Opioid-related mortality rates have escalated. Drug interactions may increase blood concentrations of the opioid. We therefore used human liver microsomes (HLMs) and cDNA-expressed human cytochrome P450s (rCYPs) to study in vitro inhibition of buprenorphine metabolism to norbuprenorphine (CYP3A4 and 2C8), oxycodone metabolism to noroxycodone (CYP3A4 and 2C18) and oxymorphone (CYP2D6), and methadone metabolism to R- and S-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP; CYP3A4 and 2B6). In this study, we have examined the inhibitory effect of 12 (mostly antifungal) azoles. These compounds have a wide range of solubility; to keep organic solvent ≤1%, there was an equally wide range of highest achievable concentration for non-CYP3A4 pathways. IC₅₀ values (μM) were determined for most CYP3A4 pathways (ranges) and other pathways as dictated by screen results: clotrimazole (0.30 – 0.35; others >30 μM); econazole (2.2 – 4.9; 2B6 R-EDDP – 9.5, S-EDDP – 6.8; 2C8 – 6.0; 2C18 – 1.0; 2D6 – 1.2); fluconazole (7.7 – 66; 2B6 – 313, 361; 2C8 – 1240; 2C18 – 17; 2D6 – 1000); itraconazole (2.5 to >5; others >5); ketoconazole (0.032 – 0.094; 2B6 – 12, 31; 2C8 – 78; 2C18 – 0.98; 2D6 – 182); miconazole (2.3 – 7.6; 2B6 – 2.8, 2.8; 2C8 – 5.3; 2C18 – 3.1; 2D6 – 5.9); posaconazole (3.4 – 20; 2C18 – 3.8; others >30); terconazole (0.48 to >10; 2C18 – 8.1; others >10) and voriconazole (0.40 – 15; 2B6 – 2.4, 2.5; 2C8 – 170; 2C18 – 13; 2D6 >300). Modeling based on estimated Kᵢ values and plasma concentrations from the literature suggest that the orally administered azoles, particularly ketoconazole and voriconazole, have the greatest potential for inhibiting CYP3A4 pathways, as does voriconazole for the CYP2B6 pathways. Azoles used for mucosal and topical applications had no significant effect. Azoles were next screened at the highest achievable concentration for non-CYP3A4 pathways. IC₅₀ values (μM) were determined for most CYP3A4 pathways (ranges) and other pathways as dictated by screen results: clotrimazole (0.30 – 0.35; others >30 μM); econazole (2.2 – 4.9; 2B6 R-EDDP – 9.5, S-EDDP – 6.8; 2C8 – 6.0; 2C18 – 1.0; 2D6 – 1.2); fluconazole (7.7 – 66; 2B6 – 313, 361; 2C8 – 1240; 2C18 – 17; 2D6 – 1000); itraconazole (2.5 to >5; others >5); ketoconazole (0.032 – 0.094; 2B6 – 12, 31; 2C8 – 78; 2C18 – 0.98; 2D6 – 182); miconazole (2.3 – 7.6; 2B6 – 2.8, 2.8; 2C8 – 5.3; 2C18 – 3.1; 2D6 – 5.9); posaconazole (3.4 – 20; 2C18 – 3.8; others >30); terconazole (0.48 to >10; 2C18 – 8.1; others >10) and voriconazole (0.40 – 15; 2B6 – 2.4, 2.5; 2C8 – 170; 2C18 – 13; 2D6 >300). Modeling based on estimated Kᵢ values and plasma concentrations from the literature suggest that the orally administered azoles, particularly ketoconazole and voriconazole, have the greatest potential for inhibiting CYP3A4 pathways, as does voriconazole for the CYP2B6 pathways. Azoles used for mucosal and topical applications did not exceed the modeling threshold.

