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Metabolism of the Anti-Tuberculosis Drug Ethionamide by Mouse and Human FMO1, FMO2 and FMO3 and Mouse and Human Lung Microsomes

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Abstract

Tuberculosis (TB) results from infection with *Mycobacterium tuberculosis* and remains endemic throughout the world with one-third of the world's population infected. The prevalence of multi-drug resistant strains necessitates the use of more toxic second-line drugs such as ethionamide (ETA), a pro-drug requiring bioactivation to exert toxicity. *M. tuberculosis* possesses a flavin monooxygenase (EtaA) that oxygenates ETA first to the sulfoxide and then to 2-ethyl-4-amidopyridine, presumably through a second oxygenation involving sulfinic acid. ETA is also a substrate for mammalian flavin-containing monooxygenases (FMOs). We examined activity of expressed human and mouse FMOs toward ETA, as well as liver and lung microsomes. All FMOs converted ETA to the S-oxide (ETASO), the first step in bioactivation. Compared to *M. tuberculosis*, the second S-oxygenation to the sulfinic acid is slow. Mouse liver and lung microsomes, as well as human lung microsomes from an individual expressing active FMO, oxygenated ETA in the same manner as expressed FMOs, confirming this reaction functions in the major target organs for therapeutics (lung) and toxicity (liver). Inhibition by thiourea, and lack of inhibition by SKF-525A, confirm ETASO formation is primarily via FMO, particularly in lung. ETASO production was attenuated in a concentration-dependent manner by glutathione. FMO3 in human liver may contribute to the toxicity and/or affect efficacy of ETA administration. Additionally, there may be therapeutic implications of efficacy and toxicity in human lung based on the FMO2 genetic polymorphism, though further studies are needed to confirm that suggestion.

Keywords

Ethionamide; tuberculosis; flavin-containing monooxygenase

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Introduction

Tuberculosis (TB) continues to be a world health problem of staggering proportions. There are an estimated nine million new cases and two million deaths annually, making TB the most common infectious cause of death (Harries and Dye, 2006). Additionally, one-third the world's population is infected with *Mycobacterium tuberculosis* without exhibiting symptoms of the disease, although 5-10% will be symptomatic at some point in their lifetime (Harries and Dye, 2006). The World Health Organization declared TB a global emergency in 1993 (WHO). Those individuals also infected with HIV are much more likely to develop active TB, further increasing incidence and mortality in areas of the world where HIV is rampant including sub-Saharan Africa (Harries and Dye, 2006; Kochi, 1994; Weyer and Kleeberg, 1992). Because of the emergence of multi-drug-resistant strains of *M. tuberculosis*, second-line drugs such as ethionamide (ETA) have become increasingly important as replacements for isoniazid and rifampicin (API Consensus Expert Committee, 2006; DeBarber et al., 2000; Kruijshaar et al., 2008; Lu et al, 2008; Shi et al., 2007; Umubyeyi et al., 2008). ETA, and other second-line anti-tuberculosis drugs, exhibit higher rates of adverse drug reactions, including hepatotoxicity (Donald et al., 1987; See et al., 1986; Wada, 1998).

Almost 20 years ago, it was recognized that S-oxygenation of the thiourea moiety of ETA was involved in bioactivation to a metabolite toxic to liver (Russ and Warring, 1991), in a manner similar to what had been shown earlier for thiocarbamides (Neal and Halpert, 1982; Krieter et al., 1984). This S-oxygenation also determines the therapeutic efficacy of ETA, i.e., ETA is a prodrug. In 2000, two separate laboratories identified the gene product Rv3855c (EtaA) of *M. tuberculosis* as the enzyme responsible for bioactivation of ETA (Baulard et al., 2000; DeBarber et al, 2000). This enzyme had properties consistent with that of a monooxygenase (Baulard et al., 2000). Subsequently, Vannelli, *et al*, (2002) cloned, expressed and characterized the product of the *M. tuberculosis* gene Rv3854c as an FAD-containing enzyme capable of bioactivating ETA to two major products, the corresponding S-oxide (ETASO), and 2-ethyl-4-amidopyridine (ETAA). ETA and ETASO exhibit equivalent therapeutic potency and hepatotoxicity, whereas, ETAA is relatively non-toxic and without therapeutic efficacy. Further oxygenation of ETASO to the sulfinic acid (which spontaneously breaks down to ETAA and other products) is the postulated pathway for *M. tuberculosis*-dependent bioactivation of ETA or ETASO. To date, the sulfinic acid has not been isolated. Further studies by Qian and Ortiz de Montellano (2006) demonstrated that the activity toward activation of ETA by *M. tuberculosis* EtaA extended to other related compounds, such as thiacetoazone, prothioamide and isoxy (4,4'-diisoamyloxydiphenylthiourea) (Dover et al., 2007; Phetsukriri et al., 2003; Wang et al., 2007).

Flavin-containing monooxygenases (FMOs) are a superfamily of flavoprotein monooxygenases active towards a wide range of xenobiotics. Humans express protein products of five genes (*FMO1* to *FMO5*) in a developmental-, sex-, and tissue-specific manner (Cashman et al., 1995; Hernandez et al, 2004; Krueger and Williams, 2005; Ziegler, 2002). Several *FMO* pseudogenes have been described that do not appear to produce functional proteins (Hernandez et al., 2004; Hines et al., 2002). FMOs catalyze oxygenation of a wide variety of endogenous and xenobiotic compounds containing soft nucleophiles (nitrogen, sulfur, and phosphorus heteroatoms) (Cashman, 2003; Krueger et al., 2005; Ziegler, 2002). FMOs contain a single FAD and require NADPH and O_2 for catalytic activity (Ziegler, 2002). The highest concentrations are found in liver, but extrahepatic tissue, including lung can express high specific concentrations in a number of mammals (Cashman, 1995; Dolphin et al., 1998; Krueger et al., 2001; Shehin-Johnson et al., 1995; Ziegler, 1988). In human fetal liver, the major FMO is FMO1; shortly after birth, expression of FMO1 is shut off and FMO3 levels increase over time and become the major hepatic FMO in adults (Hines, 2006; Koukouritaki et al., 2002). FMO1 is the major form expressed in adult intestine and kidney

(Yeung et al., 2000). FMO2 is typically expressed at high levels in lung, including those of non-human primates (Dolphin et al., 1998; Krueger et al., 2001). Humans exhibit an interesting genetic polymorphism in that both alleles of all Caucasians and Asians genotyped to date possess a C→T transition mutation resulting in a premature TAG stop codon 64 amino acids from the C-terminal (*FMO2*2*) (Dolphin et al., 1998; Whestine et al., 2000). This protein (FMO 2.2) is not detectable on western blots with antibody that cross-reacts with the truncated protein (Dolphin et al., 1998; Krueger et al., 2002a), probably due to improper folding and rapid degradation. Conversely, 27% of individuals of African descent and 2-7% (depending if they are of Mexican or Puerto Rican descent) of Hispanics possess at least one *FMO2*1* allele coding for the full length and enzymatically active enzyme (FMO 2.1) (Furnes et al., 2003; Krueger et al., 2002b; 2004; 2005; Whetstine et al., 2000).

