate concentration. TDI can be masked at lower substrate concentrations because the reversible inhibitors are able to effectively compete for the sites of metabolism.

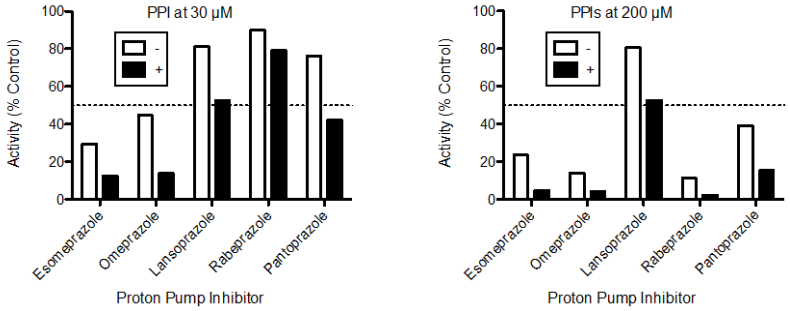

Figure 17. The effect of PPIs on metabolism of buprenorphine in HLM with PPIs at 30 and 200 µM with or without 15 minute pre-incubation.

Although the pre-incubation findings dictated the need to perform the TDI dual incubations, it is also important to have the reversible inhibition data for comparison to the other drugs. Experiments to determine the IC_{50} values for the PPIs with buprenorphine metabolism by CYP3A4 and 2C8 are shown in Figure 18.

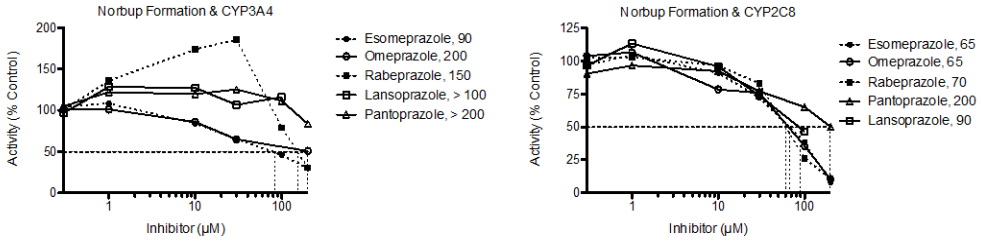

Figure 18. IC_{50} determination for PPI inhibition of buprenorphine metabolism to norbuprenorphine by A) CYP3A4 and B) CYP2C8 (IC_{50} results listed in figure key).

We then proceeded to perform the dual incubation TDI studies for the PPIs with CYP3A4-mediated buprenorphine metabolism. We were somewhat puzzled to find that only rabeprazole produced the expected time- and concentration-dependent inhibition that one would suspect (Figure 19). With other PPIs the results did not show concentration and pre-incubation time dependent loss of activity (not shown).