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 (1–4). 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 (5–8). 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 (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 more potent as a mu-opioid receptor agonist (20), whereas both R- and S-enantiomers are NMDA receptor antagonists (21). S-Methadone is the more potent blocker (≈2.5–3.5 ×) of the human ether-a-go-go-related gene (hERG) K⁺ channels that are associated with a 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-diphenylpyrrolidine (EMDP). We (23, 24), and others (25–27), 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, whereas 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 not, or only slightly glucuronidated. Oxymorphone and noroxymorphone share mu-opioid receptor activity with oxycodone, whereas 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-1990s, at which time a number of studies with selective serotonin receptor inhibitors were published, and studies with antiretrovirals were just starting to appear (32). Since that time a focus on the antiretrovirals has been apparent 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; a few examples are provided here. Many involve the testing of prototypical inhibitors of CYP2D6 and 3A4 (33) to confirm the involvement of these enzymes in the two main pathways of metabolism. A few other classic drug interactants are: rifampin (34), St. John’s wort (35) and grapefruit juice (36); and more recently, the antiretrovirals ritonavir and lopinavir/ritonavir (37). Inducers of metabolism decrease the effectiveness of oxycodone, and 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 that 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 (HLMs) and cDNA-expressed CYP450s (rCYPs). We initiated our studies with two groups of widely used compounds with varying histories of drug interactions, i.e. the H2-receptor antagonists and the proton pump inhibitors (38). While the in vitro inhibitory action of many of the compounds we study has been addressed previously in the literature, few previous studies compared large numbers of potential inhibitors. Differences in inhibition potency within each enzyme protein and incubation time to assure linearity of product formation and 10% substrate loss with substrate at 20°C are not provided. Incubation conditions were optimized for each pool does not reflect any one donor, donor demographics and liquid chromatography (LC) mobile phase was drawn from a Milli-Q filter apparatus (Millipore Corp., Billerica, MA, USA). Outdated human plasma was from the University of Utah blood bank. Silanized tubes were prepared by vapor-phase silanization using hexamethyldisilazane (Pierce, Rockford, IL, USA). Outdated human plasma was from the University of Utah blood bank.

**Materials**

Racemic methadone, racemic EDDP perchlorate, oxycodone, noroxycodone, oxymorphone, buprenorphine, norbuprenorphine and their deuterated internal standards were purchased from Cerilliant Corporation (Round Rock, TX, USA). Insect cell rCYPs 2B6, 2C8, 2C18, 2D6 and 3A4 (Supersomes) were purchased from BD Biosciences (Franklin Lakes, NJ, USA); all had co-expressed NADPH CYP reductase; rCYP2B6 and 3A4 also had co-expressed cytochrome b5, Albendazole, albendazole sulfide, clotrimazole, econazole, ketoconazole, itraconazole, miconazole, miconazole, posaconazole, terconazole, paroxetine, N,N′,N′′-trithylenetriphosphoramide (thioTEPA), troleandomycin, d-glucose 6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase, β-NADP sodium salt, EDTA disodium salt and MgCl2 were obtained from Sigma-Aldrich Chemical Corp. (St Louis, MO, USA). Fluconazole and voriconazole were obtained from Eurasia Chemicals (Mumbai, India). Gemfibrozil glucuronide was purchased from Toronto Research Chemicals (Toronto, Canada). Concentrated formic acid (88%), glacial acetic acid and ammonium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other solvents were HPLC-grade. Water used in the preparation of reagents; extraction and liquid chromatography (LC) mobile phase was drawn from a Milli-Q filter apparatus (Millipore Corp., Billerica, MA, USA). Outdated human plasma was from the University of Utah blood bank.

**Experimental methods**

**Incubations**

Incubations of buprenorphine, oxycodone or racemic methadone with HLMs and rCYPs were performed using a modification of our previously described incubation conditions (16, 43). HLMs were prepared using methods described by Nelson et al. (44), with the first centrifugation at 9,000 g the homogenization buffer containing 0.25 M sucrose and 10 strokes of homogenization. The protein content was determined by the method of Lowry et al. (45). HLMs were pooled from several sources. Since this pool does not reflect any one donor, donor demographics are not provided. Incubation conditions were optimized for enzyme protein and incubation time to assure linearity of product formation and <10% substrate loss with substrate at 20 μM. The optimization experiments and incubation conditions are discussed under results and presented in Supplementary Figures 1 and 2.
preincubation, inhibitor and the NADPH-GS were added, samples were incubated at 37°C for 15 min in a water bath shaker and then substrate was added. Time-dependent inhibition (TDI)–positive controls were included with HLM incubations. Troleandomycin at 5 μM was used with all three substrates as the CYP3A4-positive control; 10 μM thioTEPA, 0.5 μM paroxetine and 5 μM gemfibrozil glucuronide were included as the CYP2B6 (methadone), CYP2D6 (oxycodone) and CYP2C8 (buprenorphine)-positive controls, respectively (46, 47). Incubations were then started or continued for specified times, and were terminated by addition of 100 μL of ice-cold methanol and transfer of samples to an ice bath. All samples were prepared in duplicate.