M. tuberculosis EtaA appears to have properties in common with a Baeyer-Villiger monooxygenase (Fraaije et al., 2004). EtaA readily catalyzes the further oxygenation of ETASO (Vannelli et al., 2002); whereas, the human FMO 2.1 has been demonstrated to oxygenate certain thioureas only once (Henderson et al., 2004b). In this paper, we compare the activity of expressed mouse and human FMOs to ETA and demonstrate k_{cat}s greater than EtaA. Furthermore, the second oxygenation to the sulfinic acid is slower than with *M. tuberculosis* EtaA and the sulfenic acid is long-lived enough to react with glutathione (GSH). Thus, the yield of ETAA through the sulfinic acid is much reduced compared to EtaA. These findings may explain the hepatotoxicity of ETA and also raise questions about ETA metabolism in human lung.

Methods

Chemicals

Chemicals were purchased from Sigma/Aldrich (St. Louis, MO) and were of analytical grade. ETASO and ETAA were a gift from Dr. Paul R. Ortiz de Montellano (University of California, San Francisco, CA). The synthesis of these chemicals is described in Vannelli et al. (2002).

Protein Expression and Microsomes

Human FMO1 and FMO3 were a generous gift from Dr. Ronald Hines (The Medical College of Wisconsin, Milwaukee, WI). Human FMO1 was received in pENTR/SD/D-TOPO vector (Invitrogen) while FMO3 was recloned from pCR2.1 into pENTR/SD/D using 5'-CAC CAT GGG TAA GAA AGT GG and 5'-TCC TAG AGA AAA TGA TGA TTA GGT C as forward and reverse primers respectively (corresponding start and stop codons are underlined). cDNA sequences for mouse FMOs 1, 2, 3 and human FMO2 were cloned into pENTR/SD/D-TOPO as previously described (Krueger et al., 2005; Siddens et al., 2008). Human and mouse FMO sequences were integrated into BaculoDirect linear DNA using LR- mediated Clonase recombination (Invitrogen) and proteins produced by baculovirus driven expression in Sf9 cells as previously described (Henderson 2004b, Krueger et al., 2005, Siddens et al., 2008). Human FMO5 Supersomes were purchased from BD Biosciences (Woburn, MA).

Lung and liver microsomes were prepared from[mch1] frozen lungs and livers from 8- to 10-week old Swiss/Webster mice obtained from Pel-Freez (Rogers, AR). Human lung samples were obtained from donor samples and genotyped for *FMO2*1* or *FMO2*2* alleles (Krueger et al., 2002b, 2004, 2005). Microsomes were prepared as described (Krueger et al., 2002a). FAD analysis was performed by HPLC (Henderson et al., 2004a) and used to estimate FMO content. Protein was determined by the Bradford method (Bradford, 1976).

Assay Conditions

Microsomal preparations containing either expressed FMO protein (100 μg or 100 pmols) or microsomes from tissues (500 μg) were incubated at 37°C with 100 μM Tricine, 1 mM EDTA, NADPH (1 mM) and ETA (200 μM) for 5 min in a Dubnoff shaking incubator in a total volume of 250 μl. Reactions were terminated by addition of 75 μl CH_3CN on ice, then centrifuged at 12,000 × *g* for 30 min and the supernatants analyzed by HPLC. Kinetic parameters were determined by measuring velocities of ETASO formation as described by Vannelli et al. (2002), and analyzing the data using a Michaelis-Menten kinetic module in Graph Pad, Prism 5 (San Diego, CA). An alternate confirming analysis was performed with incubations as described above with the following modifications: ETA was added at 200, 150, 100, 75, and 50 μM in a total volume of 1 ml. Reactions were terminated by the addition of 2 volumes of ethyl acetate. Mixtures were vortexed 30 sec, centrifuged briefly to separate phases and the ethyl acetate extract transferred to a separate tube. The extraction was repeated, the extracts combined and taken to dryness on a Speed-Vac and redissolved in 50:50 CH_3CN/H_20 before injection onto the HPLC. Kinetic parameters were determined from Lineweaver-Burk plots.

Glutathione (GSH, 0.125 to 4.0 mM), the CYP inhibitor SKF-525A (1 mM), or the FMO competitive inhibitor thiourea (200 μM) was added to some incubations to examine their effect on ETA metabolism. Incubations were also performed for 30 min with ETASO (200 μM) as the substrate and included a NADPH-generating system (2 mM glucose-6-phosphate, 0.25 U glucose-6-phosphate dehydrogenase, and 1 mM NADPH).

Where appropriate, metabolism data was tested for statistical significance by either a t-test or one-way ANOVA, followed by Bonferonni's Multiple Comparison Test (GraphPad Prism 5, San Diego, CA).

HPLC Analysis

HPLC was performed on a Waters (Milford, MA) 2695 system equipped with a 2996 photo diode array detector and a Waters C_{18} Polarity™ 4 μm (either 3.9 × 150 mm or 3.9 × 250 mm) column. The flow rate was 0.8 and 1.3 ml/min for the different columns, respectively. The solvents consisted of 1% CH_3CN and 99% H_2O, each with 0.01% formic acid at a temperature of 35° C. Acetonitrile was increased in a linear gradient to 10% over 5 min and, after 5 min at 10%, the CH_3CN was returned to 1% in 1 min and equilibrated 4 min before the next injection. Quantitation of ETASO was by comparison to a standard curve generated with synthetic ETASO measured at 267 nm using Empower software (Waters). Spectra were collected from 210 to 400 nm for further verification of the identity of the metabolic products.

