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ACCELERATED COMMUNICATION

Expression and Characterization of Functional Dog Flavin-Containing Monooxygenase 1

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ABSTRACT

A full-length dog (beagle) flavin-containing monooxygenase 1 (FMO1) cDNA (dFMO1) was obtained from liver by reverse transcription-polymerase chain reaction. The amino acid sequence of dFMO1 was 89% homologous to human FMO1. Using a baculovirus expression system in *Sf*-9 insect cells, dFMO1 was expressed to protein levels of 0.4 nmol/mg, as determined by immunoquantitation. The flavin content of the expressed enzyme was consistent with immunodetectable dFMO1 protein levels. Expressed dFMO1 catalyzed NADPH-dependent methyl *p*-tolyl sulfide oxidation, with K_m and V_{max} values of 98.6 μM and 63.8 nmol of *S*-oxide formed/min/mg of protein, respectively. By comparison, human FMO1 showed similar values of 87.1 μM (K_m) and 51.0 nmol/min/mg (V_{max}). Activity for dFMO1 showed characteristic pH dependence, with a 4.5-fold increase in *S*-oxidase activity as the incubation pH increased from 7.6 to 9.0. Human FMO1 also showed an increase in reaction rate with pH but a somewhat lower optimum of 8.0 to 8.4. dFMO1 also catalyzed imipramine *N*-oxidation, with a K_m of 4.7 μM and a V_{max} of 82.1 nmol/min/mg of protein. This enzyme displayed other characteristics of FMO enzymes, with rapid depletion of enzyme activity upon heating in the absence of NADPH. Protein levels of 74 pmol of dFMO1/mg of microsomal protein were determined for a pooled liver microsome sample, suggesting that this enzyme is a major canine hepatic monooxygenase. In conclusion, the expression and characterization of catalytically active dFMO1 will allow the role of this enzyme in the metabolism of xenobiotics to be determined.

The flavin-containing monooxygenases (FMO) are a family of enzymes that convert nucleophilic heteroatom-containing drugs and xenobiotics to polar products that are more easily excreted (Ziegler, 1990; Hines et al., 1994; Cashman, 2000). These enzymes require the cofactor NADPH for activity, and common reactions are the formation of *N*- and *S*-oxides. In humans, FMO-dependent metabolism of endogenous or exogenous compounds can have important clinical implications. For example, FMO forms have been shown to be important for the metabolic clearance of drugs such as nicotine, chlorpromazine, and clozapine (Cashman et al., 1992; Bhamre et al., 1995; Tugnait et al., 1997). Also, FMO is responsible for trimethylaminuria, a syndrome caused by a mutation of the human *FMO3* gene and a resultant deficiency in the *N*-oxygenation of the odorous amine trimethylamine to the non-odorous *N*-oxide metabolite (Dolphin et al., 1997; Cashman et al., 2000). Multiple FMO forms have been identified in most mammalian species, including humans, with the gene family consisting of five members (FMO1–5) that exhibit at least 80% amino acid identity for orthologous forms (i.e., human FMO1 and rat FMO1) and 51 to 58% identity for homologous forms (i.e., human FMO1 and human FMO3) (Lawton et al., 1994). In humans, the expression of hepatic FMO forms changes with development. FMO1 is found in fetal liver; however, dramatic changes in enzyme expression at birth result in FMO3 as the predominant FMO form in adult human liver (Dolphin et al., 1996; Koukouritaki et al., 2002).

