Expression and characterization of functional dog flavin-containing monooxygenase 3

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Abbreviations: FMO, flavin-containing monooxygenase; P450, cytochrome P450; dFMO1, dog FMO1; hFMO1, human FMO1; dFMO3, dog FMO3; hFMO3, human FMO3; RT-PCR, reverse transcription polymerase chain reaction; Sf-9, Spodoptera frugiperda; MOI, multiplicity of infection; FAD, flavin adenine dinucleotide; HPLC, high performance liquid chromatography; tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; EDTA, (ethylenedinitrilo)tetraacetic acid; HEPES, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]); MRM, multiple reaction monitoring; DLM, dog liver microsomes.
Abstract

Mammalian flavin-containing monooxygenase (FMO) enzymes catalyze oxidation at nucleophilic, heteroatom centers and are important for drug, xenobiotic and endogenous substrate metabolism. In human liver, hFMO3 is the most abundant FMO isoform and is known to contribute to the hepatic clearance of a variety of clinical drugs. The purpose of the current study was to express and compare the dog (beagle) FMO3 (dFMO3) to hFMO3. A full-length dFMO3 cDNA was obtained from liver by reverse transcription-polymerase chain reaction. Using a baculovirus expression system in Sf-9 insect cells, dFMO3 was expressed to protein levels of 0.50 nmol/mg, as determined by LC-fluorescence detection. Expressed dFMO3 displayed Michaelis-Menten kinetics, catalyzing NADPH-dependent N-oxidation of benzydamine, with $K_m$ and $V_{max}$ values of 18.6 µM and 0.63 nmol N-oxide formed/min/nmol of enzyme, respectively. Benzydamine N-oxidation catalyzed by human FMO3 showed values of 42.6 µM ($K_m$) and 3.56 nmol/min/nmol of enzyme ($V_{max}$). Human FMO3 was observed to catalyze the S-oxidation of sulindac sulfide, with respective $K_m$ and $V_{max}$ values of 69.3 µM and 35.4 nmol/min/nmol of enzyme. dFMO3 also catalyzed sulindac sulfide S-oxidation with 6.8 nmol/min/nmol of enzyme being the highest velocity observed. Finally, Western blot analysis indicated protein expression levels of dFMO3 in pooled dog liver and lung microsomes to be 27 and 9 pmol/mg, respectively. In summary, dFMO3 appears to be a functional enzyme expressed at appreciable levels in liver, but one with some kinetic properties that are substantially different from its human homolog hFMO3.
Introduction

The flavin-containing monooxygenases (FMOs) are a family of enzymes capable of catalyzing the oxidation of various drugs, xenobiotics and endogenous substrates containing a soft nucleophile, usually nitrogen or sulfur (Cashman, 2000; Krueger and Williams, 2005). In humans, FMO-dependent drug metabolism can have important clinical implications (Cashman, 2000). Like cytochrome P450s, the FMOs are microsomal enzymes that require NADPH and O2, and FMOs have shown overlapping substrate specificity with P450s. FMOs also typically convert their xenobiotic substrates into more polar products that are less pharmacologically-active and more easily excreted, thereby enhancing their elimination from the body (Cashman, 1995). The mammalian FMO gene family includes five different isoforms (FMO1 through FMO5) (Lawton et al., 1994). In humans, as well as in a variety of preclinical species, tissue distribution patterns of FMO isoforms have been described (Cashman and Zhang, 2006; Phillips and Shephard, 2008). FMO3 is the most abundantly expressed isoform in adult human liver, existing at levels similar to the major human liver P450 isoform, CYP3A4 (Haining et al., 1997). FMO3 has been observed to contribute to the metabolic clearance of a variety of drugs, e.g. cimetidine, nicotine, and tamoxifen, as well as the diet-derived substrate trimethylamine (Cashman et al., 1992; Cashman et al., 1993; Mani et al., 1993). It has been demonstrated that FMO3 is essential for the N-oxygenation and metabolic clearance of trimethylamine (Dolphin et al., 1997; Lang et al., 1998). This led to the discovery that human FMO3 is also a highly polymorphic gene (Koukouritaki et al., 2005). Specifically, a total of 29 allelic variants of FMO3 have each been observed to be associated with the human condition known as trimethylaminuria or “fish odor syndrome” (Phillips and Shephard, 2008).