Mass Spectrometry of GSSG/GSH Ratios

Experiments, as described above with added GSH, were also analyzed by mass spectrometry to examine GSSG/GSH ratios. Incubations were performed for 15 min at 37°C with 200 pmols microsomal protein, 200 μM ETA and 200 μM GSH. Controls contained no protein, no NADPH or were stopped at 0 time. Reactions were stopped by addition of 75 μL CH_3CN on ice and centrifuged at 12,000 × *g* for 30 min. Supernatants were analyzed within 24 hr.

The mass spectrometric data was acquired on a Perkin-Elmer Sciex API 365 mass spectrometer (Applied Biosystems, Foster City, CA) in positive ion mode using TurboIon spray. Chromatographic separations were performed with a Shimadzu Prominence CBM-20 HPLC (Shimadzu Scientific Instruments, Columbia, MD) with auto sampler. The column length was the same as reported above (3.9 × 150 mm) used at a flow rate of 0.5 ml/min with a split ratio of 2:1. All analyses were run in isocratic mode with 100% aqueous formic acid (0.1%) (Burdick and Jackson, Honeywell Corp., Muskegon, MI) for 20 min. The eluant online was monitored with the mass spectrometer using MRM (multiple reaction monitoring) and Q1 full scan modes.

The mass spectrometry parameters were as follows: temperature (350°C); nebulizing gas (9); curtain gas (12); turbo heater gas (8 ml/min); declustering potential (20); and focus potential (180). The MRM transitions for GSSG were monitored at *m/z* 613.3 → *m/z* 484.1. MRM transitions for GSH were monitored at *m/z* 308.3 → *m/z* 233.2. The dwell time for each transition was 250 ms, with collision energy (21), exit potential (10), collisional cell exit potential (15), and collision cell entrance potential (GSSG, 18.09; GSH, 9.24). The corresponding peaks were identified using Q1 full scan comparing retention times and mass spectra of GSSG and GSH with those obtained for synthetic standards. Standards of varying GSSG:GSH ratios (0:1, 0.5:1, 1:1, 2:1, 3:1, 5:1, 10:1 and 1:0) were prepared and analyzed to verify the method. Experimental calculated ratios were log transformed and analyzed by a two-tailed ratio t-test, $p \leq 0.05$ (GraphPad Prism 5, San Diego, CA).

Results

We examined the activity of the major xenobiotic-metabolizing FMOs from both mouse and human, over-expressed in Sf9 insect cells following baculovirus infection. All mouse and human expressed FMOs examined, with the exception of hFMO5, oxidized ETA to ETASO. A typical chromatogram is shown in Figure 1, panel B. The identity of ETASO was verified by retention time with synthetic standard (Fig. 1, panel A) and examination of the UV spectra. Kinetic parameters for the six isoforms, calculated from velocity values obtained as described, are shown in Table 1. hFMO1 and mFmo1 had the lowest K_m's coupled with relatively high turnover numbers resulting in the highest specificity constants of the six isoforms. Although mFmo2 had a very high K_m, it also showed a very high turnover number resulting in a specificity constant almost identical to that of hFMO2. For comparison, at pH 7.5, the EtaA from *M. tuberculosis* has a K_m, k_{cat} and specificity constant for the formation of ETASO of 194 μM, 7.7 min^{-1} and 0.04, respectively (Vannelli et al., 2002).

The pH impacted the velocity of the mouse Fmos with the highest activity seen at pH 9.5 for all three isoforms (Fig. 2). However, the differences were isoform specific and were not statistically significant for mFmo1. Mouse Fmo2, activity increased significantly ($p \leq 0.05$) from pH 7.5 to 9.5. Subsequent analyses were run at pH 9.5 to maximize measurable activity for all isoforms. Incubations were also performed with all six isoforms and ETASO as substrate material. A typical chromatogram is shown in Fig. 1, panel D. Mouse and human FMOs, as with EtaA, oxygenate ETASO a second time and the product observed is ETAA. The presumed intermediate is the extremely reactive sulfinic acid (Vannelli et al., 2002). These data demonstrate that mouse and human FMOs are capable of bioactivation of the prodrug ETA, however, the velocity of ETAA formation from ETASO is slow compared to formation of ETASO (Fig. 3), especially with mouse lung microsomes.

In order to discriminate activity by FMOs from that of cytochrome P450 (CYP), incubations were performed with the general CYP inhibitor SKF-525A and with the FMO competitive inhibitor thiourea. As shown in Figure 4, SKF-525A had little effect on the appearance of ETASO with 82.9% of the activity remaining in liver and 102.4% in lung. In contrast, thiourea reduced ETA oxygenation to ETASO by an average of 73.5% in liver and 76.9% in lung. These results confirm that the conversion of ETA to ETASO in these tissues is facilitated primarily by FMOs rather than CYP.

Humans exhibit a genetic polymorphism in expression of FMO2 in lung. The active enzyme is FMO2.1, the product of *FMO2*1* (cag allele). All Caucasians and Asians genotyped to date are homozygous for the inactive, truncated FMO2.2 (product of *FMO2*2*, (tag/tag stop codon)). However our laboratory and others have shown that 27% of individuals of African descent and 2-7% of Hispanics possess at least one cag (*FMO2*1*) allele. Lung microsomes isolated from an African American tissue donor, genotyped as cag/tag, exhibited fairly robust

S-oxygenation of ETA compared to an individual of the tag/tag genotype (Figure 5). Additionally, when 200 μM thiourea was added to the incubations, the activity was decreased by 64.9 ± 1.22%, further implicating FMO as the catalyst for this oxygenation (data not shown). These data suggest that the therapeutic efficacy and/or toxicity of ETA in human lung may be a function of the FMO2 genetic polymorphism.

When GSH was added to incubations of ETA with mouse and human FMOs, it attenuated the appearance of ETASO in a dose-dependent manner (Fig. 6). This effect is manifest even when the GSH is added after a 15 min reaction time (not shown) suggesting that it is not an inhibition of activity but a conversion of ETASO back to ETA coupled with the oxidation of GSH to GSSG. S-oxygenations of thioureas to sulfenic acid, followed by reaction with GSH, and redox cycling, have been demonstrated to produce toxicity through GSH depletion and subsequent oxidative stress (Krieter et al., 1984;Neal and Halpert, 1982;Onderwater et al., 2004;Smith and Crespi, 2002).