During the drug discovery and development process, numerous safety studies must be conducted in preclinical species such as rat, dog, and monkey before a new chemical entity can be administered to humans. In addition, allometric scaling of pharmacokinetic parameters determined for preclinical species and extrapolation of in vitro results from metabolism and toxicology studies is routinely conducted before human studies, and the accuracy of these predictions depends largely on similarity of metabolic processes between species (Lave et al., 1997; Obach et al., 1997). Because differences in the expression and function of drug-metabolizing

ABBREVIATIONS: FMO, flavin containing monooxygenase; P450, cytochrome P450; dFMO1, dog FMO1; hFMO1, human FMO1; MPT, methyl *p*-tolyl; MPTS, methyl *p*-tolyl sulfide; *Sf*-9, *Spodoptera frugiperda*; tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

enzymes such as FMO have been documented and may be linked to species differences in exposure to parent drug or metabolite(s), it is important to have a thorough understanding of metabolic routes before human safety studies are performed. The assumption that an acceptable therapeutic index for a compound determined by animal studies will translate directly to safety in humans can be flawed. For example, a compound shown to undergo FMO1-dependent metabolism to a toxic product in rats may not pose a significant safety issue for humans because adult humans do not express hepatic FMO1 (Dolphin et al., 1991; Lattard et al., 2002a). FMO forms can also show overlapping substrate specificity with the cytochromes P450 and thereby complicate the interpretation of reaction phenotyping studies performed during drug development (Grothusen et al., 1996; Ring et al., 1996; Lang and Rettie, 2000).

The dog is the most common nonrodent species used for preclinical drug safety studies. The cDNA sequence and tissue distribution of dog FMO1 and FMO3 were recently published (Lattard et al., 2002b); however, the investigators were unable to demonstrate activity for the expressed enzymes. To understand the relevance of drug metabolism in dogs to the human situation, a more complete understanding of species differences in FMO characteristics and expression is necessary. Human FMO1 is commercially available; therefore, the expression of the homologous dog form is the most definitive means of comparing FMO-dependent metabolic pathways between these species. Toward this goal, we report the first heterologous expression and characterization of a dog FMO form, dog FMO1 (dFMO1).

Materials and Methods

Materials. MPT sulfide (MPTS), MPT sulfoxide, imipramine HCl, FAD, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Human FMO1 Supersomes (baculovirus insect cell expressed, ~0.5 nmol FMO/mg by FAD content), human anti-rabbit FMO1 antiserum, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Gentest Corp. (Woburn, MA). Pooled human (mixed gender and age) and male dog liver microsomes were purchased from Xenotech (Kansas City, KS). All other reagents are described below or were obtained from standard commercial sources.

DNA and Viral Constructions. Liver tissue obtained from adult male beagle dogs was cut into small pieces and immediately frozen in liquid nitrogen. Total RNA was prepared using the RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) and the concentration was determined by spectrophotometry (Sambrook et al., 1989). First-strand synthesis was performed using SuperScript II RNase reverse transcriptase (Invitrogen, Carlsbad, CA) with slight modifications to the manufacturer's suggested protocol. Oligonucleotides were designed from published sequence data and polymerase chain reaction was performed using Accuzyme DNA polymerase (Bioline Ltd, London, U.K.) to obtain the full-length dog FMO1 cDNA. The cDNA was sequenced to confirm the integrity of the clone and was in agreement with the published dog FMO1 sequence (Lattard et al., 2002b). The FMO1 cDNA was subcloned into the Bac-to-Bac baculovirus expression system (Invitrogen). Recombinant baculovirus preparation and selection were performed following the procedures recommended by the manufacturer.

Cells and Viral Infections. *Sf*-9 cells (serum-free media adapted) (Invitrogen) were maintained in SF-900 II SFM (Invitrogen) supplemented with Gentamicin (100 μg/ml; Invitrogen) at 27°C on an orbital shaker (90 rpm). Cells were transfected with recombinant dog FMO1 bacmid DNA with CellFECTIN reagent according to the manufacturer's protocol (Invitrogen). Recombinant baculovirus was harvested 72 h after transfection, amplified in suspension *Sf*-9 cultures, and virus titer was determined according to the manufacturer's protocol (Invitrogen). Cells were infected in shaker flasks at a density of 2×10^6 cells/ml and an MOI of 10 in media supplemented with FAD (10 μg/ml).