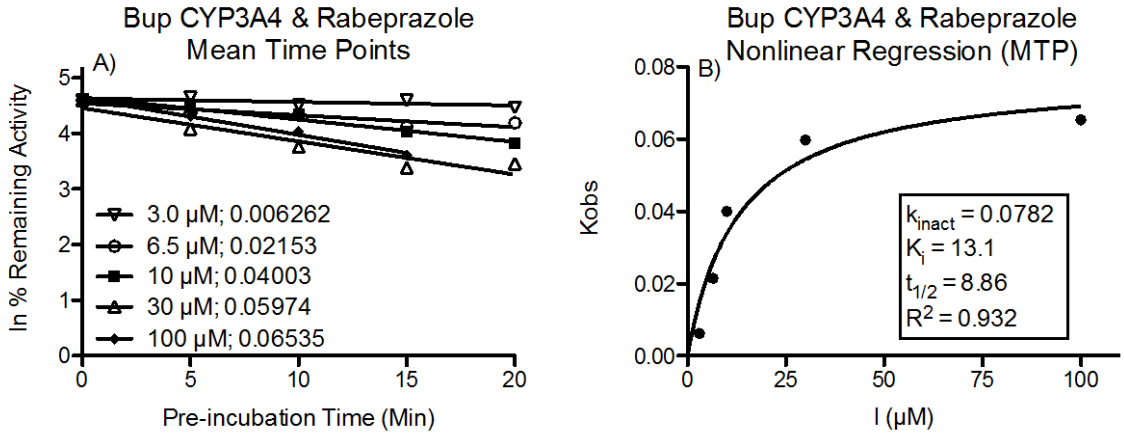

Figure 19. Time versus concentration plots for buprenorphine metabolism by CYP3A4 in the secondary incubation, where CYP3A was incubated with the noted rabeprazole in the primary incubation: A) residual activity plots to determine K_{obs}, and B) K_{obs} versus concentration plot for rabeprazole. (Results are from duplicate incubations in multiple experiments at N=3-4 for each concentration).

Considering the possibility that the TDI we saw in the HLM screen (Figure 17 above) was due to an effect on CYP2C8-mediated metabolism of buprenorphine, we conducted similar experiments with CYP2C8. Once we saw similar negative results with omeprazole and esomeprazole (data not shown), we stopped pursuing this line of investigation.

These findings were somewhat puzzling. We therefore went back to the HLM and performed the dual incubation experiments with this source of enzymes. Here we found concentration- and time-dependent inhibition of buprenorphine metabolism (Figure 20). Our rCYP450s are harvested from cells that express a single form of CYP450, such as 3A4, 2B6 or 2D6. HLM on the other hand contain the whole complement of CYP450s normally found in that part of the liver cell. This suggested that some interaction may be occurring between different CYP450s to cause this particular TDI.

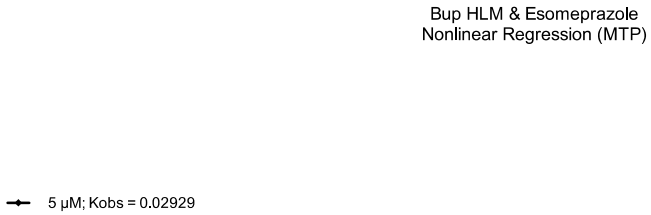

Figure 20. Pre-incubation time versus concentration (dual incubation) plots for buprenorphine metabolism by HLM with esomeprazole as inhibitor. (Results for are from duplicate incubations in multiple experiments at N=3-4 for each concentration).

These findings have lead to the hypothesis that metabolites of PPIs contribute to the inhibition of CYP3A4 metabolism. Commercially available PPI metabolites were acquired and we

commenced to test them as inhibitors of CYP3A4 and 2C8 metabolism of buprenorphine. These were conducted using our IC_{50} protocol ± a 15-minute pre-incubation of CYP3A4 or 2C8 with the tested metabolite. Examples of these results in IC_{50} format are presented in Figure 21.