To confirm the oxidation of GSH, incubations of ETA, GSH and the human FMO isoforms were analyzed by LC/MS. GSH and GSSG eluted at 5.5 and 14.3 min, respectively, as indicated by the transitions described in Methods. GSSG:GSH ratios increased dramatically in a 15 min reaction as compared to both 0 time controls (Fig. 7), no-NADPH controls and no ETA controls (data not shown).

Discussion

TB is a world-wide epidemic with one-third of the world's population infected with *M. tuberculosis* and 9 million new cases and deaths annually (Harries and Dye, 2006). The incidence of strains with resistance against multiple first-line drugs is increasing (Kruijshaar et al., 2008), making the use of the more toxic second-line drugs, such as ETA, a necessity (Lu et al., 2008). ETA is hepatotoxic in a significant percentage of patients. The toxicity is thought to occur through FMO-dependent S-oxygenation, in much the same manner as *M. tuberculosis* bioactivation of this prodrug by EtaA (Henderson et al., 2004b; Krieter et al., 1984; Neal and Halpert, 1982; Qian and Ortiz de Montellano, 2006; See et al, 1986; Smith and Crespi, 2002). The major difference is that the expressed mouse and human FMOs appear to carry out the first reaction to the sulfenic acid more readily than the bacterial enzyme, but are slow to S-oxygenate a second time, as judged by the yield of ETAA (Figs. 1 and 3). The putative precursor to ETAA, the sulfinic acid, is not seen upon incubation of FMO or EtaA with ETA or ETASO, presumably due to its high reactivity.

The rationale for examination of mouse FMOs is two-fold. The mouse has proven useful as a model for evaluation of therapeutic strategies against human *M. tuberculosis* infections in humans (Cynamon and Sklaney, 2003; Cynamon et al., 1999; Klemens, et al., 1993). In addition, the genetic tools available in mice provide important advantages as models for the human disease. One approach would be to develop lung-specific, conditional FMO knockout mice. FMO2.1, the active form of the enzyme, is expressed in a significant percentage of individuals of African descent and a smaller percent of Hispanics, but not in Caucasians or Asians. There is a complication in that mice unlike most mammals express a significant amount of Fmo1 in lung (Janmohamed et al., 2004; Siddens et al., 2008). Determination of the relative levels of FMO isoforms in mouse lung by these laboratories has yielded differing results. The Shephard laboratory in London found *Fmo1* to be expressed at relatively high levels with much lower, similar amounts of *Fmo2* and *Fmo3* (Janmohamed et al., 2004). In contrast, our laboratory found *Fmo2* to be the major isoform expressed in lung of nine different strains (Siddens et al., 2008), with lower but significant expression of *Fmo1* and much lower levels of *Fmo3*. Thus, it might prove necessary to knock out all FMO expression in mouse lung or,

at a minimum, utilize conditional *Fmo1/Fmo2* double knockouts, to model the human condition.

Kinetic analysis demonstrated (again with the exception of hFMO5) that ETA is a substrate for mouse and human FMOs. The specificity constant ranged from 0.17 to 0.86 with hFMOs and 0.21-2.62 with mFMOs (Table 1). The K_ms ranged from 105-336 and 104-2131 μM for human and mouse FMOs, respectively. Although these kinetic constants were determined at the pH optimum of 9.5, the activity at physiological pH is not much lower with either the expressed FMOs or mouse lung and liver microsomes (Figs. 2 and 3). This catalytic efficacy is greater than that of EtaA (0.04), although the K_ms are comparable (Vannelli et al., 2002). Following oral administration of therapeutic doses, concentrations of ETA reach levels of approximately 6, 2.4 and 35 μM in human plasma, lung alveolar cells and in lung epithelial lining fluid, respectively (Conte et al., 2000). Oral administration of ETA suggests that intestinal (primarily FMO1) and liver FMO (FMO3) play an important role in the pharmacokinetics of this prodrug and certainly in its demonstrated hepatotoxicity. In the human lung, the significance of FMO would depend upon the genetic polymorphism in expression of active, full-length, FMO2.

The relative yield of metabolites exhibited by bacterial EtaA and human FMOs appears distinct in that the latter do not carry out the second S-oxygenation to the sulfinic acid as readily (Figs. 1 and 3). This reactive intermediate is thought to play a major role in killing of *M. tuberculosis* and produces a number of breakdown products, including the amide, ETAA. The reactive intermediate has been demonstrated to be formed inside the bacteria, whereas other breakdown products, such as 2-ethyl-4-hydroxymethylpridine are extracellular (Hannoulle et al., 2006). Mammalian FMOs have many properties in common with CYPs (NADPH- and O_2-dependence) and often have overlapping substrate specificities, although the products are usually distinct (Cashman, 1995;Krueger and Williams, 2005;Ziegler, 1988). In the case of S-oxygenation of thioureas, the products are identical. Co-incubations with the general CYP and FMO inhibitors SKF-525A and thiourea, respectively, (Fig. 4) suggest that ETA S-oxygenation in mouse liver and lung microsomes is predominantly, FMO-mediated. S-oxygenation of ETA in human lung microsomes is markedly higher from an African American individual with a single cag (*FMO2*1*) allele, coding for the full-length and catalytically active protein, FMO2.1, compared to lung microsomes from a Caucasian possessing two *FMO2*2* alleles. These results indicate that the metabolism, therapeutic efficacy and/or toxicity of ETA in lung may be determined by the FMO2 genetic polymorphism. This observation is especially significant, given that the highest incidence of expression of the *FMO2*1* allele (up to 50%) (Phillips and Shephard, 2008) is in sub-Saharan Africa, which also exhibits the greatest incidence of *M. tuberculosis* infection and TB (McIlleron et al., 2006;Weyer and Kleeberg, 1992).

S-oxygenation of thioureas initially results in the formation of the sulfenic acid (Henderson et al., 2004b; Neal and Halpert, 1982; Ziegler, 1988). Sulfenic acids vary in degree of reactivity. In the case of ETASO, it is sufficiently stable to quantitate by HPLC and sufficiently reactive to conjugate glutathione in a concentration-dependent fashion. The addition of 4 mM GSH, following 15 minutes of incubation, removed essentially 50% of the ETASO (Fig. 6). Since Dixon, et al., 2008, estimate the concentration of GSH in rat liver microsomes at ~4.5 mM, this is consistent with a physiological concentration. The marked increase in the GSSG/GSH ratio (Fig. 7) suggests that FMO S-oxygenation of ETA leads to redox cycling with resultant depletion of GSH and NADPH producing oxidative stress and toxicity (Fig. 8A). The *M. tuberculosis* EtaA monooxygenase appears to be more efficacious in carrying out the second S-oxygenation to the putative sulfinic acid intermediate which may be a more direct route for the toxic actions of this prodrug on the bacteria (Fig. 8B).