Membrane Fraction Preparation. Insect cells were harvested at 72 h after infection and washed with sucrose buffer containing 280 mM sucrose, 25 mM HEPES, pH 7.5, 1 mM EDTA, pH 7.5, 10 μg/ml FAD, and protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Cells were centrifuged, resuspended in sucrose buffer, homogenized on ice with a glass-glass homogenizer, and microsomes prepared by differential centrifugation. The protein pellets were resuspended in 3 pellet volumes of sucrose buffer supplemented with FAD (10 μg/ml) and the protein concentration was determined using bovine serum albumin as a standard (Bradford, 1976). Microsomal FAD levels were measured by heat treatment of the samples followed by reversed-phase high-performance liquid chromatography with fluorometric detection as described previously (Lang et al., 1998). Control *Sf*-9 cells did contain endogenous FAD; therefore, flavin levels for dFMO1 microsomes were adjusted for levels observed in mock-transfected control *Sf*-9 cells.

Immunoquantitation of FMO1. Microsomes from infected insect cells, control insect cells, dog liver and expressed hFMO1 were separated by SDS polyacrylamide (10% acrylamide) electrophoresis (Laemmli, 1970) and transferred to nitrocellulose. The membrane was blocked, and then incubated with human anti-rabbit FMO1 antiserum. According to the manufacturer (Gentest Corp., Woburn MA), this monoclonal antibody was made to the peptide -EEIN-ARKENKPS- of human FMO1 (amino acid sequence position 408–419) and does not cross-react with human FMO3. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG and proteins detected by chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway NJ). After film development, the optical density of the immunoreactive protein band was determined.

Enzyme Activity. MPT *S*-oxidation has been shown to be a high velocity reaction catalyzed by various FMO forms (Sadeque et al., 1992; Rettie et al., 1994; Yeung et al., 2000). An achiral assay method was conducted according to the data sheets provided by Gentest, with some modifications. Based on preliminary determinations of the linearity of metabolite formation with protein concentration and incubation time, incubation conditions were established using 50 μg (tissue microsomes) or 25 μg (*Sf*-9 cell microsomes) protein, 1 mM NADPH, and 0.1 M tricine in a total volume of 0.25 ml. Samples were incubated for 3 min at 37°C followed by the addition of substrate (in 1% methanol) to start the reaction. Incubations were terminated after 10 min by the addition of 50 μl of acetonitrile, and samples were centrifuged to precipitate protein. The supernatant was analyzed by high-performance liquid chromatography, using a 4.6- × 250-mm, 5-μm C_{18} analytical column (Luna $C_{18(2)}$; Phenomenex, Torrance CA) and a 1 ml/min gradient elution of 40:60 to 60:40 methanol/water over 8 min, followed by substrate elution with 95% methanol. The MPT sulfoxide product was detected by absorbance at 240 nm and quantitated using the authentic standard. Imipramine *N*-oxidation was measured by substrate-dependent oxidation of NADPH at 340 nm (Wyatt et al., 1998), using 25 to 50 μg of microsomal protein in a total volume of 750 μl. For both imipramine *N*- and MPT *S*-oxidase assays, heat inactivation (3 min, 45°C) and NADPH-protection (1 mM during heat treatment) were evaluated using the methods described above. Kinetic parameters (K_m and V_{max}) were determined using Prism 3.0 (GraphPad Software, San Diego, CA) and a one-site binding model (best-fit) of 7 to 10 data points. All incubations were performed in duplicate unless otherwise indicated.

Results and Discussion

Expression of Dog FMO1. The application of RT-polymerase chain reaction to a beagle dog liver cDNA library