Preclinical species, e.g. mouse, rat, dog, monkey, serve as a valuable tool for the drug discovery and drug development process. Data obtained from in vivo metabolism and toxicology studies in these models are essential for scaling and prediction of pharmacokinetic and pharmacodynamic behavior of drug candidates to be potentially administered to humans. Importantly, the accuracy of such predictions greatly depends on similarity of metabolic processes between species. This makes it particularly important to have a complete characterization of the metabolic pathways of a candidate compound in a given species prior to determination of safe doses for humans.
The dog is the most widely used nonrodent species in preclinical drug safety studies (Gad and Gad, 2003). We suggest that to gain a definitive understanding of the relevance of drug metabolism in dogs to that in humans, a characterization of species differences in FMOs is necessary. The cDNA sequence for dog FMO1 (dFMO1) (Lattard et al., 2002) and a characterization of functional dFMO1 have been reported (Stevens et al., 2003). While the cDNA sequence of dog FMO3 (dFMO3) has also been published previously, the investigators were unable to demonstrate activity following recombinant expression of the enzyme (Lattard et al., 2002). Therefore, the objective of this study was to quantify the protein expression levels of dFMO3 in various tissues and perform a brief functional characterization of a recombinant dFMO3, a highly homologous canine form of the major human liver FMO, hFMO3.
Materials and Methods

**Materials.** Benzydamine hydrochloride, benzydamine N-oxide, sulindac sulfide, sulindac sulfoxide, clozapine, clozapine N-oxide, FAD, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Human FMO3 Supersomes (1.0 nmol FMO/mg by FAD content), WB-FMO3 (rabbit anti-human FMO3 antiserum), and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from BD Gentest Corp. (Woburn, MA). Pooled human liver microsomes (mixed gender and age), and pooled microsomes from beagle dog tissues (liver, lung, kidney, and intestine) were purchased from Xenotech, LLC (Lenexa, KS).

**DNA and Viral Constructions.** Total RNA was isolated from liver tissue freshly obtained from a single adult female beagle dog using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. First-strand cDNA synthesis was performed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with slight modifications to the manufacturer's suggested protocol. Oligonucleotide primers including sense (5'-GTAACTATGGGGAAAAGAGTGGC-3') and antisense (5'-GGAATGATAATTAGATCAATGCGAGGA-3') were designed based on the published dog FMO3 sequence (Lattard et al., 2002) and used in a polymerase chain reaction with first-strand cDNA synthesis product and Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The resulting ~1600-bp PCR product was gel-purified by agarose gel electrophoresis and ligated into pGEM-T Easy vector (Promega Corp., Madison, WI). The cDNA was sequenced on both strands using a series of 10 primers and Gene Codes Sequencher v4.7 (Ann Arbor, MI). Sequence analysis indicated a 1599-bp sequence identical to the published dog FMO3 sequence (Lattard et al., 2002). The dog FMO3 cDNA was digested with NotI restriction endonuclease and ligated into NotI-cleaved pFastBac1 of the Bac-to-Bac Baculovirus Expression System (Invitrogen). Recombinant baculovirus preparation and selection were performed as recommended by the manufacturer.

**Viral Infection of Sf-9 Cells and Sf-9 Microsome Isolation.** Sf-9 insect cells were maintained in SF-900 II Serum Free Media at 27°C on an orbital shaker (90 rpm). Cells were transfected with recombinant dog FMO3 bacmid DNA using Cellfectin reagent according to the manufacturer’s protocol.
At 72 h post-transfection, the transfection broth containing recombinant baculovirus was harvested and amplified in Sf-9 suspension cultures. The viral titer of the amplified baculovirus stock was determined using the BaculoELISA assay (Clontech, Mountain View, CA). Cells were infected in shaker flasks at a density of 1.5 x 10^6 cells/ml and an MOI of 1 in media supplemented with FAD (10 μg/ml). Insect cells were harvested 72 h after infection and washed with sucrose buffer (pH 7.5) containing 280 mM sucrose, 25 mM HEPES, 1 mM EDTA, and protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Cells were pelleted, resuspended in sucrose buffer, homogenized on ice with a glass-glass homogenizer, and microsomes prepared by differential centrifugation. The protein concentration was determined by BCA Protein Assay (Pierce Biotechnology, Rockford, IL).

