In Vitro Hepatic Oxidative Biotransformation of Trimethoprim

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NONSTANDARD ABBREVIATIONS

Idiosyncratic adverse drug reactions: IADRs

Trimethoprim-Sulfamethoxazole: TMP-SMX
Trimethoprim (TMP) has been widely used since the 1960s, both alone and in combination with sulfamethoxazole (SMX). Unfortunately, information regarding the role that cytochrome P450 enzymes (P450s) play in the formation of TMP primary metabolites is scarce. Hence, we undertook *in vitro* studies to identify and more fully characterize the P450s that catalyze formation of six TMP primary metabolites: TMP 1- and 3-N-oxides (1-NO-TMP and 3-NO-TMP), 3′- and 4′-desmethyl-TMP, a benzylic alcohol (Cα-OH-TMP) and an N-acetyl cysteine (NAC) adduct of TMP (Cα-NAC-TMP). Formation kinetics for each TMP metabolite in human liver microsomes (HLMs) were consistent with single enzyme Michaelis-Menten kinetics and Km values were markedly above (≥10-fold) the therapeutic concentrations of TMP (50 µM). The combined results from correlation studies between rates of metabolite formation and marker P450 activities in a panel of HLMs along with inhibition studies utilizing selective P450 inhibitors incubated with pooled HLMs suggested that 1-NO-TMP, Cα-NAC-TMP and Cα-OH-TMP were predominantly formed by CYP3A4. In contrast, 3-NO-TMP was formed predominantly by CYP1A2 in HLMs and inhibited by α-naphthoflavone. 4′-desmethyl-TMP, which is believed to be a reactive TMP metabolite precursor, was formed by several P450s including CYP3A4, correlated with multiple P450 activities, but was inhibited primarily by ketoconazole (up to 50%), suggesting that CYP3A4 makes a major contribution to TMP 4′-demethylation. TMP 3′-demethylation was catalyzed by multiple P450s including CYP2C9, correlated with CYP2C9 activity and was inhibited by sulfaphenazole (up to 40%). Overall, CYP2C9 and CYP3A4 appear to be the most significant contributors to TMP primary metabolism.
INTRODUCTION

Trimethoprim-sulfamethoxazole (TMP-SMX) is a frequently prescribed antimicrobial for the treatment of urinary tract, skin and soft tissue infections, and the prevention of opportunistic infections in the immune-compromised host (Copp et al., 2011; Hersh et al., 2008; Mofenson et al., 2009). This drug combination is also one of the most common causes of unpredictable, undesired and potentially severe idiosyncratic adverse drug reactions (IADRs) with symptoms that include rash, fever, lymphadenopathy, cytopenias, liver injury or severe skin detachment, such as Stevens Johnson Syndrome or toxic epidermal necrolysis (Roujeau et al., 1995; Clarkson and Choonara, 2002). Historically, SMX has been suspected as the inciting agent in these IADRs, largely based on its ability to form a nitroso intermediate capable of covalent modification of proteins, which results in subsequent immunogenicity and antigenicity (Manchanda et al., 2002; Naisbitt et al., 2002). However, administration of TMP alone has also been associated with severe and even fatal IADRs (Hawkins et al., 1993; Mortimer et al., 2005; Nwokolo et al., 1988). Recently, we identified N-acetyl cysteine (NAC) adducts of TMP metabolites in the urine of children taking TMP-SMX, confirming bioactivation of TMP to reactive metabolite species in vivo, thus raising the possibility that TMP may also contribute to IADRs with TMP-SMX use (van Haandel et al., 2014). Incubations containing human liver microsomes (HLMs), NAC and an NADPH-generating system formed all of the TMP-NAC adducts observed in urine, suggesting the possibility that cytochrome P450 enzymes (P450s) may be responsible for TMP bioactivation.

TMP is largely excreted unchanged in the urine. Several stable human urinary metabolites have previously been identified: the N-oxides, 1-NO-TMP and 3-NO-TMP, which are generated by oxidation of nitrogen atoms in the pyrimidine ring; the benzylic alcohol, Ca-
OH-TMP, formed via oxidation of the methylene bridge; and 3’ and 4’-desmethyl-TMP resulting from oxidative O-demethylation (Figure 1) (Brooks et al., 1973; Sigel et al., 1973). Further in vitro data generated by Damsten et al, suggest that multiple O-demethylation reactions may occur, resulting in up to three free hydroxyl groups on the benzylic ring (Damsten et al., 2008).

In addition to the formation of these stable metabolites, evidence of TMP bioactivation has been demonstrated in vitro through the trapping of bioactivated TMP metabolites with small molecular weight thiols, such as NAC. TMP reactive metabolites are believed to form through both direct oxidation of TMP to an imino-quinone methide and via sequential, secondary oxidation of primary, demethylated TMP metabolites to form a para-quinone methide (Damsten et al., 2008; Lai et al., 1999). Consistent with the NAC-TMP adducts observed in vitro, we detected six NAC-TMP conjugates in the urine of children taking TMP-SMX (van Haandel et al., 2014).

In vitro results have demonstrated that TMP bioactivation is catalyzed by P450s, although the specific enzymes involved in generating both the stable and reactive metabolites have not been well defined. Damsten et al described the formation of several NAC adducts and stable metabolites from TMP utilizing HLMs, rat liver microsomes, heterologously expressed P450 enzymes and P450 BM3 mutants. Based only on data from incubations containing heterologously expressed human P450s 1A2, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4, Damsten et al suggested that CYP1A2 and CYP3A4 were capable of forming the imino-quinone methide, CYP2D6 was primarily responsible for catalyzing both the demethylation and subsequent secondary oxidation step leading to the para-quinone-methide species, whereas CYP3A4 catalyzed multiple TMP demethylation reactions leading to quinone methide intermediates (Damsten et al., 2008). Formation of reactive intermediates, such as the imino-quinone-methide...
and the para-quinone methide species, are believed to be the initiating step in IADRs.