Although ETA is currently administered orally, as with other drugs targeted to lung disease, including TB (Chimote and Banerjee, 2008; Hwang et al., 2008), administration through inhalation may be a more effective delivery. In the case of ETA, it could potentially reduce hepatotoxicity associated with oral dosing. In the case of *FMO2*2/2*2* individuals, FMO would not impact prodrug delivery to *M. tuberculosis* and one would expect maximum bacterial killing with little FMO2-associated toxicity to lung cells through oxidative stress. Conversely, for those individuals with at least one *FMO*2.1* allele, it is possible that less ETA prodrug would be available to *M. tuberculosis*, reducing therapeutic efficacy while at the same time enhancing lung toxicity (Fig.8B). In any case, interpretation of the results (with ETA and other FMO substrates) in animal models expressing FMO in lung need to be interpreted in light of the human FMO2 genetic polymorphism.

The results from this study raise some issues to be addressed in future studies. Do individuals expressing active FMO (at least one FMO2*1 allele, a genotype present at high incidence in regions of Africa where TB is epidemic (Phillips and Shephard, 2008) in lung exhibit therapeutically or toxicologically distinct responses to ETA compared to individuals of the *FMO2*2/2*2* genotype? Does FMO oxygenation in lung cells enhance or reduce the therapeutic efficacy of EtaA-dependent bioactivation of the prodrug ETA in the *M. tuberculosis* organism? These are important questions to address as second-line TB drugs, such as ETA, become increasingly important in treating multi-drug resistant *M. tuberculosis*.

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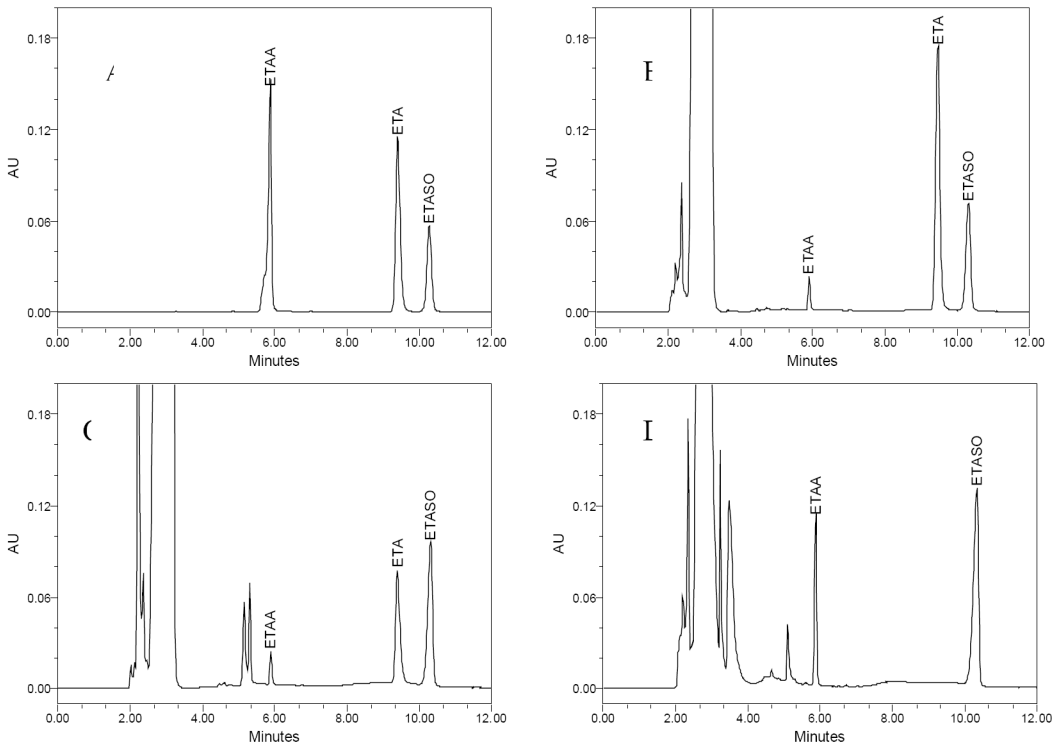

Figure 1.
Chromatograms of standards (A); ETA and hFM01 (B); ETA and mouse lung microsomes (C); ETASO and mFmo1 (D).

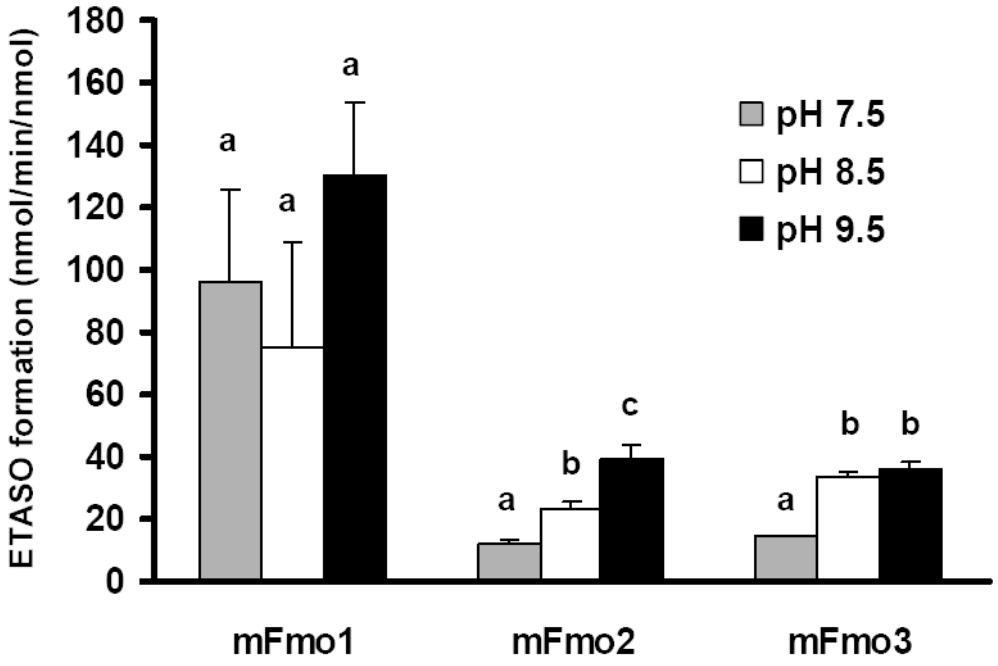

Figure 2.
Effect of pH on ETA (200 μM) catalytic activity of mFmos. Results are means of three determinations of velocity of ETASO formation ± SD. Bars within each mFmo group labeled with different letters are statistically different from each other, $p \leq 0.05$.