Sample preparation and LC–electrospray ionization-tandem mass spectrometry (LC–ESI-MS-MS)

The enantiomer-specific method for methadone (48) and the method for oxycodone and metabolites (29) were performed as described previously; they are only briefly described at this time.

R and S-methadone and R- and S-EDDP determination used deuterated internal standards and liquid : liquid extraction with methyl t-butyl ether. The m/z 510 (MH⁺) to 265, m/z 313 (MH⁺) to 268, m/z 278 (MH⁺) to 234 and m/z 281 (MH⁺) to 234 selected ion transition monitoring were used to analyze methadone, methadone-d₃, EDDP and EDDP-d₃, respectively. Enantiomer separation was achieved with a chiral column (Chiral AGP 50 × 2 mm, 5 μm, currently from Chiral Technologies, Inc., West Chester, PA, USA) with a mobile phase gradient of methanol : 4:1 n-butyl chloride : acetonitrile. The method for buprenorphine and norbuprenorphine low (0.250 ng/mL) 98.0%/8.2%, 106%/6.0%; medium (20 ng/mL) 101%/3.4%, 104%/2.9%; high (70 ng/mL) 101%/3.1%, 95.1%/2.4%; R and S-methadone, R-EDDP and S-EDDP low (5.0 ng/mL) 95.6%/11.7%, 96.0%/11.0%, 104%/14.2%, 104%/13.5%; medium (100 ng/mL) 101%/5.9%, 101%/5.9%, 102%/13.7%, 103%/12.6%; high (425 ng/mL) 99.3%/4.3%, 98.8%/4.0%, 101%/6.7%, 102%/7.4%; oxycodone, noroxycodone and oxymorphone low (0.60 ng/mL) 100%/7.5%, 100%/11.2%, 95.8%/6.6%; medium (10.0 ng/mL) 96.9%/2.9%, 96.9%/3.7%, 93.8%/3.5%; high (200 ng/mL) 96.5%/4.1%, 99.5%/4.0%, 97.5%/4.1%.

Results

Enzyme assay optimization

Assays were optimized to meet three criteria recommended by the United States Food and Drug Administration for in vitro inhibition studies: substrates were kept below Km (to the extent possible), substrate loss did not exceed 10% and product formation was linear over the time of incubation. In addition, experiments were performed to test for TDI (49). In our previous study, oxycodone and methadone metabolism were optimized at 2 μM using 0.5 mg/mL of HLMs (38). We found that buprenorphine at this concentration was metabolized rapidly; a concentration of 20 μM with HLM concentration reduced to 0.05 mg/mL was required to achieve optimal incubation conditions with a 10-min incubation; protein content of CYP3A4 and 2C8 were likewise optimized to 2.5 and 10 pmol/mL with 10 min incubations (Supplementary Figure 3). Methadone and oxycodone incubation conditions were therefore also adjusted to use of substrate at 20 μM with appropriate reduction in enzyme protein and selection of incubation time (Supplementary Figure 4). Methadone incubations were for 15 min with HLM, CYP3A4 and CYP2B6 at 0.2 mg/mL, 5 pmol/mL and 10 pmol/mL. Oxycodone incubations with HLM were for 60 min at 0.3 mg/mL; those with CYP3A4, 2D6 and 2C18 were for 30 min with 5, 5 and 25 pmol/mL (see also Supplementary Table IV).