**Characterization of FMO3-Containing Insect Cell Membranes.** Expression levels of the recombinant dog FMO3 enzyme were assessed by measurement of both holoenzyme and apoprotein. First the FAD content of microsomes isolated from the dFMO3-baculovirus-infected and mock-infected Sf-9 cells was quantified. Accordingly, heat treatment of the samples was followed by reversed-phase high-performance liquid chromatography with fluorometric detection as described previously (Lang and Rettie, 2000). Given that naïve Sf-9 cells contain endogenous FAD, flavin levels for dFMO3 microsomes were adjusted for levels observed in mock-infected Sf-9 cells. In addition, immunoreactive dFMO3 of these Sf-9 microsomes, as well as pooled microsomes (n=8) from dog liver, kidney, intestine (duodenum/jejunum) and lung (Xenotech, LLC) were quantified by Western blot. Following separation of protein bands on a 10% SDS polyacrylamide gel (120 V, 2h), proteins were transferred to a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen). Antibody dilutions included a 1:500 dilution of primary antibody WB-FMO3 (BD Gentest) and a 1:10,000 dilution of the secondary antibody, IRDye 680 Goat Anti-Rabbit IgG, (LI-COR Biotechnologies, Lincoln, NE). According to the manufacturer (BD Gentest), WB-FMO3 is a polyclonal anti-peptide antisera antibody that was generated against amino acids 265-282 (KHENYGLMPNGVLRKEP) of the human FMO3 protein and does not cross-react with human FMO1. Additionally, although 11 of the 21 amino acids in the hFMO3 peptide antigen correspond to the sequence for dFMO1 (Lattard et al., 2002), the Western blot analysis with WB-FMO3, which included 1 μg of dFMO1 microsomes (Stevens et al., 2003) in lane 6 of Fig. 1, indicated there is no cross-reactivity with this homolog. Protein-antibody complexes were detected by measuring fluorescent signal on the
Odyssey Infrared Imaging System (LI-COR Biotechnologies, Lincoln, NE) and quantified using a standard curve composed of hFMO3 microsome standard (BD Gentest).

**Enzyme Kinetics.** Based on preliminary determinations of the linearity of metabolite formation with protein concentration and incubation time, all incubation conditions were established in 0.1 M tricine buffer and 1 mM NADPH in a total volume of 500 µl. Kinetic experiments for benzydamine and clozapine N-oxidation were determined at pH, 7.4 and 8.4, respectively. On the other hand, kinetic experiments for sulindac sulfide metabolism were conducted at pH 9.0 to maintain sufficient substrate solubility. All incubations included negative controls that did not contain NADPH. All negative control incubations were quenched at time zero. Background metabolite levels were always subtracted from the level of metabolite produced in corresponding NADPH-containing incubations.