Unfortunately, additional studies designed to more fully characterize the quantitative importance of individual P450s, such as correlation analyses with marker P450 activities in a panel of HLMs and inhibition studies with selective P450 isoform inhibitors, were not performed. We sought to fill this knowledge gap by more completely characterizing the role of P450s in the formation of TMP primary stable and reactive metabolites at therapeutically relevant concentrations of TMP (5-50 µM, based on plasma C_max). Defining the metabolic pathways leading to the bioactivation of TMP is a critical first step to better understand factors that may contribute to variability in reactive metabolite formation, and potentially, the risk of developing IADRs.
MATERIAL AND METHODS

Chemicals. LC-MS Optima® grade acetonitrile, methanol, and water were purchased from Fisher Scientific (FairLawn, NJ, USA). EDTA, formic acid (LC-MS grade), glucose-6-phosphate dehydrogenase, magnesium chloride, N-acetyl-L-cysteine (NAC), NADP, potassium phosphate dibasic, potassium phosphate monobasic, TMP, danazol, 4-methylpyrazole, montelukast, α-naphthoflavone, quinidine, sulfaphenazole, tranylcypromine, and Triton X-100 were obtained from Sigma Aldrich Chemical Co. (St. Louis, MO). Benzylnirvanol, and thiotepa were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Ketoconazole was obtained from Research Biochemicals International (Natick, MA). All reagents were reagent grade quality or higher. 1-NO-TMP, 3-NO-TMP, 4’-desmethyl-TMP, Ca-OH-TMP, and Ca-NAC-TMP were custom synthesized by Artis-Chem Co. Ltd (Shanghai, China). Purity of the standards was determined to be ≥98% by H-NMR, LCMS and LCUV at 214nm.

Human Liver Microsomes and cDNA-Expressed Enzymes. A reaction phenotyping kit (Version 8; containing HLMs prepared from 16 individuals; 4 females, 12 males), pooled HLMs, and EasyCYP Bactosomes (manufactured by Cypex Ltd, Scotland, UK) were purchased from XenoTech, LLC (Lenexa, KS). The EasyCYP bactosomes included the following cDNA-expressed human P450 enzymes: P450s 1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, 3A7 and 4A11. Each of the human P450 enzymes were co-expressed with P450 reductase and CYPs 2B6, 2C8, 2C9, 2E1, 3A5 and 3A7 were co-expressed with cytochrome b5. Low reductase containing microsomes were utilized where available. Microsomes were stored at -80°C and placed on ice prior to use.
**In vitro incubation conditions.** Standard *in vitro* enzyme incubations (200-µl) contained HLMs (20 µg of microsomal protein) or baculovirus cDNA-expressed P450 enzymes (4 pmol), potassium phosphate buffer (50mM, pH 7.4), MgCl₂ (3mM), EDTA (1mM), NAC (5mM) and TMP (0-500 µM) dissolved in water. Reactions were initiated by the addition of a NADPH-generating system consisting of NADP (1 mM), glucose 6-phosphate (1 U/ml), and glucose-6-phosphate dehydrogenase (5mM). Following the addition of the NADPH generating system, reactions were incubated in a shaking water bath at 37 ± 1°C for 0-30 minutes, and were terminated by the addition of 200 µL of ice-cold methanol. Protein was precipitated by centrifugation at 10,000 g for 10 minutes and an aliquot of the supernatant was analyzed by HPLC/MS via direct injection.

Preliminary experiments with TMP (0.5, 0.75, 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, 250, 300, 400, 500 µM) and pooled HLMs (0.1, 0.5, 1.0 mg protein/ml) suggested that the rates of TMP metabolite formation were directly proportional to protein concentration (1.0 mg/ml) and time (60 min). Under these conditions, TMP biotransformation was less than 15%. Experiments utilizing HLMs that were warmed to 45°C for 5 minutes, placed on ice to cool and then incubated with TMP (5 and 50 µM), NAC and the NADPH-generating system (as described previously) were conducted to determine if flavin-containing monooxygenases (FMOs) could catalyze TMP primary metabolite formation. Similarly, HLMs treated with Triton X-100 (1 per cent final concentration) were added to incubations containing TMP (5 and 50 µM), NAC and the NADPH-generating system as described to determine the contribution of total P450 to TMP primary metabolite formation. Control incubations contained HLMs that were not subjected to heat nor treated with Triton X-100, TMP (5 and 50 µM), NAC and the NADPH-generating system, as described above. Correlation experiments with HLMs were performed in triplicate
with two replicate samples per condition. Kinetic experiments and experiments performed with cDNA-expressed P450s were conducted in duplicate.

**Chemical-Inhibition Experiments.** Conversion of TMP (50 µM) to the primary metabolites of interest (1-NO-TMP, 3-NO-TMP, 3’-desmethyl-TMP, 4’-desmethyl-TMP, Ca-OH-TMP, and Ca-NAC-TMP) by pooled HLMs was determined in the presence or absence of selective, competitive P450 inhibitors at four concentrations encompassing the denoted ranges including: α-Naphthoflavone (CYP1A2, 0.15-5 µM), tranylcypromine (CYP2A6, 0.2-6 µM), thiotepa (CYP2B6, 5-150 µM), montelukast (CYP2C8, 0.15-5 µM), sulfaphenazole (CYP2C9, 0.3-10 µM), benzylnirvanol (CYP2C19, 0.3-10 µM), quinidine (CYP2D6, 0.15-5 µM), 4-methylpyrazole (CYP2E1, 2-60 µM), danazol (CYP2J2, 0.02-0.6 µM), and ketoconazole (CYP3A4, 0.03-1 µM). These ranges of inhibitor concentrations were based on reported Ki values with the highest concentration of inhibitor approximately 30 times Ki (range: Ki, 3 Ki, 10 Ki and 30 Ki). In theory, the highest concentration of inhibitor used in these studies should virtually abolish (>90%) product formation by the inhibited enzyme. Inhibition experiments were conducted in triplicate with replicate incubations.

**UPLC MS/MS Analysis:** Sample analyses were performed by UPLC MS/MS, as described by van Haandel et al (van Haandel et al., 2014). Optimization of the analysis protocol included the injection of 3µL of sample to minimize band broadening, and the custom synthesis of reference compounds. The availability of reference standards allowed us to construct calibration curves for 1-NO-TMP, 3-NO-TMP, 4’-desmethyl-TMP, Ca-OH-TMP, and Ca-NAC-TMP. 3’-desmethyl-TMP was quantitated from the 4’-desmethyl-TMP standard because of its structural similarity and due to the absence of available reference material. Calibration curves were constructed in
MeOH quenched incubations containing HLMs and 1, 3, 10, 30, 100, 300 or 400 µM concentrations of standard reference compounds.