The use of substrate at 20 μM (i.e., 6,200 to 9,360 ng/mL) had an impact on the mass spectrometric conditions. With substrate at such high concentration, it was not possible to keep both substrate and metabolite within the linear range of the assay. Only the metabolite was quantitated. The substrate peak area ratio was evaluated to assure no large fluctuations. The linear range for the metabolite was dictated in part by incubation volume (e.g., for norbuprenorphine, the 0.2-mL incubate was calculated as a 5-fold dilution and the analytical range thereby adjusted from 0.1–100 to 0.5–500). This also resulted in background detection of metabolites (Supplementary Figure 5) ranging from 0.006 to 0.48% of substrate added. Background samples that contained only substrate at 20 μM were routinely included with all batches, and the amounts detected subtracted from study samples.
Impact of azole polarity

It is also highly recommended that in vitro inhibition studies keep the final concentration of organic solvent ≤1% (49). 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 1).

Azoles in HLM with or without preincubation

The 11 azole drugs, albendazole, clotrimazole, econazole, fluconazole, itraconazole, ketoconazole, metronidazole, miconazole, posaconazole, terconazole and voriconazole, and the metabolite of albendazole, albendazole sulfoxide, were first incubated in HLM with either buprenorphine, racemic methadone or oxycodone, with or without a 15-min preincubation in the presence of inhibitor and NADPH-GS (Figure 2). Controls included those with no inhibitor, those with no inhibitor plus 1% DMSO (solvent control) (Supplementary Figure 6) and positive controls for TDI (Figure 3). Preincubation always lead to significantly lower control activity; addition of 1% DMSO had variable effects (Supplementary Figure 6). These controls were not pooled. Most azole compounds caused >50% inhibition for norbuprenorphine, R- and S-EDDP as well as noroxycodone formation. Metronidazole and albendazole (data not shown) were notable exceptions (Figure 2). Only econazole and miconazole produced >50% inhibition of oxymorphine formation in HLM. Positive controls were indicative of TDI for CYP3A4, 2D6 and 2B6 reactions (Figure 3). Only posaconazole incubated with methadone demonstrated TDI (Figure 2). We have not further explored mechanisms for this TDI.

Screen for rCYP inhibition

CYP3A4 is the major CYP present in HLM, and the magnitude of inhibition found in HLM is a good indicator of inhibition of CYP3A4. All azoles were selected for IC50 determinations with CYP3A4 pathways according to the findings in Figure 2, except that albendazole sulfoxide and metronidazole were first screened using CYP3A4 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 a maximum concentration of azole to determine which would require IC50 determinations (Figure 4). Based on these studies, we decided that CYP3A4 IC50 determinations would include albendazole sulfoxide with methadone; CYP2B6, 2C8, 2C18 and 2D6 IC50 determinations would include econazole, fluconazole, ketoconazole, miconazole, and voriconazole (except 2D6); and CYP2C18 IC50 determinations would also include terconazole and posaconazole (Figure 4).

IC50 determinations for azoles in rCYPs

IC50 determinations for selected azoles were carried out as described above. rCYPs were incubated with the selected substrates and azoles at six concentrations (each in duplicate) based on the findings with the screen and the upper limit of solubility of the compound. For most compounds, the IC50 was determined using nonlinear regression (see Supplementary Figure 7 for examples). For a few azoles, extrapolation from where the curve intercepted the 50% inhibition point was used. A summary of the IC50 determinations for each CYP-dependent pathway is presented in Table I. Comparison of the IC50 determinations for the CYP3A4 pathways is shown in Figure 5. In general, there was a fair agreement in the rankings. In about one-half of the comparisons, the IC50 determinations are inversely proportional to the reported $K_m$ for the respective pathways, with oxycodone highest, methadone intermediate and buprenorphine lowest (15, 28, 50), as expected for competitive inhibition.

Estimates of in vivo potency

One of the simpler equations to extrapolate in vitro to in vivo inhibition is:

$$\frac{\text{AUC}_i}{\text{AUC}_n} = 1 + \frac{[I]}{K_i}$$

(1)

where $\text{AUC}_i$ and $\text{AUC}_n$ are the area under the time versus plasma concentration curve with ([I]) and without ([n]) inhibitor, $[I]$ is the in vivo concentration of inhibitor and $K_i$ is the inhibition constant. Ratios of $\text{AUC}_i/\text{AUC}_n \geq 2$ are considered to be potentially significant inhibitions. This equation is discussed in numerous articles; Mao et al. (51) is a recent example that studied some azoles along with other inhibitors. When one assumes competitive inhibition, the $K_i$ can be estimated from the IC50 using the Cheng-Prusoff equation (52):

$$K_i = \frac{\text{IC50}}{1 + S/K_m}$$

(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 calculated IC50 values (Table II), 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.