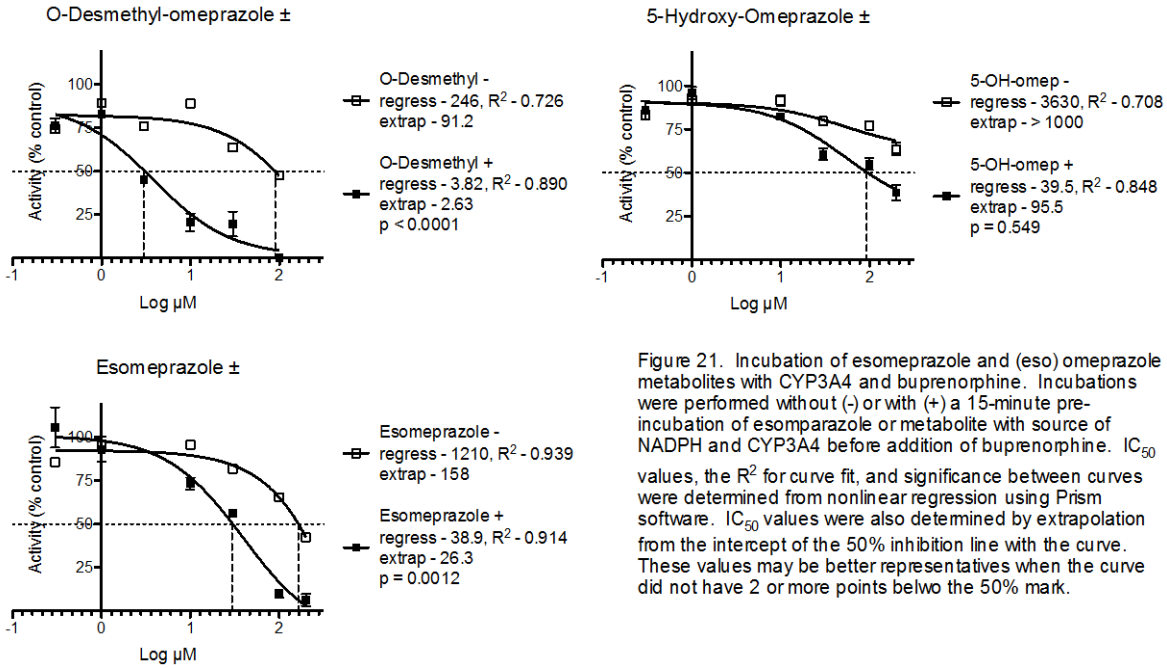

Figure 21. Incubation of esomeprazole and (eso) omeprazole metabolites with CYP3A4 and buprenorphine. Incubations were performed without (-) or with (+) a 15-minute pre-incubation of esomparazole or metabolite with source of NADPH and CYP3A4 before addition of buprenorphine. IC_{50} values, the R^2 for curve fit, and significance between curves were determined from nonlinear regression using Prism software. IC_{50} values were also determined by extrapolation from the intercept of the 50% inhibition line with the curve. These values may be better representatives when the curve did not have 2 or more points belwo the 50% mark.

The inhibitory effect of these metabolites compared to parent PPI compounds is shown in Table 9. Fairly potent inhibition of CYP3A4 is found for the O-desmethyl metabolite of omeprazole, the 5-hydroxy metabolite of lansoprazole and the sulfone N-oxide metabolite of pantoprazole, the former being significantly enhanced by pre-incubation. Under these conditions the inhibition

Table 9. IC_{50} Determination for Proton Pump Inhibitors (PPIs) and Their Metabolites on Buprenorphine Metabolism: Effect of Pre-incubation with Inhibitor

PPI or PPI Metabolite	CYP3A4 IC_{50} (µM) - Preinc	CYP3A4 IC_{50} (µM) + Preinc	CYP3A4 p-value	CYP2C8 IC_{50} (µM) - Preinc	CYP2C8 IC_{50} (µM) + Preinc	CYP2C8 p-value
Esomeprazole	158	26.3	0.0012	166	83.2	NS
Omeprazole	123	22.8	0.0414	53.0	84.7	NS
5-OH-Omeprazole	> 1000	95.5	NS	> 200	> 200	NS
Omeprazole sulfone	200	> 200	NS	77.6	132	NS
O-Desmethyl-Omeprazole	91.2	3.82	< 0.0001	> 200	115	NS
Lansoprazole	> 200	100	NS	> 200	> 200	NS
5-OH-Lansoprazole	50.9	9.05	< 0.0001	33.0	17.6	NS
Pantoprazole	> 200	> 200	NS	> 200	> 200	NS
Pantoprazole Sulfone N-oxide	29.4	64.6	NS	158	> 200	NS
Rabeprazole	120	8.63	0.0475	15.0	17.0	NS
Rabeprazole sulfide	> 200	> 200	NS	97.7	112	NS
NS – not significant						

of CYP3A4 by omeprazole, esomeprazole and rabeprazole was enhanced by pre-incubation. The hydroxyl metabolite of lansoprazole and rabeprazole itself were the only potent (IC_{50} < 50 µM) inhibitors of CYP2C8 (Table 9). The enhancement of inhibition by pre-incubation suggests that metabolites generated by CYP3A4 may also contribute, or are consistent with CYP3A4 being able to also, but in a more limited fashion, produce metabolites normally attributed to CYP2C19. Since these experiments use only a single incubation with enzyme, substrate and inhibitor combined, the effect of both reversible and irreversible inhibitors is measured (i.e., reversible inhibitors are not diluted out due to dilution of primary incubation aliquot into secondary incubation).