yielded a full-length coding sequence for dFMO1. The deduced amino acid sequence for dFMO1 was identical to that described previously, and 89, 84, and 56% similar to sequences for human FMO1, rat FMO1, and dog FMO3, respectively (Lattard et al., 2002b). *Sf*-9 insect cell cultures were subsequently transformed with dFMO1 using a baculovirus expression system (Itagaki et al., 1996; Krueger et al., 2002). Microsomal preparations from transfected cells showed high levels of dFMO1 protein expression as determined by Western blot analysis using a peptide-specific antibody to human FMO1 (Fig. 1). Specifically, preparations where a MOI (virus to cell ratio) of 10 was used produced 0.4 nmol dFMO1/mg of microsomal protein (Fig. 1, lanes 10 and 11). The expression level was somewhat lower than levels reported for other FMOs (Haining et al., 1997; Krueger et al., 2001), but similar to expression levels of commercially available hFMO1 produced by baculovirus expression system technology (Gentest Corp., recent data sheets). Simply increasing the MOI did not increase the levels of expressed dFMO1 (Fig. 1, lane 12); therefore, all subsequent experiments were conducted using the MOI-10 preparation. To ensure that the expression process included the incorporation of the requisite flavin into the protein, FAD levels of dFMO1 were analyzed. The content of 0.35 nmol of FAD/mg of protein agreed closely with protein levels (data not shown), suggesting that only a small percentage of the enzyme existed in the form of apoprotein. In contrast, although flavin was detected in mock-transfected *Sf*-9 cells (~0.15 nmol/mg), these preparations did not show immunoreactive dFMO1 protein (lane 9, limit of detection of 0.05 pmol).

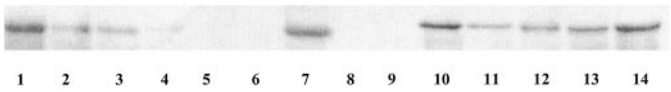

Fig. 1. Western blot of expressed FMO1 microsomal preparations, beagle dog liver microsomes, and human liver microsomes using anti-human FMO1 antibody. Lanes 1 to 5, expressed hFMO1 (1.0, 0.5, 0.25, 0.1, and 0.05 pmol of protein, respectively); lane 6, blank; lane 7, 2 μg of expressed hFMO1; lane 8, 10 μg of pooled human liver microsomes; lane 9, 10 μg of *Sf*-9 control cell microsomes; lane 10, 2 μg of dFMO1 MOI-10; lane 11, 0.5 μg of dFMO1 MOI-10; lane 12, 2 μg of dFMO1 MOI-100; lane 13, 2 μg of dFMO1 MOI-0.5; lane 14, 10 μg of pooled dog liver microsomes.