**Data Analysis:** Correlation coefficients (r) between the rates of 1-NO-TMP, 3-NO-TMP, 3'-desmethyl-TMP, 4'-desmethyl-TMP, Ca-OH-TMP, and Ca-NAC-TMP formation with the marker activities of P450 enzymes in the HLM reaction phenotype kit were determined using least-squares regression analysis. Significance was determined by Pearson's regression analysis using a two-tailed t-test with $\alpha = 0.05$ (JMP® 10). Effects of the selective P450 inhibitors were determined by comparing the rate of individual metabolite formation as per cent of control by inhibitor concentration (Microsoft Excel 2007®). Rate calculations with the heterologously expressed enzymes were corrected for metabolite formation by microsomes containing vector only. Data derived from kinetic incubation studies using HLMs and selected c-DNA expressed enzymes (CYP2C9 and 3A4) were analyzed by nonlinear regression to estimate $K_m$ and $V_{max}$ values. These data were visually inspected via Eadie–Hofstee and Lineweaver-Burk plots and subsequently fit to a one-enzyme Michaelis-Menten model (GraphFit 5; Erithacus Software Ltd., Surrey, UK).
RESULTS:

Biotransformation of TMP by HLMs

In the presence of oxygen, NAC and an NADPH-generating system, HLMs converted TMP (5 and 50 µM) to six primary metabolites resulting from oxidation of TMP, namely, 1-NO-TMP, 3-NO-TMP, 3'-desmethyl-TMP, 4'-desmethyl-TMP, Cα-OH-TMP, and Cα-NAC-TMP (Figure 1). The identities of the metabolites formed were consistent with our previous in vitro and in vivo observations (van Haandel et al., 2014). 3'- and 4'-Desmethyl-TMP were the most abundant metabolites formed and accounted for approximately 65 and 25 percent of total metabolite formation, respectively, regardless of substrate concentration examined. Of the remaining metabolites, only 1-NO-TMP had an abundance approaching 5 per cent; each of the other minor metabolites constituted less than 2 per cent of the total metabolite formation (Figure 2).

In order to determine if flavin-containing monooxygenases (FMOs) might contribute to the formation of these metabolites, HLMs heat-treated at 45°C for 5 minutes (causing heat inactivation of FMOs) were evaluated for their ability to convert TMP (5 and 50 µM) to primary metabolites. There was no difference in metabolite formation between heat treated- and control HLMs (results not shown). In contrast, incubations containing 1% Triton X-100 (which inhibits P450 activity), HLMs and TMP (5 and 50 µM) demonstrated that Triton X-100 virtually eliminated TMP metabolite formation relative to control incubations (results not shown).

The kinetics of formation for the six primary TMP metabolites were estimated in pooled adult HLMs and in microsomes containing cDNA-expressed CYP2C9 and CYP3A4 enzymes (Table 1). CYP2C9 and CYP3A4 were selected because both enzymes were shown to
significantly contribute to either direct bioactivation of TMP (Cα-NAC-TMP) or result in the abundant formation of primary metabolites (3’ and 4’-desmethyl-TMP) that could potentially undergo further bioactivation to potential reactive species (experimental results supporting these findings are described below). The aqueous solubility of TMP limited final incubation concentrations to 500 µM or less. Direct plots of substrate concentration versus rate of TMP metabolite formation indicated that substrate saturation did not occur for the formation of any of the TMP primary metabolites (i.e., the plateau phase was not achieved). Although this precluded an accurate estimation of K_m and V_max parameters, Eadie-Hofstee plots were constructed for the available data. While there was a slight indication of substrate activation in the formation of Cα-OH-TMP, visual inspection of the Eadie-Hofstee plots for each of the six primary metabolites suggested that they could be fit to a single-enzyme Michaelis-Menten equation. Data were then fit to a single enzyme model using the software, Grafit 5, to estimate the kinetic parameters K_m and V_max, which are shown in Table 1. Of note, all of the determined K_m values for the six primary metabolites were above 450 µM, and as high as 2 mM, markedly higher than therapeutic concentrations of TMP observed in vivo.

**TMP N-oxidation (1 and 3-NO-TMP)**

Incubations with microsomes containing one of seventeen cDNA-expressed human P450 enzymes were conducted at two TMP concentrations spanning the therapeutic range (5µM and 50 µM) to evaluate the ability of these enzymes to catalyze formation of TMP N-oxides. The selected TMP concentrations were based on observed in vivo TMP C_max measurements (Hoppu and Arjomaa, 1984; Rylance et al., 1985). Several heterologously expressed P450 enzymes, including CYP1A1, CYP1B1, CYP2C19, CYP3A4, CYP3A5 and CYP3A7, were capable of catalyzing 1-NO-TMP formation (Figure 3, panel A, 5µM data shown) whereas CYP1A1, and
CYP1B1 were the heterologously expressed enzymes that catalyzed the highest rates of 3-NO-TMP formation.

A panel of HLMs prepared from 16 individual donors and characterized for P450 and FMO activities using marker substrate reactions was examined for the ability to catalyze the 1- and 3-\textit{N}-oxidation of TMP. At a substrate concentration of 5 µM, each of the microsomal preparations catalyzed the formation of both \textit{N}-oxides and the rate of TMP \textit{N}-oxide formation varied >6-fold; 1-\textit{N}-oxide formation (rates ± S.D.) ranged from 0.08 ± 0.02 to 0.46 ± 0.20 pmol/mg protein/min whereas 3-\textit{N}-oxide formation ranged from 0.01 ± 0.005 to 0.08 ± 0.04 pmol/mg protein/min (Fig 2, panel A; Figure 3, panel B). Rates of TMP 1- and 3-\textit{N}-oxide formation for each of the microsomal samples increased proportionally (approximately) with a 10-fold increase in substrate concentration (50 µM; results not shown). The sample-to-sample variation in the rates of 1-NO-TMP formation, correlated significantly ($P < 0.01$) with CYP2A6, CYP2C8, CYP2C9, and CYP3A4 activities whereas 3-NO-TMP formation correlated significantly with CYP1A2, CYP2C8 and CYP2C9 activities, but not with any other P450 or FMO activities (Table 2).