Graphical representations:

Figure 1. Plot of upper concentration of inhibitor used to not exceed 1% DMSO in incubation versus log of the octano-water partition coefficient (X Log P3). X Log P3 values are from PubChem.
Figure 2. Inhibition of metabolism of buprenorphine to norbuprenorphine (Norbup), methadone to R-EDDP (R-EDDP) and S-EDDP (S-EDDP), and oxycodone to noroxycodone (Norox) and oxymorphone (Oxymor). HLMs were incubated with three concentrations of antifungal azole either without (open bars) or with a 15-min preincubation of HLM with an inhibitor and an NADPH-GS. A crude approximation of an IC_{50} was made and is shown next to the respective condition. Albendazole and metronidazole did not exceed 50% inhibition and are not shown.
metabolism of oxycodone (Table II). When equation (1) is then used, we find 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 is also seen with a number of the CYP3A4 pathways, as well as ketoconazole and CYP2B6 metabolism (Table II). 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 10-fold to mimic a serious overdose situation (Table II).

Several modifications can be made to equation (1) as increasingly complex models are derived for in vitro to in vivo extrapolations. This is also discussed in numerous articles; again see Mao et al (51) for a recent example. 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 data are available in the literature (51), but (f_{u,HLM} or f_{u,rCYP}) for the azoles are not available. With the low protein content used in our incubations, non-specific binding is relatively reduced. Also, non-specific binding to membranes is often less than to plasma protein [e.g., we found that f_{u,HLM} for buprenorphine was 0.42, while f_{u,pl} is 0.01 (55)]. We therefore have used a hypothetical range of f_{u,rCYP} of 1.0–0.5. These unbound fractions have now been used to show how protein binding effects the ratios of AUC_{i}/AUC_{n} (Table III). When f_{u,pl} is small and f_{u,rCYP} is negligible, the ratio decreases dramatically. When f_{u,pl} ≈ f_{u,rCYP}, the ratio is essentially the same as when binding was not considered.

Discussion

The overriding aim of our studies is to characterize the inhibitory potential of groups of drugs based on their in vitro inhibition of metabolic pathways of oxycodone, methadone and buprenorphine. While many of the potential inhibitors we are studying have been characterized to a certain extent, this has generally been done using model substrates for various CYP gene products. Here, we are using mass spectrometric quantification to study specific pathways in the metabolism of opioids, which are among those contributing to a decade long epidemic of opioid-related fatalities (1–4).

At this time, we have now examined the in vitro effect of a group of anti-protozoan azoles on the metabolism of buprenorphine, methadone and oxycodone. The pathways studied include a CYP3A4-mediated N-dealkylation for each drug, a CYP2C8-, 2B6- and 2C18-mediated dealkylation of buprenorphine, methadone and oxycodone, respectively, and a CYP2D6-mediated O-demethylation of oxycodone (15, 16, 24, 27–29). This study included 11 azole drugs and 1 predominant metabolite. The focus was on the seven azoles administered by oral and/or iv routes, which are associated with 1–10 μM plasma concentrations; three representative azoles used to treat mucosal oral and/or vaginal cavities, which are associated with ≈20 nM plasma concentrations,

Figure 3. Effect of preincubation on inhibition by the positive controls 5 μM troleandomycin (Trol), 5 μM gemfibrozil glucuronide (Gem), 0.5 μM paroxetine (Parox) and 10 μM ThioTEPA.
Figure 4. Inhibition of the CYP450-mediated metabolism using the highest achievable concentration for non-CYP3A4 pathways, and highest concentration used in IC_{50} determinations for CYP3A4 pathways.
and one representative topical application azole, which is associated with 0.3–3 nM plasma concentrations (40–42).