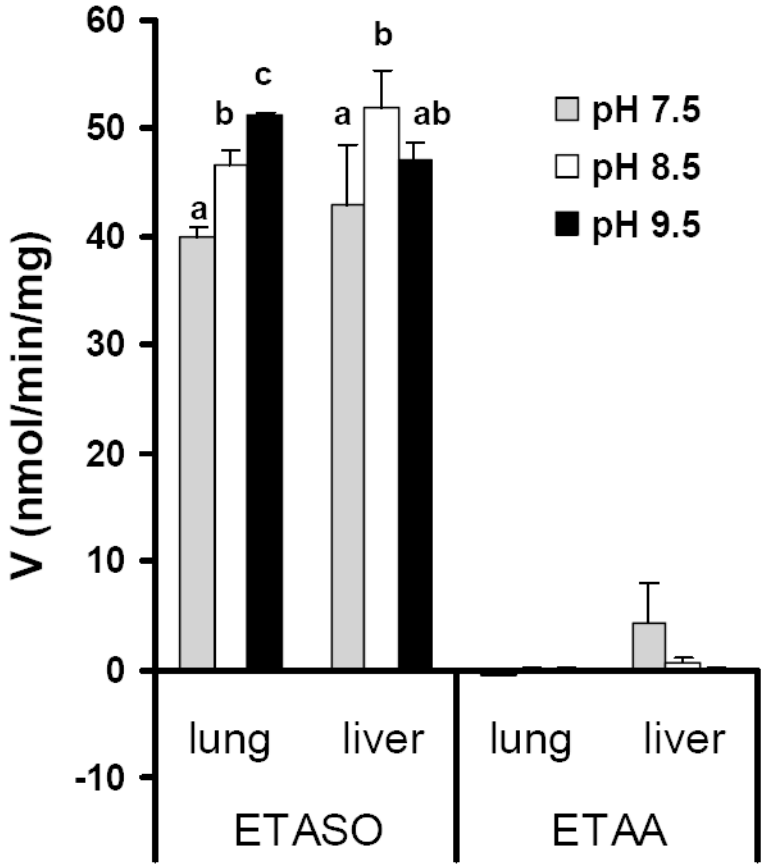

Figure 3.
Velocity of ETASO and ETAA formation from mouse lung and liver microsomes incubated with 200 μM ETA and the effect of pH. Results are means of three determinations ± SD. Bars within each tissue group labeled with different letters are statistically different from each other, $p \leq 0.05$. There were no statistically significant effects of pH on ETAA velocities.

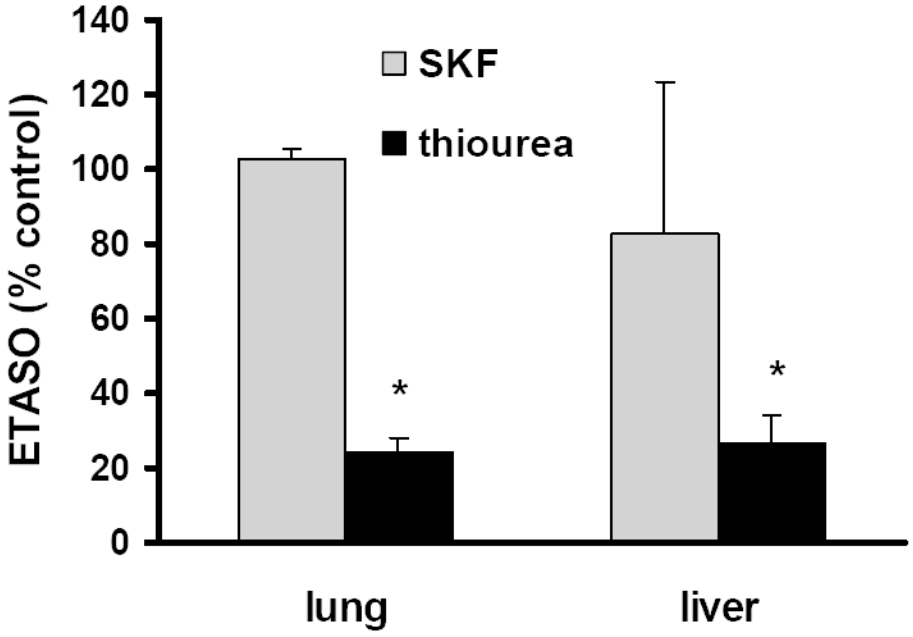

Figure 4.
Effect of SKF-525A (CYP inhibitor) or thiourea (FMO inhibitor) on S-oxidation of 200 μM ETA by mouse liver and lung microsomes. Bars represent means ± SD of three determinations. Controls were without any chemical additions. *Significantly different from controls at $p \leq 0.001$.