Studies on the contribution of metabolites to reversible and irreversible inhibition have grown over the past few years. One example is the inhibition of CYP2D6 metabolism by bupropion where bupropion metabolites have been shown to have a contributory effect [70]. In work partially funded by this grant we have presented a case study on a moderate but insidious inhibition of the CYP2D6 substrate metoprolol by bupropion [71].

3.4.4 Establishing MBI

Initial protocols to establish mechanism-based inhibition have now been established as shown for cimetidine and rabeprazole in Figure 22. In brief, the control contains CYP3A4 and NADPH generating system, but no inhibitor in the primary incubation and shows buprenorphine metabolism when a 10-fold diluted aliquot is transferred to the secondary incubation system with the substrate buprenorphine and additional NADPH generating system. Cimetidine (right) or rabeprazole (left) or TAO, a positive control for MBI of CYP3A4 included in both experiments, produce about 50% loss of enzyme activity at the concentrations used (i.e., their metabolism inactivates the enzyme, so reduced activity is measured when the enzyme is incubated with substrate and the inhibitors have been

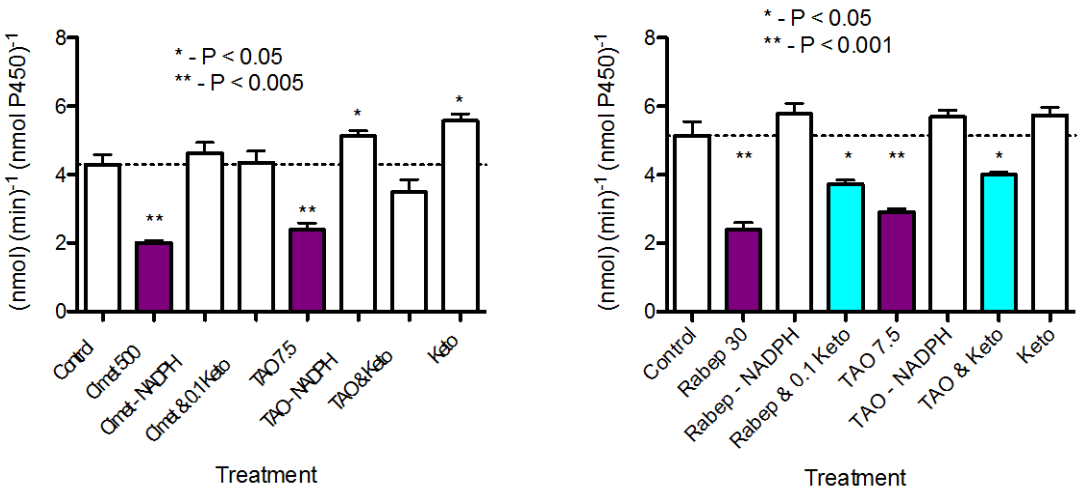

Figure 22. Effect of cimetidine, troleandromycin and ketoconazole, when pre-incubated with CYP3A4 and an NADPH generating system before dilution into a secondary incubation system, on metabolism of buprenorphine in the secondary system.

diluted 10-fold). No enzyme activity is lost when NADPH is omitted from the primary incubation system, which establishes that this is an NADPH driven reaction. Enzyme loss is eliminated for cimetidine, but only lessened for the more potent inhibitors TAO and rabeprazole when co-incubated with the CYP3A4 reversible inhibitor ketoconazole. The concentration of ketoconazole was chosen as one close to its IC_{50}, so when diluted 10-fold it has little or no inhibitory effect. This was established when ketoconazole at this concentration (0.1 μM) is added to the primary incubation system without another inhibitor (last bar), there is no damage to the enzyme and the ketoconazole is diluted sufficiently to not cause inhibition in the secondary incubation system.

Additional protocols will address the effect of co-incubation with radical scavengers and potassium ferricyanide to test for metabolic complex inhibitors. Other protocols will test the reversibility of the reaction by ultracentrifugation and re-suspension of the primary incubation, and the potential for MIC formation by potassium ferricyanide [41].