Inhibition studies were conducted to corroborate the identities of the relevant enzymes involved in the formation of TMP \textit{N}-oxides. As shown in Figure 3, panel C, the formation of 1-NO-TMP was inhibited markedly (greater than 20% inhibition) and in a concentration dependent manner by thiotepa (CYP2B6 inhibitor) and ketoconazole (CYP3A inhibitor). However, cDNA-expressed CYP2B6 did not appear to catalyze 1-NO-TMP formation, nor was CYP2B6 activity correlated significantly ($P < 0.01$) with 1-NO-TMP formation in the panel of HLMs, suggesting that CYP2B6 may not be a major catalyst of this reaction. \textit{α}-Naphthoflavone (CYP1A inhibitor) was the only inhibitor examined that was found to inhibit 3-NO-TMP formation. The other
competitive P450 inhibitors resulted in little or no inhibition of either 1-NO-TMP or 3-NO-TMP formation. Cumulatively, this evidence suggests that in HLMs, 1-NO-TMP and 3-NO-TMP formation are catalyzed primarily by CYP3A4 and CYP1A, respectively.

**TMP Demethylation (3’ and 4’-desmethyl-TMP)**

Several of the heterologously expressed P450 isoforms examined in this study demonstrated the capability to catalyze the formation of 3’ or 4’-desmethyl-TMP at substrate concentrations of 5µM (Figure 4, panel A) and 50 µM TMP (data not shown). Unlike other P450 enzymes, CYPs 2B6, 2C19, 2D6, 2J2 and 3A4 were capable of catalyzing both the 3’ and 4’-demethylation of TMP at the concentrations examined. At a substrate concentration of 5µM, there was nearly 3-fold variation in 3’-desmethyl-TMP formation (range: 1.5 ± 0.14 to 4.8 ± 0.49 pmol/mg protein/min) and 5-fold variation in 4’-desmethyl-TMP formation (range: 0.4 ± 0.02 to 2.3 ± 0.35 pmol/mg protein/min) in the panel of HLMs (Figure 2, panel B; Figure 4, panel B). The sample-to-sample variation in the rates of formation were mostly highly correlated \((P< 0.01)\) with CYP2C8 and CYP2C9 activities for both 3’- and 4’-desmethyl-TMP formation, although several other P450 activities including CYP2J2 and CYP3A4/5 activities were observed to have significant correlations with TMP 3’- or 4’-demethylation (Table 2). Interestingly, CYP2C8 and CYP2C9 activities were significantly correlated with one another \((r=0.668; P< 0.01)\), as were CYP2J2 and CYP3A activities \((r=0.648; P< 0.01)\) among the panel of HLMs.

The CYP2C9 inhibitor, sulfaphenazole, markedly inhibited TMP 3’-demethylation in a concentration-dependent manner, but had little or no effect on the 4’-demethylation of TMP. In contrast, the CYP3A inhibitor, ketoconazole, had little or no effect on TMP 3’-demethylation, but markedly inhibited TMP 4’-demethylation in a concentration manner. All of the other
competitive P450 inhibitors tested resulted in little or no inhibition of TMP 3' or 4'-demethylation.

**TMP α-bridge Oxidation (Cα-OH-TMP and Cα-NAC-TMP)**

Incubations containing cDNA-expressed human P450 enzymes and TMP (5 µM) revealed that CYPs 1A1, 3A4, 3A5 and 3A7 had the capability to catalyze Cα-OH-TMP formation, whereas Cα-NAC-TMP formation appeared to be exclusively catalyzed by CYP3A4 (Figure 5, panel A). Similar results were obtained when heterologously-expressed P450s were incubated with 50 µM TMP (results not shown). Considerable variability in the rates of oxidation of the α-bridge carbon of TMP was observed among the sixteen different preparations of HLMs at a substrate concentration of 5µM (Figure 2, panel C; Figure 5, panel B); nearly 10-fold variation in Cα-OH-TMP formation (range: 0.02 ± 0.004 to 0.18 ± 0.03 pmol/mg protein/min) and 17-fold variation in Cα-NAC-TMP formation (range:0.007 ± 0.006 to 0.12 ± 0.07 pmol/mg protein/min) was observed. The sample-to-sample variation in the rates of Cα-OH-TMP and Cα-NAC-TMP formation by the panel of HLMs were most highly correlated with CYP3A4/5 activity (P < 0.01), but were significantly correlated with the activities of a number of other P450 enzymes (Table 2). Inhibition studies revealed that the formation of both Cα-OH-TMP and Cα-NAC-TMP were inhibited markedly by the CYP3A inhibitor, ketoconazole, and moderately by the CYP2B6 inhibitor, thiopeta but not by any other selective P450 inhibitors (Figure 5, panel C).
DISCUSSION

Trimethoprim undergoes biotransformation to a number of metabolites by humans, and Fig. 1 depicts the primary oxidative metabolites formed from TMP. Many TMP metabolites are stable, but a few reactive intermediates, which can be trapped with NAC, such as an iminoquinone methide species, have also been identified (Damsten et al., 2008; van Haandel et al., 2014). To date, little information is available on the roles of individual oxidative enzymes, including P450s, involved in the formation of these metabolites. Only one previous in vitro study (Damsten et al., 2008) has provided insight into the enzymes involved in the formation of TMP metabolites. Based on their experiments with heterologously expressed human P450s and the average expression content of these enzymes in human livers, Damsten et al inferred the identities of the P450 enzymes they believed were responsible for TMP biotransformation. Unfortunately, the activities of heterologously-expressed P450 enzymes do not necessarily reflect the activities of native P450s, even when relative abundance is taken into consideration. Differences in P450 reductase and cytochrome b\textsubscript{5} expression levels as well as differences in lipid membrane composition of the utilized expression system can contribute to alterations in P450 activities (Parkinson, 1996). By combining additional kinetic, correlation and inhibition experimental approaches with experiments utilizing cDNA-expressed enzymes, a more accurate assessment of the identities and roles of P450s involved in TMP biotransformation can be obtained. Therefore, we undertook these in vitro studies to further characterize the roles of P450s involved in those TMP primary metabolite biotransformation pathways observed in vivo.