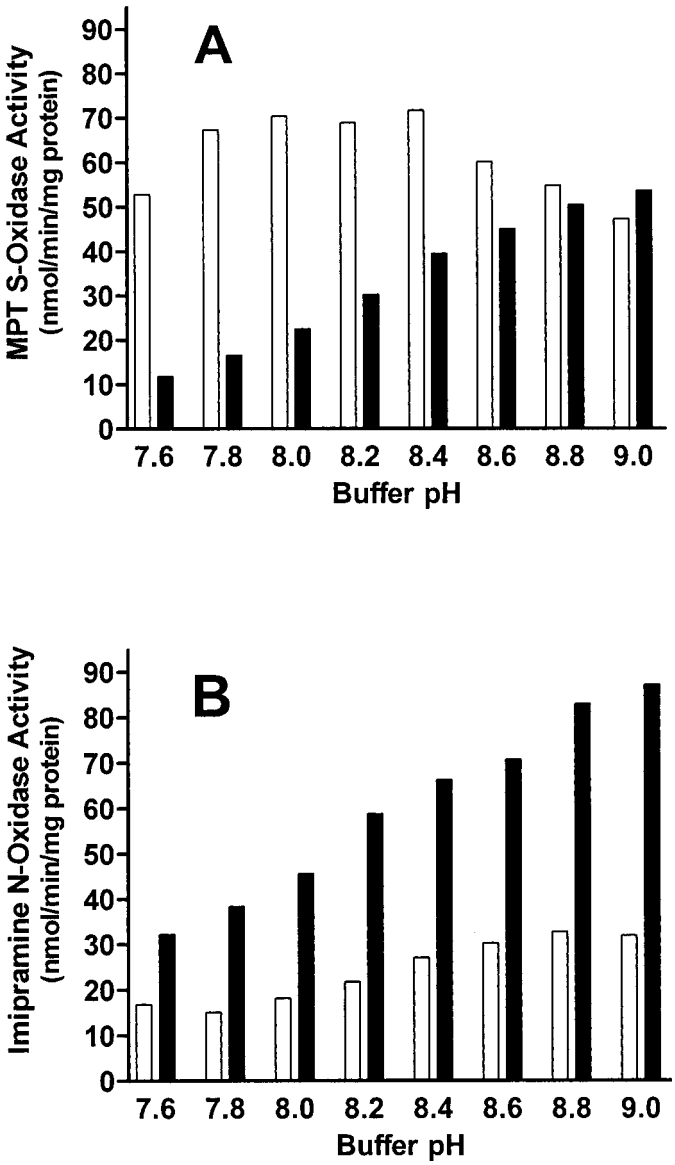

Fig. 2. Effect of buffer pH on MPT *S*-oxidation (A) and imipramine *N*-oxidation (B) by expressed dFMO1 (■) and hFMO1 (□). Microsomes (25 μg of total protein) were incubated with MPTS (1 mM) or imipramine (50 μM) and NADPH for 10 min in 0.1 M tricine buffer over the effective pH range of the buffer.

Antibody Reactivity and Immunoblot Analysis. The antibody used for the detection and quantitation of dFMO1 reacts with a peptide corresponding to amino acid positions 408 to 419 of hFMO1. This peptide matched exactly with the corresponding amino acid sequence for dFMO1, with the exception of a conservative valine (dog)-to-isoleucine (human) substitution. This antibody would not be expected to show cross-reactivity with dFMO3, based on only 25% homology (3 of 12 amino acids) of the hFMO1 peptide antigen with the corresponding sequence for dog FMO3 (Lattard et al., 2002b). Also, expressed dFMO1 had the same electrophoretic mobility as an immunoreactive protein in dog liver microsomes (lane 14), consistent with a previous report in which dFMO1 was detected in liver microsomes using an antibody to the homologous rat form (Lattard et al., 2002b). By immunoquantitation, protein levels of 74 pmol of FMO1/mg of protein were determined for a pooled (n = 3) dog liver microsome sample, clearly demonstrating appreciable hepatic expression of dFMO1. Therefore, based on the peptide antigen used for antibody generation, the strong reactivity with an FMO protein in dog liver microsomes, and the lack of reactivity with expressed human FMO3 (data not shown) or adult human liver microsomes (Fig. 1, lane 8), this anti-human FMO1 peptide antibody seems to be a specific and useful probe for the detection of dFMO1. Finally, consistent with the results of Lattard et al. (2002b), dog FMO3 was detected by immunoblot analysis of liver microsomes, using expressed human FMO3 and the corresponding antibody. Protein levels were estimated at 40 pmol/mg of microsomal protein (data not shown).

Enzyme Activity. A general characteristic of FMO forms is that maximal enzyme activity is typically observed for pH ranges of 8.5 to 10.0, substantially higher than the pH used for the determination of activity for other microsomal enzymes such as the cytochromes P450 (Itagaki et al., 1996; Krueger et al., 2001; Lattard et al., 2001; Krueger et al., 2002). Therefore, the effect of tricine buffer pH on expressed dog and human FMO1-mediated MPT *S*-oxidase and imipra-

TABLE 1

Kinetic parameters for MPT *S*-oxidase and imipramine *N*-oxidase activity for expressed dFMO1, hFMO1, and dog liver microsomes. MPT *S*-oxidase assays were conducted using a substrate concentration range of 1 to 1000 μM and high-performance liquid chromatography analysis as described under *Materials and Methods*. Imipramine *N*-oxidase activity was measured by the rate of NADPH oxidation ($\Delta A_{340\ nm}$) with a substrate range of 3 to 100 μM imipramine. Assays with dog liver microsomes were conducted at pH 9.0 (0.1 M tricine) and assays with expressed enzyme were performed at the pH value previously determined to produce maximal activity (see Fig. 2). Duplicate determinations were made for each substrate concentration. Kinetic parameters were determined using non-linear one-site binding analysis of the Michaelis-Menten curves. Statistical analysis entailed a *t* test of K_m values and the 95% confidence intervals from the curve fit.