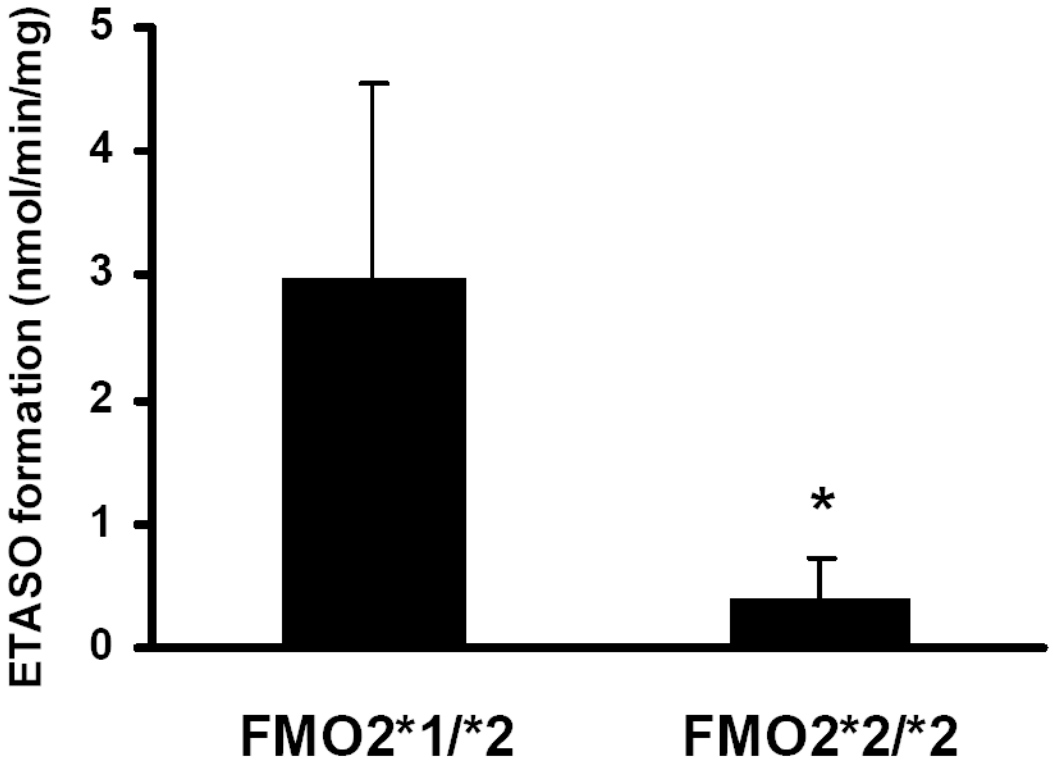

Figure 5.
S-Oxygenation activity toward 200 μM ETA by human lung microsomes from individuals genotyped as *FMO2*1/ *2 FMO2*2/ *2*. Bars represent means ± SD of three determinations from a single individual. * Significantly different at $p \leq 0.02$.

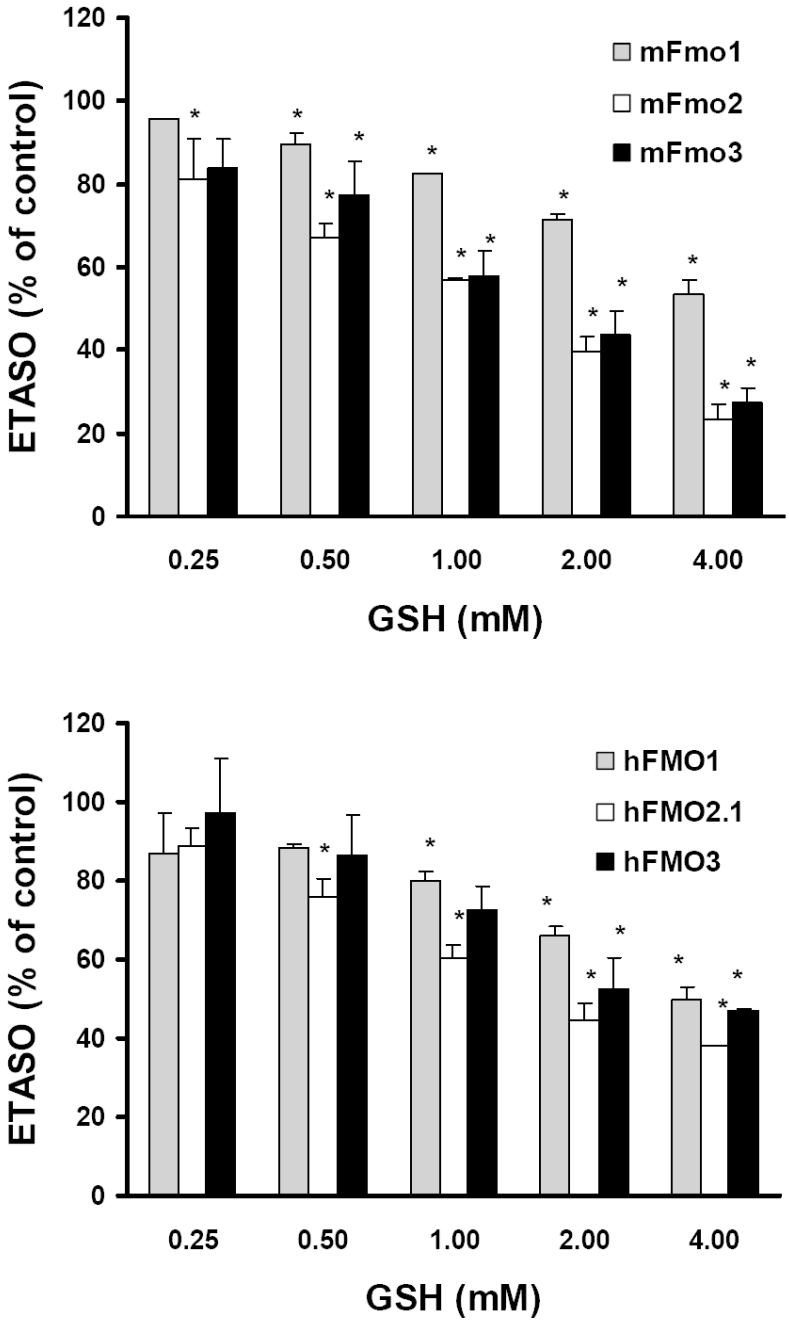

Figure 6.
Effect of GSH on S-oxygenation of 200 μM ETA by mFmos and hFMOs. * Significantly different from control (no GSH) at $p \leq 0.05$.