Benzydamine N-oxidation has been shown to be a high-velocity reaction catalyzed by hFMO3 (Lang and Rettie, 2000; Stormer et al., 2000; Yeung et al., 2007). Samples were incubated for 3 min at 37°C prior to addition of substrate to initiate the reaction. Incubations were terminated after 10 minutes by addition of three volumes of cold acetonitrile. The quenched reactions were centrifuged for 5 min at 3700g. LC-MS/MS analysis was performed using a Shimadzu LC-20 AD HPLC pump (Shimadzu, Columbia, MD), and a CTC PAL autosampler (Leap Technologies, Carrboro, NC). Analytical separation was achieved using binary gradient chromatography conditions (5.5 min run time; 0-95%) utilizing a Zorbax xDB C-18 (30 x 2.1, 3.5 µm) analytical column. The mobile phases used for analysis were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. A sample injection volume of 10 µl was introduced to the 5-µl loop, and the mobile phases were delivered at a 0.45 ml/min flow rate. An Applied Biosystems API 3000 triple-quadrupole mass spectrometer in the positive mode, equipped with a Turbo Ion Spray source was used. The probe was operated at a sprayer voltage of 5000 V and an electrospray source temperature of 400°C. All parameters were optimized for each component in a multiple reaction monitoring (MRM) method. Benzydamine N-oxide (collision energy of -22 eV) and tolbutamide as the internal standard (collision energy of -20 eV) were monitored using the m/z transitions 310 → 102 and 271 → 155, respectively. Metabolite formation was quantified by comparing peak area ratios (metabolite/internal standard) in incubations to ratios obtained from a standard curve containing known amounts of metabolite. Sulindac S-oxidation has likewise been shown to be a high-velocity reaction
catalyzed by hFMO3 (Hamman et al., 2000). Incubation conditions and workup of samples for analysis were similar to those performed with benzydamine, and the LC-MS/MS analytical conditions were identical as well, with the following two exceptions: collision induced dissociation was conducted at a collision energy of 65 eV; and sulindac S-oxide was detected using the $m/z$ transition 357→233.

Clozapine $N$-oxidation has been shown to be a high-velocity reaction catalyzed by hFMO3 (Tugnait et al., 1997). LC-MS/MS analytical conditions were identical to those used for benzydamine $N$-oxide and sulindac sulfoxide, except that collision induced dissociation was conducted at collision energy of 61 eV; and clozapine $N$-oxide was detected using the $m/z$ transition 343→192. Kinetic parameters ($K_m$ and $V_{max}$) were determined using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and a nonlinear regression (least squares) curve fit. Incubations were performed in triplicate.

**Results and Discussion**

**Expression of Dog FMO3.** The application of RT-polymerase chain reaction to a beagle dog liver cDNA library yielded a full-length coding sequence for dFMO3. The deduced amino acid sequence for dFMO3 was identical to that described previously, and 83, 82, and 56% similar to sequences for human FMO3, rat FMO3, and dog FMO1, respectively (Lattard et al., 2002). Sf-9 insect cell cultures were subsequently transformed with dFMO3 using a baculovirus expression system. Microsomal preparations from transfected cells showed high levels of dFMO3 protein expression as determined by Western blot analysis using a peptide-specific antibody to human FMO3 (Fig. 1). Specifically, infection of Sf-9 cells using a MOI of 1.0 resulted in dFMO3 expression levels of 1.07 nmol/mg of microsomal protein (Fig. 1., lane 7). This expression level was slightly lower than the level (1.36 nmol/mg) reported for expression of the human FMO3 using the same expression system and cell line (Yeung et al., 2007). However, it is important to note that the actual dFMO3 holoenzyme content, i.e. the recombinant enzyme with the incorporated FAD prosthetic group, was 0.50 nmol/mg, indicating that 46% of the dFMO3 protein could be expected to be catalytically active. This level of holoenzyme relative to total protein expression level is comparable to that observed by Yeung et al. when expressing hFMO3 (Yeung et al., 2007). Notably, FAD was detectable in mock-transfected Sf-9 cells at a concentration of only 0.016 nmol/mg (data not shown).