Although there were some differences, many of the results from our study corroborate observations by Damsten et al (Damsten et al., 2008). 3'-Desmethyl-TMP and 4'-desmethyl TMP were the most abundant metabolites formed by HLMs and accounted for approximately 65
and 25 percent of total metabolite formation, respectively, whereas TMP N-oxides constituted minor metabolites (≤5%) and the benzylic metabolites, Cα-OH-TMP and Cα-NAC-TMP, were in even lower abundance. Damsten et al. concluded that CYPs 2C9 and 3A4 were primarily responsible for the formation of 3’-desmethyl-TMP and 4’-desmethyl-TMP, respectively. On the basis of results from our correlation, inhibition and cDNA-expressed enzyme experiments, we reached the same conclusions.

CYP3A4 appears to play a major role in the formation of 4’-desmethyl TMP. Although several heterologously expressed P450s, including CYPs 2C19, 2D6, 2J2 and 3A4, demonstrated the capability to catalyze TMP 4’-demethylation, the only effective inhibitor of TMP 4’-demethylation examined in HLMs was the CYP3A inhibitor, ketoconazole, which inhibited ~50% of TMP 4’-demethylase. The highest concentration of inhibitor used in this study should eliminate >90 percent of the targeted P450 isoform activity, which suggests that although CYP3A4 appears to be a major contributor to 4’-desmethyl TMP formation, it is likely that other P450 enzymes may contribute to the formation of this metabolite at therapeutically relevant concentrations of TMP.

Heterologously-expressed CYP2D6 was the most prolific enzyme catalyzing TMP 3’-demethylation, corroborating the observation by Damsten et al. (Damsten et al., 2008), but the CYP2D6 inhibitor quinidine caused no appreciable inhibition of TMP 3’-demethylation and inter-individual CYP2D6 activity in HLMs correlated poorly with rates of TMP 3’-demethylation (r=0.481). The low abundance of CYP2D6 in HLMs is likely responsible for the lack of apparent contribution by CYP2D6 to the formation of 3’-desmethyl TMP and the lack of inhibition in product formation by quinidine. These results suggest that in vivo, CYP2D6 will make little or no contribution to the formation of 3’-desmethyl TMP.
In contrast, heterologously expressed CYP2C9 catalyzed a much lower rate of TMP 3’-demethylation than did CYP2D6, but CYP2C9 appeared to play a major role in TMP 3’-demethylation in HLMs based on the observations that the CYP2C9 inhibitor, sulfaphenazole, substantially inhibited the formation of 3’-desmethyl TMP (up to 40 percent) in pooled HLMs and the rate of 3’-desmethyl TMP formation significantly correlated with CYP2C9 and CYP2C8 activities (enzymes that are co-regulated) in the panel of HLMs (Chen and Goldstein, 2009). Similar to the case with TMP 4’-demethylation, it is likely that other P450 enzymes may contribute to the formation of 3’-desmethyl TMP.

Human liver microsomes formed only small amounts (≤5%) of 1- and 3-TMP-N-oxides and TMP-3-NO was found to be primarily catalyzed by CYP1A2, observations also previously noted (Damsten et al., 2008). Damsten et al. concluded that TMP-1-NO was also primarily formed by CYP1A2. However, the results of our study suggest that CYP3A4 is the enzyme primarily responsible for catalyzing TMP 1-N-oxidation. It is important to note that both the 1- and 3-N-oxides are found in much greater abundance in human urine (approximately 30% of total recovered metabolites), suggesting that these metabolites are formed by other processes, potentially by extra-hepatic P450 enzymes or by other oxidative enzymes. (Sigel et al., 1973)

We also identified CYP3A4 as the primary catalyst forming both Cα-NAC-TMP and Cα-OH-TMP. Heterologously-expressed CYP3A4 catalyzed high rates of Cα-NAC-TMP and Cα-OH-TMP formation, CYP3A4/5 activity was significantly correlated with rates of formation for Cα-NAC-TMP and Cα-OH-TMP ($r^2 \geq 0.990$), and the potent CYP3A inhibitor, ketoconazole, inhibited formation of both metabolites in a concentration dependent manner. Interestingly, CYP3A5 and CYP3A7, enzymes that are expressed in hepatic tissue (Hakkola et al., 1998) also catalyzed Cα-OH-TMP formation. CYP3A7 is the predominant P450 expressed in fetal and
neonatal livers, but rapidly declines postnatally, whereas CYP3A5 is expressed at relatively low levels in fetal tissue, but is expressed at constant amounts postnatally (Hines, 2007). Thiotepa, a CYP2B6 inhibitor, inhibited Cα-OH-TMP formation at the highest concentration used, and to a much lesser extent than did the CYP3A inhibitor, ketoconazole. However, heterologously expressed CYP2B6 did not appear to catalyze Cα-OH-TMP formation. Hence, it is possible that the inhibition caused by the highest concentration of thiotepa was not due to inhibition of CYP2B6, but a loss in selectivity of the inhibitor at this concentration.

It is conceivable that Cα-NAC-TMP and Cα-OH-TMP arise from a single intermediate, but the factors that determine whether the intermediate forms a reactive species capable of forming an adduct or a stable metabolite are currently unknown. It is possible that these two metabolites arise via different mechanisms or pathways. P450s in addition to CYP3A4, such as CYP3A5 and CYP3A7, are capable of catalyzing the formation of Cα-OH-TMP but not Cα-NAC-TMP. In addition, we observed that the ratio of Cα-OH-TMP to Cα-NAC-TMP varied between individual HLMs nearly 2-fold, suggesting that other factors play a role in the balance of formation of the two metabolites.