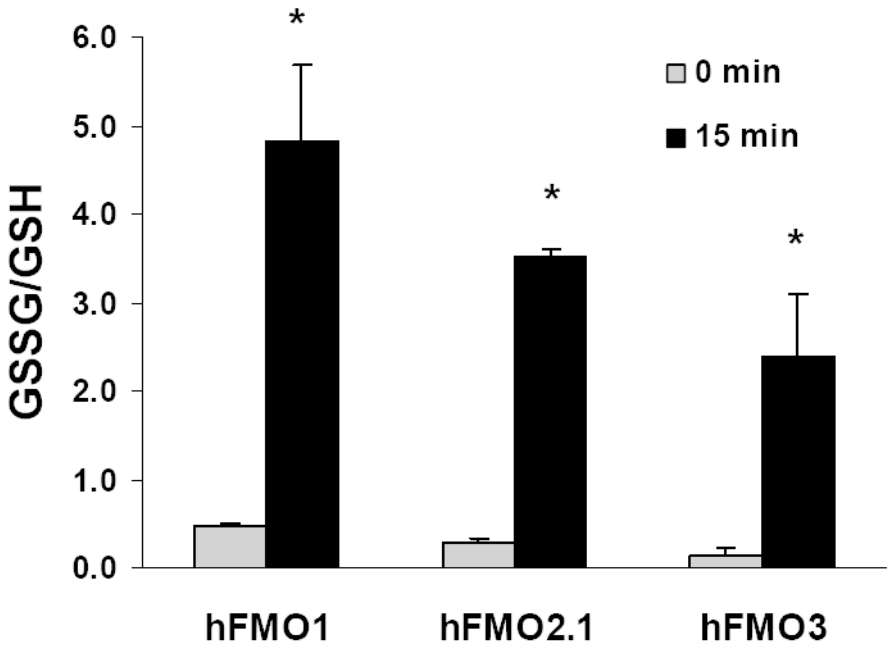

Figure 7.
Effect of ETA (200 μM) metabolism by human FMOs on GSSG/GSH ratios. Bars represent averages of ratios from three experiments ± SD. Ratios were log transformed and analyzed by a two-tailed ratio t-test as described in Methods. * Significantly different from 0 time; $p \leq 0.001$.

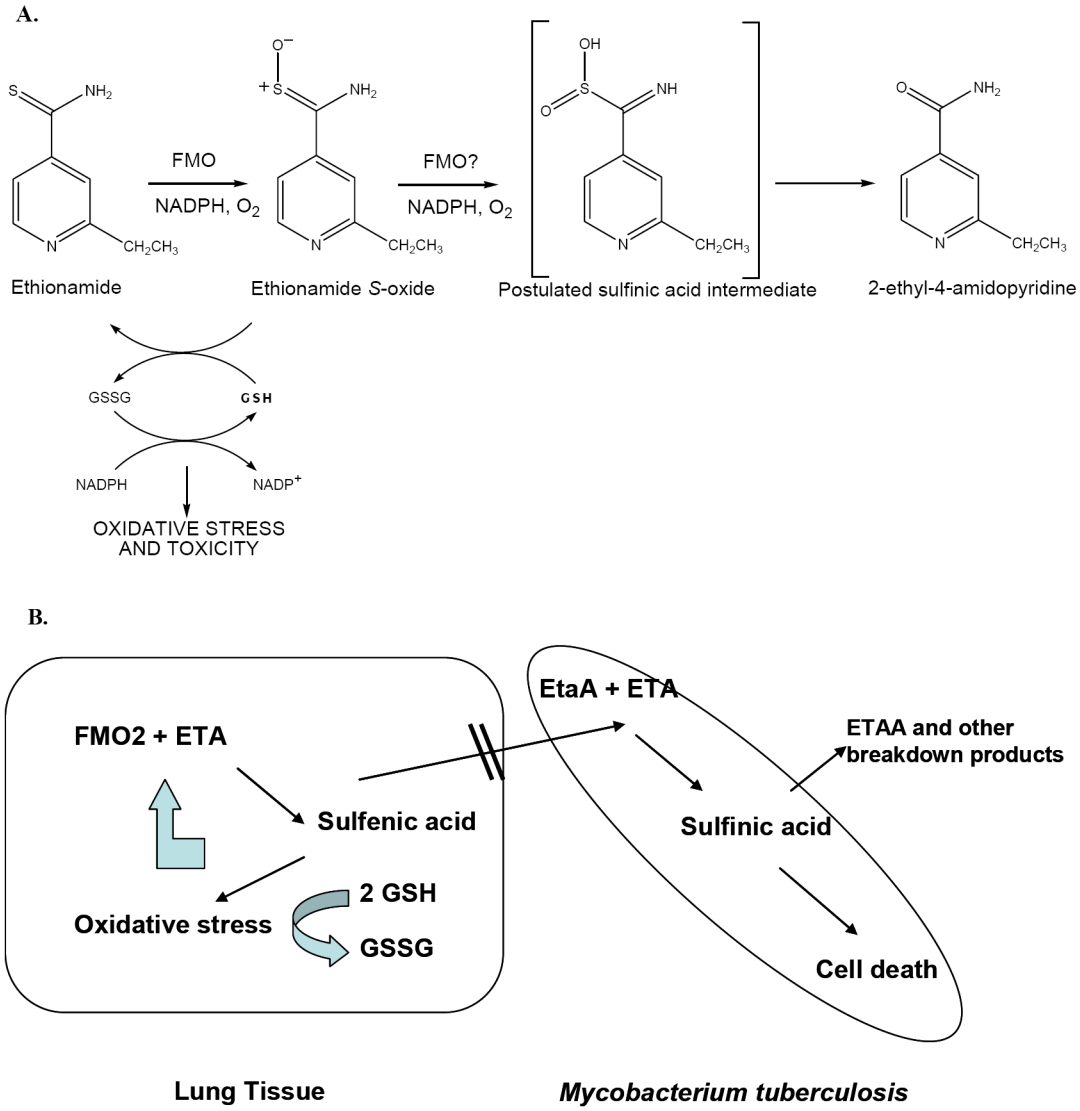

Figure 8.

(A) Proposed scheme of ETA metabolism by FMOs; (B) Cartoon depicting proposed scheme of ETA metabolism in lung of TB patients with active pulmonary infection and expressing the active FMO2.1 protein.

Table 1

Kinetic values for mouse and human FMOs and ETA[1].

Protein	K_m (μM)	k_{cat} (min^{-1})	Specificity Constant (k_{cat}/ K_m)
hFMO1	105	89.9	0.86
hFMO2	261	48.3	0.19
hFMO3	336	58.4	0.17
mFmo1	104	271	2.62
mFmo2	2131	567	0.21
mFmo3	114	60.6	0.53

[1] Assays performed at pH 9.5 on at least two expressed protein preparations with ETA concentrations of 50 to 500 μM. Kinetic values determined with the Michaelis-Menton Module of GraphPad Prism 5. No activity was detected with expressed hFMO5.