4.0 Conclusions

4.1 Summary of Accomplishments

We have made considerable progress on achieving our specific aims. We have completed optimization of the incubation conditions in HLM (Aim 1A), and have established positive TDI/MBI controls for about 2/3 of the metabolic pathways we are studying (Aim 1B). The HLM screen has now been performed for three classes of inhibitors, the H_2-receptor antagonists, the PPIs and the azole antifungal agents (Aim 2). IC_{50} values have been determined for the respective pathways and those of significance have been extrapolated to estimate in vivo potency (Aim 3). We have found evidence for TDI inhibition with cimetidine and a number of PPIs and are well underway in determining mechanisms of TDI, which include MBI for cimetidine and rabeprazole, and contribution of inhibitor metabolites for some of the PPIs. Five manuscripts related to these studies have already been published and our results have been presented in five platform and 4 poster presentations to forensic toxicologists and related disciplines (see section 7.0 for details).

4.2 Implications for Policy and Practice

4.2.1 What is the impact of the project on the criminal justice system?

The impact of drug interactions spreads beyond the field of forensic toxicology to encompass general medicine, pharmacy and other areas involved in drug use and dispensing. These can all directly and indirectly impact the criminal justice system, as many in these professions are called upon as consultants and expert witnesses for cases going through the criminal justice system. We anticipate that information we provide on potential drug interactions with methadone, buprenorphine and oxycodone is of benefit in these fields.

4.2.2 How has it contributed to crime laboratories?

At this early stage of the project our impact has been modest. We would hope that our presentations to the forensic toxicology community, along with publications, have provided some thought among the audience of the potential impact drug interactions may have on the toxicity of other drugs. As crime laboratory personal often testify in regard to plasma concentrations of drugs, these findings will hopefully enhance the interpretation of these testimonies.

4.3 Implications for Further Research

We will focus on further studies on TDI by cimetidine and some of the PPIs. This will include follow-up experiments listed in our revised aims. Experiments will be included with positive controls for TDI. The initial experiments for all three of these areas have been discussed above. With further 3-year funding now awarded from NIJ, we will continue these experiments. We will next screen another set of inhibitor class listed in Table 1. We will continue in this manner, either simply determining the IC_{50} values and extrapolating to in vivo potency if there is no evidence of TDI, or testing for mechanisms if evidence of TDI is found. We have now set in place most of the protocols to perform these studies, and look forward to continuing our progress in determining the potential for inhibition of the metabolism of the opioids, buprenorphine, methadone and oxycodone.

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6.0 Training and Professional Development Opportunities

While training and professional development are not stated aims of the project, it is a scientific study being undertaken in a collaborative fashion by 3 scientists with different levels of experience and expertise. In such an environment, training and professional development is an unwritten outcome of the collaboration. The major activity has been the training of Fenyun in the LC-MS/MS and HLM and CYP incubation techniques. Her training in this area was recognized at the 2014 Annual meeting of SOFT where Ms. Liu was awarded the Young Scientist Meeting Award (YSMA). As mentioned above, we have had the opportunity to interact and train two undergraduate students. Kimberly Kalp spent about 3 months with the group in the summer of 2014; Caleb Ham has now received an Undergraduate Research Opportunities Program (UROP) award to work with our group, and is pursuing experiments under the tutelage of Drs. Moody and Fang.

Professional development has occurred for all 3 scientists in literature review of the inhibitors being studied and discussions about such and the mechanisms of inhibition. Our department and other departments within the University offer weekly seminars during the fall and spring semesters. The PI attends most of the departmental seminars and a few extra-departmental seminars. Staff members attend very specific seminars, 1 or 2 per year.

7.0 Dissemination of Results

Publications (PDFs for these publications have been provided in previous reports and are not attached)

Moody, D.E. Metabolic and toxicologic considerations of opioid replacement therapy and analgesic drugs: methadone and buprenorphine. *Exp. Opin. Drug Metab. Toxicol.* **9**: 675-697, 2013.