**Quantification of dFMO3 in Various Dog Tissues by Immunoblot Analysis.** The antibody used for the detection and quantification of dFMO3 reacts with a peptide corresponding to amino acid
positions 265-282 of human FMO3. Quantification of dFMO3 protein levels using the WB-FMO3 antibody requires that the affinity of the antibody for hFMO3 and dFMO3 be identical, and this peptide matched exactly with the corresponding amino acid sequence for dFMO3, with the exception of a conservative valine (human)-to-threonine (dog) substitution. An immunoreactive protein in microsomes from a number of dog tissues had the same electrophoretic mobility as the FMO3 microsome standard and the expressed dFMO3, with migration indicating a protein size of ~56 kDa (Fig. 1). Consistent with a previous report (Lattard et al., 2002), dFMO3 was determined to be expressed in liver and lung but was below the lower limit of detection in kidney. dFMO3 expression was also below the lower limit of detection in intestine (Fig. 1). According to the standard curve, dFMO3 expression levels in pooled microsomes (n=9) of liver and lung were 27 and 9 pmol/mg, respectively (Fig. 1). Given that there are known gender differences in liver FMO expression in other species, a Western blot was performed on pooled, gender-specific microsomes of male (n=11) and female (n=12) beagle dog liver (Falls et al., 1995; Ripp et al., 1999). This revealed that male DLMs have less than half as much dFMO3 (16.3 pmol/mg) as female DLMs (36.7 pmol/mg) (figure not shown). In adult human liver, FMO3 protein expression has been reported to vary considerably among individuals, with levels being reported as low as 12.5 pmol/mg and as high as 117 pmol/mg (Haining et al., 1997; Overby et al., 1997; Koukouritaki et al., 2002). For comparison, the 27 pmol/mg level in mixed-gender DLMs that we report here is approximately half the quantity of hFMO3 we detected in pooled (n=50) human liver microsomes, 50 pmol/mg (Fig. 1).

**Comparison of dFMO3 and hFMO3 Enzyme Activity.** Kinetic analysis of benzydamine N-oxidation, clozapine N-oxidation and sulindac sulfide S-oxidation was performed with expressed dFMO3 and hFMO3. These compounds were selected because they are well-established as FMO substrates and because the authentic standard metabolite for each substrate is commercially available, enabling us to take advantage of LC/MS/MS analysis. For benzydamine N-oxidation, the $K_m$ values for dFMO3 and hFMO3 were 18.6 and 42.6 µM, respectively (Table 1). Statistical analysis (t test) indicated that the $K_m$ value for dFMO3 is significantly lower than that for hFMO3. The rates of enzyme activity for benzydamine N-oxidation by dFMO3 and hFMO3 also differed significantly, with the $V_{max}$ of dFMO3 being 5.6-fold lower than that of the human ortholog. While there is some information in the literature on interspecies
differences in FMO-mediated metabolism of select substrates, there appear to have been relatively few investigations to date.

This led us to investigate the \(N\)-oxidation of clozapine by both human and dog FMO3. While human FMO3-mediated catalysis of clozapine \(N\)-oxidation was similar to values reported previously by Tugnait et al. (data not shown), dFMO3-mediated \(N\)-oxidation did not display saturability (maximal observed activity of 1.5 nmol/min/nmol of enzyme) (Tugnait et al., 1997). Comparisons of recombinant FMO3 from mouse and human have demonstrated relatively similar kinetics of methimazole \(S\)-oxidation, and methimazole \(S\)-oxidation by these two isoforms can be inhibited by a variety of known \(N\)- and \(S\)-containing substrates to a similar extent, as well (Falls et al., 1997). Additionally, Stevens et al. have demonstrated that the \(K_m\) values for \(N\)-oxidation of imipramine by dFMO1 (4.7 \(\mu\)M) and hFMO1 (7.8 \(\mu\)M) are also relatively similar (Stevens et al., 2003).