In terms of exploring in vitro inhibition by both a number of azoles and a number of CYP pathways, this study exceeds previous studies. Zhang et al. studied eight CYP pathways, but only with clotrimazole and miconazole (56), Niwa et al. studied six CYP pathways, but with only four azoles (57, 58), Jeong et al. studied eight CYP pathways, but only with voriconazole (59), and Zientek et al. (60) studied five CYP pathways, but only with miconazole. Several studies have focused on the effect of selected azoles on single CYPs. A number of studies examined the effects of 3–4 azoles on CYP3A4; cumulatively, data on the effect of eight azoles on CYP3A4 have been reported (56, 58, 61–66). Additional studies have provided information on specific CYPs: four azoles with CYP2B6 (67), five azoles with CYP2C8 (68), six azoles with CYP2C9 (69) and five azoles with CYP19 (aromatase) (70).

Prior to starting experiments on inhibition, it was necessary to optimize incubation conditions to keep substrate loss < 10% and product formation linear over the time course of the incubation. While we found we could readily meet these conditions with a relatively low substrate concentration (2 μM at 0.5 mg/mL HLM) for oxycodone and methadone (38), the turnover rate for buprenorphine in HLMs or rCYPs was too high at this concentration. A 10-fold increase in substrate concentration along with a 10-fold decrease in protein was required to meet the substrate depletion criteria. To conduct oxycodone and methadone incubations at a comparable substrate concentration, HLM and rCYP protein concentrations were also lowered for these drugs. Reducing enzyme protein had the benefit of minimizing the impact of non-specific substrate binding to the microsomes (71, 72).

While we were able to keep the oxycodone and methadone concentrations considerably lower than their reported Km (28, 50), assuring minimal substrate depletion for buprenorphine resulted in a trade-off to use of a substrate concentration almost twice the reported Km values for CYP3A4 and 2C8 (15). As seen, this was reflected in generally higher IC50 values for inhibition of buprenorphine compared with oxycodone and methadone.

For the two most potent inhibitors, ketoconazole and clotrimazole, there was little difference among the IC50 values with buprenorphine compared with oxycodone and methadone.

We now report novel findings that a number of the azoles inhibit the orphan CYP2C18 with relative potency of ketoconazole > econazole > miconazole > posaconazole > terconazole > voriconazole > fluconazole. For CYP2D6, we found econazole > miconazole > ketoconazole > flavonazole. The potent inhibition of CYP2D6 by miconazole has been noted in other studies (56, 58, 60), as has the relative ineffectiveness of fluconazole (58). This is the first study to report an IC50 for CYP2D6 with econazole and ketoconazole.

Clinical studies have been performed studying drug interaction of anti-fungal azoles with oxycodone and methadone. The clinical studies performed with methadone were for interactions with fluconazole and voriconazole. With fluconazole, the change in racemic methadone AUC was 1.35 (73); this closely matches the extrapolated value for fluconazole and CYP3A4 (Table III). In our study, fluconazole was about a 15-fold more potent inhibitor of CYP3A4 than CYP2B6. It should be noted that Kharasch et al. (74, 75) have demonstrated examples where CYP3A4/5 is inhibited in vitro but methadone N-demethylation is not affected. These and other findings lead them to suggest that CYP2B6 is more important in controlling methadone clearance. While this is not clearly evident from the fluconazole study, the results from the interaction with voriconazole support a significant role for CYP2B6. In vitro inhibition by voriconazole has about an equal effect on CYP2B6 and CYP3A4. However, when voriconazole was co-administered with methadone, the change in AUC of R-methadone was only 1.37, while that of S-methadone was 2.16 (76). The greater inhibition of S-methadone clearance is consistent with a greater impact on CYP2B6.