Enzyme Source	MPT *S*-Oxidase Activity		Imipramine *N*-Oxidase Activity	
	$K_{m,\ app}$[a]	V_{max}	$K_{m,\ app}$[a]	V_{max}
	μM	*nmol/min/mg*	μM	*nmol/min/mg*
dFMO1	98.6	63.8	4.7	82.1
hFMO1	87.1	51.0	7.8	26.0
Dog liver microsomes[c]	70.6	36.2	3.4	22.4

[a] Relative errors in K_m values (based on S.D. of 1-site curve fit) were 21, 12, and 17% for dFMO1, hFMO1, and dog liver microsomes, respectively.
[b] Relative errors in K_m values (based on S.D. of 1-site curve fit) were 17, 23, and 24% for dFMO1, hFMO1, and dog liver microsomes, respectively.
[c] 74 pmol FMO1/mg protein by immunoquantitation.

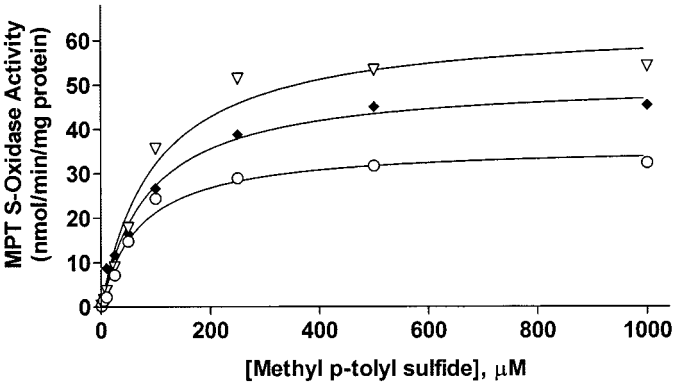

Fig. 3. MPT sulfoxide formation as a function of substrate concentration. Pooled dog liver microsomes (○), expressed dFMO1 (▽), and expressed hFMO1 (◆) were incubated with increasing concentrations of MPTS and activity determined as described under *Materials and Methods*. The curve fit was performed using a one-site binding model and calculated apparent K_m and V_{max} values are provided in Table 1.

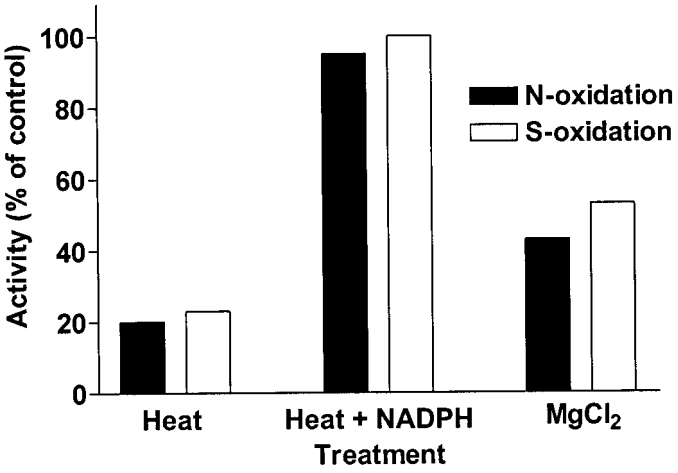

Fig. 4. Effect of heat treatment, NADPH and magnesium chloride on dFMO1-dependent imipramine *N*-oxidase (■) and MPT *S*-oxidase (□) activities. Microsomes were treated for 3 min at 45°C in the absence (left) or presence (center) of 1 mM NADPH. Alternatively, microsomes were incubated in the presence of 0.1 M magnesium chloride (right). Activities are presented relative to the respective control values (no heat treatment, no heat treatment and NADPH added, and absence of magnesium chloride, respectively).