Fang, W.B., Lofwall, M.R., Walsh, S.L. and Moody, D.E. Determination of oxycodone, noroxycodone and oxymorphone by high performance liquid chromatography-electrospray ionization -tandem mass spectrometry in human matrices: In vivo and in vitro applications. *J. Anal. Toxicol.* **37**: 337-344, 2013.

Moody, D.E., Liu, F., and Fang, W.B. In vitro inhibition of methadone and oxycodone cytochrome P450-dependent metabolism: Reversible inhibition by H_2-receptor agonists and proton pump inhibitors. *J. Anal. Toxicol.* **37**: 476-485, 2013.

Moody, D.E. and Backman, R.L. Metoprolol and bupropion interaction: A case study. *Clin. Exp. Pharmacol.* **4:** 165-166, 2014.

Moody, D.E., Liu, F., and Fang, W.B. Azole antifungal inhibition of buprenorphine, methadone and oxycodone in vitro metabolism. *J. Anal. Toxicol.* **39**: 374-386, 2015.

Oral Presentations

Moody, D.E. Invited Speaker, Prediction of Drug Interactions with Methadone, Buprenorphine and Oxycodone from In vitro Inhibition of Metabolism; *Presented at NIJ Conference*, Arlington, VA. June 19, 2012.

Moody, D.E., Fang, W.B. Prediction of drug interactions with methadone, buprenorphine and oxycodone from in vitro inhibition of metabolism: Study design. *Presented at the 42nd Annual Meeting of the Society of Forensic Toxicologist's*, Boston, MA, July 1-6, 2012.

Moody, D.E. Invited Speaker, "In vitro Inhibition of Oxycodone Oxidative Metabolism by H_2-Antagonists and Proton Pump Inhibitors"; *Presented at The 2013 NIJ Grantees Seminar, February 19, 2013* "The Research Behind Eureka! - How NIJ Funded Research Supports the Science of Forensics" in conjunction with *the 65th Annual Meeting of the American Academy of Forensic Sciences, Washington, DC, February 18-23, 2013.* (Note: this presentation was also provided by live on-line broadcast on June 6 and June 13, 2013).

Liu, F., Fang, W.B., Moody, D.E. In vitro reversible inhibition of oxycodone cytochrome P450-dependent metabolism by azole antifungal agents. (Young Investigator Awardee Presentation). *Presented at the 44th Annual Meeting of the Society of Forensic Toxicologist's*, Grand Rapids, MI, October 20-24, 2014.

Moody, D.E., Liu, F., Fang, W.B. Inhibition of buprenorphine metabolism by proton pump inhibitors: enigmatic time-dependent effect of esomeprazole. *Presented at the 44th Annual Meeting of the Society of Forensic Toxicologist's,* Grand Rapids, MI, October 20-24, 2014.

Poster Presentations

Moody, D.E., Liu, F., Fang, W.B. In vitro inhibition of buprenorphine cytochrome P450-dependent metabolism by H_2-receptor antagonists: Cimetidine is a time-dependent inhibitor. *Presented at the 43nd Annual Meeting of the Society of Forensic Toxicologist's,* Orlando, FL, October 28 – November 1, 2013.

Liu, F., Fang, W.B., Moody, D.E. In vitro inhibition of methadone and oxycodone cytochrome P450-dependent metabolism: Reversible inhibition by H_2-receptor agonists and proton pump inhibitors. *Presented at the 43nd Annual Meeting of the Society of Forensic Toxicologist's,* Orlando, FL, October 28 – November 1, 2013.

Moody, D.E., Liu, F., Fang, W.B. Inhibition of buprenorphine metabolism by proton pump inhibitors: enigmatic time-dependent effect of esomeprazole. *Presented at Experimental Biology 2014,* San Diego, CA, April 26-30, 2014.

Moody, D.E., Fang, W.B., Liu, F. Involvement of proton pump inhibitor metabolites in the inhibition of buprenorphine metabolism. *Presented at the 45th Annual Meeting of the Society of Forensic Toxicologist's,* Atlanta, GA, October 19-23, 2015.