Our results suggest that CYP2C9 and CYP3A4 are key enzymes in the biotransformation pathways responsible for metabolic clearance of TMP and the formation of both stable metabolites and reactive intermediates. Genotypic variation in CYP2C9 and CYP3A4 alleles can lead to alterations in the activity of these enzymes (Kirchheiner and Brockmoller, 2005; Werk and Cascorbi, 2014), which has the potential to alter the balance between bioactivation and detoxification, and thus alter the relative abundance of reactive and stable TMP metabolites. Further studies assessing in vivo TMP bioactivation are needed to better understand the extent and variability of TMP bioactivation in humans.
Interestingly, previous data propose that SMX undergoes CYP2C9-mediated bioactivation to a hydroxylamine. This hydroxylamine undergoes subsequent auto-oxidation resulting in a reactive nitroso intermediate that can form haptenated cellular proteins capable of triggering a cellular immune response.(Callan et al., 2009; Naisbitt et al., 2002) The results of the present study demonstrate that CYP2C9 contributes to formation of the most abundant primary TMP metabolite (3'-desmethyl TMP) \textit{in vitro}. Consequently, the potential exists for either TMP or SMX to directly influence the extent of metabolism and possible bioactivation of the other component when administered concomitantly at a combined fixed dose. For example, an individual who extensively metabolizes TMP to the 3'-desmethyl TMP metabolite could conceivably experience a relative decrease in the amount of SMX hydroxylamine that is formed. There is no evidence of mechanism-based inhibition for either trimethoprim or sulfamethoxazole; however the impact of simultaneous biotransformation of the two substrates on each other, and the potential impact on bioactivation of one component on the other, has not been evaluated (Wen et al., 2002).

The work presented here characterizes only the role that P450s play in the primary biotransformation pathways of TMP. Hence, reactive metabolites that were previously observed (van Haandel et al., 2014) but that are formed via multiple biotransformation reactions, such as the imino-quinone- or para-quinone-methides, were not detected in our incubations nor were they expected to be produced under the conditions utilized in these experiments. Identification of the determinants leading to the formation of the imino-quinone methide intermediate is crucial, as this may serve as a potential risk factor for IADR development. It is possible that variability in CYP3A4 activity determines the extent of primary TMP bioactivation \textit{in vivo} and further studies are underway to assess the extent of primary TMP metabolism variability \textit{in vivo}. Hence, factors
modulating activity of these contributing enzymes may affect the risk of ADRs (Gill et al., 1999).

Detection of 3’- and 4’-demethylated TMP glutathione or NAC adducts *in vitro* and *in vivo* implies that 3’-desmethyl and 4’-desmethyl TMP undergo further oxidation leading to reactive intermediates (Leblanc et al., 2010; van Haandel et al., 2014). Subsequent bioactivation of stable, primary metabolites to reactive intermediates has been demonstrated with other drugs commonly associated with IADRs, such as phenytoin and carbamazepine (Cuttle et al., 2000; Pearce et al., 2008). Hence, additional studies are needed to investigate the enzymes responsible for converting 3’-desmethyl and 4’-desmethyl TMP to reactive species capable of forming protein adducts.

In summary, our *in vitro* studies implicate CYP3A4 as the predominant cytochrome P450 enzyme involved in the direct formation of a reactive metabolite (i.e. Cα-NAC-TMP) from TMP. In addition, CYP 3A4 plays a major role in the generation of 4’-desmethyl TMP, which is believed to be a precursor in a second bioactivation pathway. Characterization of the isoforms responsible for secondary TMP bioactivation is critical to understand all potential sources of inter-individual variation involved in the generation of reactive intermediates capable of haptenating proteins. Although TMP has historically been overlooked as a probable contributor of IADRs, evidence of TMP bioactivation suggests that it too may contribute to these well-recognized, unpredictable reactions associated with TMP-SMX. Therefore, a better understanding of bioactivation may provide insight into means of identifying individuals at risk for developing an IADR prior to its occurrence.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Goldman and Pearce

Conducted experiments: Goldman, Van Haandel, Pearce

Performed data analysis: Goldman, Van Haandel, Leeder and Pearce

Wrote or contributed to the writing of the manuscript: Goldman, Van Haandel, Leeder and Pearce
REFERENCES


Mofenson LM, Brady MT, Danner SP, Dominguez KL, Hazra R, Handelsman E, Havens P, Nesheim S, Read JS, Serchuck L, Van Dyke R, Centers for Disease C, Prevention,


FIGURE LEGEND

**Figure 1.** Metabolic scheme for the conversion of TMP to its primary oxidative metabolites.

**Figure 2.** Variability in the formation rates of trimethoprim primary metabolites. TMP (5 µM) was incubated with HLMs prepared from individual donors (n=16) as described under Materials and Methods. Microsomes from each donor were incubated in triplicate. Box and whisker plots were constructed for the rate of formation for each metabolite. The box extends from the 25th to the 75th percentile with the line in the middle at the median. The whiskers extend to the minimum and maximum values respectively. Panel A: TMP N-oxidation (1 and 3-NO-TMP), Panel B: TMP demethylation (3’ and 4’-desmethyl-TMP), and Panel C: TMP Cα-oxidation (Cα-OH-TMP and Cα-NAC-TMP).

**Figure 3.** N-oxidation of TMP to primary metabolites. Panel A: 1- and 3-NO-TMP formation by human cDNA-expressed P450 enzymes. TMP (5 µM) was incubated with cDNA-expressed P450 enzymes as described under Materials and Methods. Rates shown are corrected for rates from microsomes containing vectors alone. Each bar represents the mean of the duplicate incubations. Panel B: Sample-to-sample variation in the rates of TMP 1- and 3 N-oxidation by HLMs at a substrate concentration of 5 µM. Each bar represents the mean of the triplicate experiments ± S.D. Panel C: Effects of various P450 isoform-selective inhibitors on the formation of 1- and 3-NO-TMP by pooled HLMs at a substrate concentration of 50 µM, as described under Material and Methods. Each bar represents the mean of the triplicate incubations. Uninhibited mean rates of 1- and 3-NO-TMP formation were 8.2 and 1.6 pmol/mg protein/min, respectively.
Figure 4. Demethylation of TMP to primary metabolites. Panel A: 3’ and 4’-desmethyl-TMP formation by human cDNA-expressed P450 enzymes. TMP (5 µM) was incubated with cDNA-expressed P450 enzymes as described under Materials and Methods. Rates shown are corrected for rates from microsomes containing vectors alone. Each bar represents the mean of the duplicate incubations. Panel B: Sample-to-sample variation in the rates of TMP 3’ and 4’-desmethyl-TMP by HLMs at a substrate concentration of 5 µM. Each bar represents the mean of the triplicate experiments ± S.D. Panel C: Effects of various P450 isoform-selective inhibitors on the formation of 3’ and 4’-desmethyl-TMP by pooled HLMs at a substrate concentration of 50 µM, as described under Material and Methods. Each bar represents the mean of the triplicate incubations. Uninhibited rates of 3’ and 4’-desmethyl-TMP formation were 48.1 and 29.2 pmol/mg protein/min, respectively.