In studies with oxycodone, the respective changes in plasma AUC after treatment with ketoconazole were 1.84, 0.86 and 3.46 for oxycodone, noroxycodone and oxymorphone, respectively (33). Another study reported a 2.46-fold increase in oxycodone plasma AUC (77). The respective changes in plasma AUC after treatment with voriconazole were 3.57, 0.52 and 6.97 for oxycodone, noroxycodone and oxymorphone, respectively (78). These changes are consistent with a strong inhibition of CYP3A4 with little or no effect on CYP2D6. The magnitude of the changes is relatively close to those seen in our extrapolations after accounting for unbound concentrations (Table III). The respective changes in plasma AUC after treatment with itraconazole were 2.25, 0.52 and 3.20 for oxycodone, noroxycodone and oxymorphone, respectively (79). This is also consistent with strong inhibition of CYP3A4, but was not consistent with our extrapolations. This may be explained, in part, as the metabolites of itraconazole have been found to contribute to its inhibition of CYP3A4 pathways (80). The respective changes in plasma AUC after treatment with miconazole oral gel were 1.64, 1.27 and 0.25 for oxycodone, noroxycodone and oxymorphone (81). This is consistent with a modest inhibition of both CYP3A4 and 2D6. While we found miconazole to be a relatively good inhibitor of both of these CYPs, the plasma concentrations reported following oral gel use are too low to indicate these amounts of inhibition with our extrapolations. An almost 1000-fold increase would be required to extrapolate the magnitude of inhibition seen in the Gronlund et al. study. Miconazole plasma concentrations were not reported in that study (81). While we note this discrepancy between anticipated miconazole plasma concentrations from use as an oral gel and the potential for interaction with oxycodone, the fact that an interaction may occur should not be
**Table I**

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

<table>
<thead>
<tr>
<th>Antifungal Azole</th>
<th>CYP pathways and products*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R-EDD/P</td>
</tr>
<tr>
<td><strong>Albendazole sulfoxide</strong></td>
<td>224&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Clotrimazole</strong></td>
<td>0.348</td>
</tr>
<tr>
<td><strong>Econazole</strong></td>
<td>2.38</td>
</tr>
<tr>
<td><strong>Fluconazole</strong></td>
<td>16.1</td>
</tr>
<tr>
<td><strong>Itraconazole</strong></td>
<td>2.48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Ketoconazole</strong></td>
<td>0.0793</td>
</tr>
<tr>
<td><strong>Miconazole</strong></td>
<td>&gt;500</td>
</tr>
<tr>
<td><strong>Methotrexate</strong></td>
<td>2.34</td>
</tr>
<tr>
<td><strong>Posaconazole</strong></td>
<td>3.44</td>
</tr>
<tr>
<td><strong>Terconazole</strong></td>
<td>1.70</td>
</tr>
<tr>
<td><strong>Voriconazole</strong></td>
<td>2.28</td>
</tr>
<tr>
<td><strong>Posaconazole</strong></td>
<td>14.6</td>
</tr>
</tbody>
</table>

*Pathways studied: CYP3A4 and 2B6—methadone conversion to R-EDD/P and S-EDD/P; CYP3A4 and 2C8—buprenorphine conversion to norbuprenorphine (Norbup); CYP3A4 and 2C19—oxycodone conversion to noroxycodone (Norox) and CYP2D6—oxycodone conversion to oxymorphone (Oxymor).

<sup>a</sup>Determined by extrapolation.

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**Table II**

Extrapolation of IC<sub>50</sub> Determinations to K<sub>i</sub> and Inhibitory Potential at Average and 10 x Average Plasma Concentration of Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>[I]p ([μM])&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ([μM])</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; ([μM])&lt;sup&gt;b&lt;/sup&gt;</th>
<th>AUC&lt;sub&gt;0&lt;/sub&gt;/AUC&lt;sub&gt;∞&lt;/sub&gt;</th>
<th>AUC&lt;sub&gt;0&lt;/sub&gt;/AUC&lt;sub&gt;∞&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ([μM])</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; ([μM])</th>
<th>AUC&lt;sub&gt;0&lt;/sub&gt;/AUC&lt;sub&gt;∞&lt;/sub&gt;</th>
<th>AUC&lt;sub&gt;0&lt;/sub&gt;/AUC&lt;sub&gt;∞&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluconazole</strong></td>
<td>4.24</td>
<td>7.69</td>
<td>7.44</td>
<td>1.57</td>
<td>6.70</td>
<td>1000</td>
<td>666</td>
<td>1.01</td>
<td>1.06</td>
</tr>
<tr>
<td><strong>Itraconazole</strong></td>
<td>1.86</td>
<td>3.62</td>
<td>3.70</td>
<td>1.50</td>
<td>6.03</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Ketoconazole</strong></td>
<td>0.793</td>
<td>0.0679</td>
<td>0.067</td>
<td>1.12</td>
<td>0.0853</td>
<td>0.0622</td>
<td>102</td>
<td>102</td>
<td>—</td>
</tr>
<tr>
<td><strong>Posaconazole</strong></td>
<td>0.202</td>
<td>0.303</td>
<td>0.293</td>
<td>1.10</td>
<td>1.99</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Miconazole</strong></td>
<td>0.024</td>
<td>0.373</td>
<td>3.61</td>
<td>1.01</td>
<td>1.07</td>
<td>5.89</td>
<td>3.92</td>
<td>1.01</td>
<td>1.06</td>
</tr>
<tr>
<td><strong>Terconazole</strong></td>
<td>0.0029</td>
<td>0.481</td>
<td>0.485</td>
<td>1.04</td>
<td>1.41</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>Econazole</strong></td>
<td>0.71</td>
<td>2.28</td>
<td>2.02</td>
<td>1.00</td>
<td>1.01</td>
<td>1.22</td>
<td>0.812</td>
<td>1.00</td>
<td>1.04</td>
</tr>
</tbody>
</table>