mine *N*-oxidase activities was evaluated before additional activity characterization. As shown in Fig. 2A, MPT *S*-oxidase activity for dFMO1 increased 4.5-fold over the pH range tested, with maximal activity at pH ≥9.0. In contrast, hFMO1 *S*-oxidase activity increased only 44% from pH 7.6 to 8.4 (maximal) and decreased above pH 8.4. For imipramine *N*-oxidation, activity increased 2.7-fold for dFMO1 within the pH range tested whereas hFMO1 showed a narrower range of enzyme activity (Fig. 2B). Together, these data demonstrate both the pH dependence of enzyme activity between orthologous FMO forms and substrate-dependent pH effects for dog and human FMO1.

Kinetic analysis of MPT *S*-oxidation and imipramine *N*-oxidation was performed with expressed hFMO1, dFMO1, and dog liver microsomes. For MPT *S*-oxidation, apparent K_m values ranged from 70.6 μM for dog liver microsomes to 98.6 μM for expressed dFMO1 (Table 1). Statistical analysis (*t* test) showed no difference between the K_m values for MPT *S*-oxidation based on the calculated confidence intervals from the curve fit analysis (Fig. 3). The high rates of enzyme activity for expressed dog and human FMO1 (calculated on a per milligram of protein or nanomoles of FMO basis) are consistent with rates reported for other FMO-dependent *S*-oxidation reactions (Sadeque et al., 1992; Itagaki et al., 1996; Krueger et al., 2002). Dog liver microsomes also showed high activity, particularly when adjusted for the level of expression of FMO1. Regarding imipramine *N*-oxidation, apparent K_m values were similar (not statistically different at the 95% confidence level) for orthologous dog and human forms; however, V_{max} was approximately 3-fold higher for dFMO1 compared with hFMO1. Dog liver microsomes also displayed high activity, consistent with previous reports of efficient imipramine catalysis by several FMO forms and high levels of hepatic expression of FMO1 (Kubo et al., 1997; Wyatt et al., 1998).

FMO-dependent activity has been shown to be effected by various incubation parameters. For example, heat treatment of tissue microsomes or enzyme preparations can dramatically inactivate FMO forms and thus can serve as a convenient biochemical marker for identifying the involvement of these enzymes during in vitro reaction phenotyping studies (Grothusen et al., 1996; Ring et al., 1996; Rawden et al., 2000). In addition, the effect of heat is often form- and species-dependent (Krueger et al., 2002). As shown in Fig. 4, a consistent decrease in MPT *S*- and imipramine *N*-oxidase activities (23 and 20% of control, respectively) was observed after treatment of dFMO1 microsomes for 3 min at 45°C in the absence of NADPH. In contrast, heat treatment of the

enzyme in the presence of NADPH resulted in virtually no change in activity. Heat treatment had a similar effect on enzyme activity in dog liver microsomes, with MPT *S*-oxidase activity decreased 69% and imipramine *N*-oxidation decreased 85% compared with controls (data not shown). Finally, the presence of high levels of magnesium has been shown to alter FMO-dependent activities, with FMO2-catalyzed activities stimulated 2- to 3-fold with the increase in ionic strength (Lawton and Philpot, 1993; Krueger et al., 2002). For dFMO1, the opposite effect was observed, with *S*- and *N*-oxidase activities decreased by 47 and 57%, respectively, in the presence of 0.1 M magnesium chloride.

In summary, our study has demonstrated that dFMO1 is expressed at appreciable levels in control male beagle dog liver and that this enzyme can catalyze the oxidation of sulfur and nitrogen containing compounds at extremely high rates. Thus, expressed dog FMO1 represents an additional tool for identifying hepatic monooxygenase-dependent drug metabolism in this important preclinical species. Clearly, the contribution of dFMO1 to total in vivo metabolism and the implications of hepatic FMO1 expression in dogs given the lack of expression of the orthologous form in adult human liver remain to be investigated. Further studies on the expression, substrate specificity, and metabolic capacity of additional dog FMO forms are necessary to evaluate the contribution of this enzyme family to overall in vivo metabolism.

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