Figure 5. Cα-oxidation of TMP to primary metabolites. Panel A: Cα-OH-TMP and Cα-NAC-TMP formation by human cDNA-expressed P450 enzymes. TMP (5 µM) was incubated with cDNA-expressed P450 enzymes as described under Materials and Methods. Rates shown are corrected for rates from microsomes containing vectors alone. Each bar represents the mean of the duplicate incubations. Panel B: Sample-to-sample variation in the rates of Cα-OH-TMP and Cα-NAC-TMP formation by HLMs at a substrate concentration of 5 µM. Each bar represents the mean of the triplicate experiments ± S.D. Panel C: Effects of various P450 isoform-selective inhibitors on the formation of Cα-OH-TMP and Cα-NAC-TMP by pooled HLMs at a substrate concentration of 50 µM, as described under Material and Methods. Each bar represents the mean of the triplicate incubations. Uninhibited rates of Cα-OH-TMP and Cα-NAC-TMP formation were 4.6 and 2.1 pmol/mg protein/min, respectively.
Table 1. Estimated kinetic parameters for the formation of TMP primary metabolites in human liver microsomes and cDNA-expressed human P450 enzymes

<table>
<thead>
<tr>
<th></th>
<th>Km (^a)</th>
<th>Vmax (^b,c)</th>
<th>Cl(_{int}) (^d,e)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pooled HLMs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-NO-TMP</td>
<td>1050 ± 120</td>
<td>69 ± 6</td>
<td>0.07</td>
</tr>
<tr>
<td>3-NO-TMP</td>
<td>690 ± 76</td>
<td>5 ± 0.3</td>
<td>0.007</td>
</tr>
<tr>
<td>3'-desmethyl-TMP</td>
<td>647 ± 55</td>
<td>453 ± 25</td>
<td>0.70</td>
</tr>
<tr>
<td>4'-desmethyl-TMP</td>
<td>482 ± 42</td>
<td>146 ± 7</td>
<td>0.30</td>
</tr>
<tr>
<td>Ca-OH-TMP</td>
<td>2180 ± 400</td>
<td>108 ± 17</td>
<td>0.05</td>
</tr>
<tr>
<td>Ca-NAC-TMP</td>
<td>1310 ± 160</td>
<td>18 ± 2</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>CYP 2C9</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-NO-TMP</td>
<td>531 ± 264</td>
<td>23.3 ± 6.87</td>
<td>0.044</td>
</tr>
<tr>
<td>3-NO-TMP</td>
<td>428 ± 253</td>
<td>5.97 ± 1.95</td>
<td>0.014</td>
</tr>
<tr>
<td>3'-desmethyl-TMP</td>
<td>1330 ± 400</td>
<td>2030 ± 470</td>
<td>1.53</td>
</tr>
<tr>
<td>4'-desmethyl-TMP</td>
<td>I.D.</td>
<td>I.D.</td>
<td>I.D.</td>
</tr>
<tr>
<td>Ca-OH-TMP</td>
<td>I.D.</td>
<td>I.D.</td>
<td>I.D.</td>
</tr>
<tr>
<td>Ca-NAC-TMP</td>
<td>I.D.</td>
<td>I.D.</td>
<td>I.D.</td>
</tr>
<tr>
<td><strong>CYP 3A4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-NO-TMP</td>
<td>971 ± 188</td>
<td>1320 ± 190</td>
<td>1.36</td>
</tr>
<tr>
<td>3-NO-TMP</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>3'-desmethyl-TMP</td>
<td>1470 ± 510</td>
<td>2060 ± 571</td>
<td>1.40</td>
</tr>
<tr>
<td>4'-desmethyl-TMP</td>
<td>1040 ± 231</td>
<td>1430 ± 240</td>
<td>1.38</td>
</tr>
<tr>
<td>Ca-OH-TMP</td>
<td>754 ± 122</td>
<td>1020 ± 110</td>
<td>1.35</td>
</tr>
<tr>
<td>Ca-NAC-TMP</td>
<td>784 ± 143</td>
<td>137 ± 18</td>
<td>0.17</td>
</tr>
</tbody>
</table>

N.D. metabolite not detected
I.D. insufficient data to determine kinetic parameters

Value ± S.E. \(^a\)micromolar, \(^b\)pmol/mg protein per minute for pooled HLMs, \(^c\)pmol/nmol P450/min for heterologously expressed enzymes, \(^d\)µl/mg protein per minute for pooled HLMs, \(^e\)µl/nmolP450 per minute for heterologously expressed
Table 2. Correlation analysis (r) of the relationship between the rates of TMP primary metabolite formation with the sample-to-sample variation in cytochrome P450 activities in human liver microsomes