<sup>a</sup>Potassium concentrations ([K]+) for the oral azoles were taken from literature C<sub>max</sub> values as follows: albendazole sulfoxide (53), fluconazole, itraconazole and voriconazole (41), ketoconazole (40) and posaconazole (54); the mucosal formulations clotrimazole, miconazole and terconazole were calculated from an estimated C<sub>max</sub> of 10 ng/mL and the topical econazole from an estimated C<sub>max</sub> of 1.0 ng/mL.

<sup>b</sup>Extrapolated from the Cheng-Prusoff, equation, where K<sub>i</sub> = IC<sub>50</sub> ([I]/1 + S/K<sub>m</sub>), K<sub>m</sub> 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 (119) and 2B6 (112.8), (10) and buprenorphine by CYP3A4 (13.6) and 2D6 (12.4) (15).

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**Table III**

Haemodynamic changes during oral administration of the two drugs should be avoided. Indeed, with warfarin, case reports have also been presented for interactions with topicaly applied miconazole (83) and econazole (84).
Small changes in plasma concentrations of the opioids we studied may not be as clinically important as those involving warfarin, with its narrow therapeutic index; still, this should serve as a cautionary note.

While our extrapolations have not used the more detailed equations that take into account fraction metabolized by different CYPs, and contribution of metabolism in the gastrointestinal system (51), good agreement was found with several clinical studies. The exception being itraconazole, which has contributing inhibitory metabolites, and oral gel miconazole, where the anticipated plasma concentrations from gel delivery did not seem sufficient to cause an in vivo interaction. Non-CYP factors have been proposed to contribute to the in vivo effects of the azoles on drug disposition. These include inhibition of drug transports (85), antagonism of PXR receptors (86) and inhibition of UDP-glucuronosyl transferases (87). The contribution of these and other factors cannot be ruled out, but a good correlation to the effect of azoles on CYP activity is a consistent finding.

Conclusions

Many of theazole agents inhibited the in vitro metabolism of oxycodone, methadone and buprenorphine. Ketoconazole and clotrimazole had sub-micromolar IC₅₀ values for the CYP3A4-mediated pathways, as did terconazole and voriconazole for oxycodone metabolism by CYP3A4. Several azoles had IC₅₀ values between 1 and 10 μM for the CYP3A4 pathways. Two to three azoles also had IC₅₀ values in this range for the CYP2B6, 2C8, 2C19 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 contributing inhibitory metabolites, and with oral gel use of miconazole. The impact of orally administered azoles is likely to be far more significant as our correlations show. Potential impact from mucosal and topical azoles was minimal in our calculations, but some caution must also be applied based on case reports of toxicity in the literature. While opioid fatalities most often arise from the toxic effect of the opioid itself, our studies suggest that further considerations may be required if evidence is found for co-use of drugs such as the azoles that can cause pharmacokinetic interactions.

Supplementary data

Supplementary data are available at Journal of Analytical Toxicology online.

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