In the case of sulindac sulfide \(S\)-oxidation, the highest velocity observed for dFMO3 was 6.8 nmol/min/nmol of enzyme. Attempts to determine the \(K_m\) value of dFMO3 for sulindac sulfide were unsuccessful, as the enzyme did not display saturability at concentrations as high as 1000 \(\mu\)M (substrate solubility could not be achieved at greater concentrations). On the other hand, kinetic parameters of sulindac sulfide \(S\)-oxidation by hFMO3 were defined, by a \(K_m\) of 69.3 \(\mu\)M and a \(V_{max}\) of 35.4 nmol/min/nmol of enzyme (Table 1). This \(K_m\) value was considerably higher than those reported previously for the respective (R)- (16 \(\mu\)M) and (S)-sulindac sulfoxide (51 \(\mu\)M) metabolites (Hamman et al., 2000). This may be due to the different pH conditions at which each of the experiments were performed. Regarding \(V_{max}\), we are not able to compare the value shown in Table 1 to those reported previously since activity levels reported by Hamman et al. were not described in terms of activity per unit of FMO3 enzyme (Hamman et al., 2000). As mentioned above, for both dFMO3 and hFMO3, all reactions had to be performed at pH 9.0, making it difficult to infer the physiologic relevance of the respective kinetic parameters of dFMO3 and hFMO3 for sulindac sulfide \(S\)-oxidation. However, we report that sulindac sulfide \(S\)-oxidation was observable at pH 7.4 at 100 \(\mu\)M for both hFMO3 (3.3 nmol/min/nmol) and dFMO3 (39.4 pmol/min/nmol). There is also clear evidence from experiments with human liver microsomes that FMO3 is capable of sulindac sulfide \(S\)-oxidation at physiologic pH (Hamman et al., 2000).
In summary, our study has demonstrated that dFMO3 is expressed at appreciable levels in beagle dog liver as well as in lung. Given that FMO3 is the major form expressed in human liver, expressed dog FMO3 could be an important preclinical tool when it comes to comparing pathways of hepatic drug metabolism. Our data demonstrates that dFMO3 is capable of catalyzing the N- and S-oxidation of prototypical FMO substrates. Importantly, given that the kinetic data for dFMO3 displayed some significant differences when compared with hFMO3, additional characterization of the enzyme will be necessary in order to properly interpret data from preclinical experiments where FMO3 appears to have a role. While there is considerable information on species differences in tissue distribution and expression profiles of FMOs, there is relatively little information describing metabolism of specific FMO substrates (or substrates with specific structural features) across all species. Conversely, studies that compare not just enzyme activity but also the effect of structural properties on kinetic parameters may provide greater insight and appreciation for the catalytic function of the dog FMO3 isoform and other FMO3 orthologs.

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References


Footnotes

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Legends for figures

**Fig. 1.** Western blot of FMO3 microsomal preparations, human liver microsomes, and beagle dog microsomes from several tissues using anti-human FMO3 antibody. Lanes 1 to 5, expressed hFMO3 standard (0.25, 0.5 pmol, 1.0, 2.5 and 5.0 pmol of human FMO3 protein); lane 6, expressed dog FMO1 microsomes, 1 µg; lane 7, expressed dog FMO3 microsomes, 0.1 µg; lane 8, pooled human liver microsomes, 5 µg; lane 9, pooled dog liver microsomes, 5 µg; lane 10, pooled dog intestine microsomes, 5 µg; lane 11, pooled dog kidney microsomes, 30 µg; lane 12, pooled dog lung microsomes, 30 µg.
Table 1. Kinetics of benzydamine and sulindac sulfide metabolism by dFMO3 and hFMO3.

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<tr>
<th>Enzyme</th>
<th>Benzydamine $N$-Oxidase Activity</th>
<th>Sulindac $S$-Oxidase Activity</th>
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<tr>
<td></td>
<td>$K_m$ (±SE)</td>
<td>$V_{max}$ (±SE)</td>
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<tr>
<td>dFMO3</td>
<td>18.6 (±4.5)</td>
<td>0.63 (±0.03)</td>
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<tr>
<td>hFMO3</td>
<td>42.6 (±7.7)</td>
<td>3.56 (±0.16)</td>
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Table 1. Kinetic parameters for benzydamine $N$-oxidase and sulindac $S$-oxidase activity for expressed dFMO3 and hFMO3. Benzydamine $N$-oxidase assays were conducted at pH 7.4 (0.1 M tricine) using a substrate concentration range of 1 to 1000 μM. Sulindac $S$-oxidase activity assays were conducted at pH 9.0 (0.1 M tricine) using a substrate concentration range of 1 to 200 μM for hFMO3 and 1 to 1000 μM for dFMO3. Kinetic parameters were determined in GraphPad Prism 5.0 using a Michaelis-Menten least squares (ordinary) fit. This statistical analysis included determinations of standard error for both $K_m$ and $V_{max}$. (n = 3) n.d. – The $K_m$ value for dFMO3 could not be determined, as the enzyme did not display saturability at concentrations through 1000 μM of sulindac sulfide. * Activity levels were normalized to nmol of catalytically active enzyme (FAD content) used in the assay, as determined by LC-fluorescence.
Figure 1.