<table>
<thead>
<tr>
<th>Cytochrome P450 Concentration</th>
<th>1-NO-TMP</th>
<th>3-NO-TMP</th>
<th>3'-desmethyl-TMP</th>
<th>4'-desmethyl-TMP</th>
<th>Cα-OH-TMP</th>
<th>Cα-NAC-TMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2 (5uM)</td>
<td>0.444</td>
<td>0.710**</td>
<td>0.578*</td>
<td>0.437</td>
<td>0.247</td>
<td>0.232</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.434</td>
<td>0.785**</td>
<td>0.555*</td>
<td>0.409</td>
<td>0.235</td>
<td>0.221</td>
</tr>
<tr>
<td>2A6 (5uM)</td>
<td>0.633**</td>
<td>0.435</td>
<td>0.554*</td>
<td>0.566*</td>
<td>0.506*</td>
<td>0.493</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.587**</td>
<td>0.391</td>
<td>0.512*</td>
<td>0.558*</td>
<td>0.488</td>
<td>0.488</td>
</tr>
<tr>
<td>2B6 (5uM)</td>
<td>0.499*</td>
<td>0.145</td>
<td>0.055</td>
<td>0.352</td>
<td>0.521*</td>
<td>0.510*</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.404</td>
<td>0.077</td>
<td>0.000</td>
<td>0.316</td>
<td>0.459</td>
<td>0.457</td>
</tr>
<tr>
<td>2C8 (5uM)</td>
<td>0.734**</td>
<td>0.884**</td>
<td>0.750**</td>
<td>0.930**</td>
<td>0.302</td>
<td>0.292</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.612*</td>
<td>0.807**</td>
<td>0.694**</td>
<td>0.867**</td>
<td>0.303</td>
<td>0.286</td>
</tr>
<tr>
<td>2C9 (5uM)</td>
<td>0.742**</td>
<td>0.629**</td>
<td>0.873**</td>
<td>0.759**</td>
<td>0.502*</td>
<td>0.499*</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.763**</td>
<td>0.616*</td>
<td>0.888**</td>
<td>0.809**</td>
<td>0.537*</td>
<td>0.528*</td>
</tr>
<tr>
<td>2C19 (5uM)</td>
<td>0.429</td>
<td>0.152</td>
<td>0.145</td>
<td>0.239</td>
<td>0.500*</td>
<td>0.502*</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.379</td>
<td>0.152</td>
<td>0.122</td>
<td>0.255</td>
<td>0.424</td>
<td>0.428</td>
</tr>
<tr>
<td>2D6 (5uM)</td>
<td>0.110</td>
<td>0.167</td>
<td>0.436</td>
<td>0.190</td>
<td>0.032</td>
<td>0.045</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.167</td>
<td>0.190</td>
<td>0.481</td>
<td>0.251</td>
<td>0.063</td>
<td>0.055</td>
</tr>
<tr>
<td>2E1 (5uM)</td>
<td>0.110</td>
<td>0.032</td>
<td>0.226</td>
<td>0.000</td>
<td>0.190</td>
<td>0.192</td>
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<tr>
<td>(50uM)</td>
<td>0.197</td>
<td>0.000</td>
<td>0.167</td>
<td>0.000</td>
<td>0.237</td>
<td>0.251</td>
</tr>
<tr>
<td>2J2 (5uM)</td>
<td>0.510*</td>
<td>0.089</td>
<td>0.438</td>
<td>0.390</td>
<td>0.548*</td>
<td>0.571*</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.540*</td>
<td>0.095</td>
<td>0.514*</td>
<td>0.518*</td>
<td>0.573*</td>
<td>0.571*</td>
</tr>
<tr>
<td>3A4/5 (5uM)</td>
<td>0.853**</td>
<td>0.148</td>
<td>0.399</td>
<td>0.509*</td>
<td>0.991**</td>
<td>0.990**</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.905**</td>
<td>0.138</td>
<td>0.466</td>
<td>0.632*</td>
<td>0.994**</td>
<td>0.995**</td>
</tr>
<tr>
<td>4A11 (5uM)</td>
<td>0.389</td>
<td>0.512*</td>
<td>0.458</td>
<td>0.489</td>
<td>0.164</td>
<td>0.179</td>
</tr>
<tr>
<td>(50uM)</td>
<td>0.423</td>
<td>0.544*</td>
<td>0.511*</td>
<td>0.511*</td>
<td>0.217</td>
<td>0.219</td>
</tr>
</tbody>
</table>

* significant at P < 0.05,  ** significant at P < 0.01
Figure 2

A

B

C

rate of metabolite formation (pmol/mg protein/min)

1-NO-TMP 3-NO-TMP

3'-desmethyl-TMP 4'-desmethyl-TMP

C_{12}-OH-TMP C_{12}-NAC-TMP

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Figure 3

**A**

TMP N-oxidation (pmol/nmol P450/min) for cDNA-Expressed P450 Enzymes.

**B**

TMP N-oxidation (pmol/mg protein/min) for Human Liver Microsomal Samples.

**C**

 TMP N-oxidation (percent of control) for Cytochrome P450 Inhibitors (μM).

- α-Naphthoflavone (CYP1A)
- Tranilcyromine (CYP2A6)
- Thiotepa (CYP2B6)
- Montelukast (CYP2C8)
- Sulfaphenazole (CYP2C9)
- Benzylnirvanol (CYP2C19)
- Quinidine (CYP2D6)
- 4-methylpyrazole (CYP2E1)
- Danazol (CYP2J2)
- Ketoconazole (CYP3A)
**A**

3'-desmethyl-TMP

4'-desmethyl-TMP

cDNA-Expressed P450 Enzymes

**B**

3'-desmethyl-TMP

4'-desmethyl-TMP

Human Liver Microsomal Samples

**C**

3'-desmethyl-TMP

4'-desmethyl-TMP

Cytochrome P450 Inhibitors (μM)

- α-Naphthoflavone (CYP1A)
- Tranylcypromine (CYP2A6)
- Thiotepa (CYP2B6)
- Montelukast (CYP2C8)
- Sulfaphenazole (CYP2C9)
- Benzylnirvanol (CYP2C19)
- Quinidine (CYP2D6)
- 4-methylpyrazole (CYP2E1)
- Danazol (CYP2J2)
- Ketoconazole (CYP3A)
Figure 5

A

Cα-TMP oxidation (pmol/nmol P450/min)

cDNA-Expressed P450 Enzymes

B

Cα-TMP oxidation (pmol/mg protein/min)

Human Liver Microsomal Samples

C

Cα-TMP oxidation (percent of control)

Cytochrome P450 Inhibitors (μM)

α-Naphthoflavone (CYP1A)
Tranylcypromine (CYP2A6)
Thiopeta (CYP2B6)
Montelukast (CYP2C8)
Sulfaphenazole (CYP2C9)
Benzylninanol (CYP2C19)
Quinidine (CYP2D6)
4-methylpyrazole (CYP2E1)
Danazol (CYP2J2)
Ketoconazole (CYP3A)

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