EVIDENCE FOR POLYMORPHISM IN THE CANINE METABOLISM OF THE CYCLOOXYGENASE 2 INHIBITOR, CELECOXIB

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ABSTRACT:

The pharmacokinetics of celecoxib, a cyclooxygenase-2 inhibitor, was characterized in beagle dogs. Celecoxib is extensively metabolized by dogs to a hydroxymethyl metabolite with subsequent oxidation to the carboxylic acid analog. There are at least two populations of dogs, distinguished by their capacity to eliminate celecoxib from plasma at either a fast or a slow rate after i.v. administration. Within a population of 242 animals, 45.0% were of the EM phenotype, 53.5% were of the PM phenotype, and 1.65% could not be adequately characterized. The mean (±S.D.) plasma elimination half-life and clearance of celecoxib were 1.72 ± 0.79 h and 6.4 ml/min/kg for EM dogs and 5.18 ± 1.29 h and 7.15 ± 1.41 ml/min/kg for PM dogs. Hepatic microsomes from EM dogs metabolized celecoxib at a higher rate than microsomes from PM dogs. The cDNA for canine cytochrome P-450 (CYP) enzymes, CYP2B11, CYP2C21, CYP2D15, and CYP3A12 were cloned and expressed in sf9 insect cells. Three new variants of CYP2D15 as well as a novel variant of CYP3A12 were identified. Canine rCYP2D15 and its variants, but not CYP2B11, CYP2C21, and CYP3A12, readily metabolized celecoxib. Quinidine (a specific CYP2D inhibitor) prevented celecoxib metabolism in dog hepatic microsomes, providing evidence of a predominant role for the CYP2D subfamily in canine celecoxib metabolism. However, the lack of a correlation between celecoxib and bufuralol metabolism in hepatic EM or PM microsomes indicates that other CYP subfamilies besides CYP2D may contribute to the polymorphism in canine celecoxib metabolism.

Celecoxib is an inhibitor of the inducible form of cyclooxygenase, cyclooxygenase-2, and does not inhibit cyclooxygenase-1 at plasma concentrations that are therapeutic in osteoarthritis and rheumatoid arthritis (Isakson et al., 1998). Celecoxib is extensively metabolized in animals (Paulson et al., 1997) and humans (Karim et al., 1997) and is excreted primarily in the form of metabolites (Fig. 1). Excretion of intact celecoxib is a very minor route of elimination. Celecoxib is metabolized via a single oxidative pathway in dog and humans. The methyl group of celecoxib is first oxidized to the hydroxymethyl metabolite, followed by further oxidation of the hydroxyl metabolite to the carboxylic acid analog (Paulson et al., 1997; Karim et al., 1997).

In the present study, both in vivo and in vitro canine models were used to characterize the disposition of celecoxib and a polymorphism in the metabolism of celecoxib was discovered. Beagle dogs are used extensively for discovery and safety assessment of new drug candidates. Until recently, little was known about the cytochrome P-450 (CYP) enzymes associated with canine xenobiotic metabolism. Several canine CYPs have been identified by cloning techniques including members of the CYP1A (Uchida et al., 1990; Fukuta et al., 1992), CYP2B (Graves et al., 1990), CYP2C (Komori et al., 1989; Uchida et al., 1990; Blaisdell et al., 1998), CYP2D (Sakamoto et al., 1995; Tasaki et al., 1998a,b), and CYP3A (Ciaccio et al., 1991; Fraser et al., 1997) families. A unique canine cytochrome in the CYP2C subfamily was recently described and designated CYP2C41. The gene coding for this cytochrome was present in approximately 15% of the canine population studied, revealing a unique polymorphism (Blaisdell et al., 1998).

Recently, variants of CYP2D15 have also been described (Roussel et al., 1998). Roussel et al. (1998) used pooled liver RNA from several beagles to generate a CYP2D15 protein (CYP2D15WT2) that exhibited three amino acid changes (S186 → G; I250 → F; and I307 → V) that rendered it distinct from the published CYP2D15 sequence (Sakamoto et al., 1995). Furthermore, Roussel et al. (1998) reported a variant (CYP2D15V1) that differs from CYP2D15WT2 with three base changes that include a silent mutation, I138 → V; and K407 → E. The sequence published by Sakamoto et al. (1995) was not detected in the canine liver cDNA sequences analyzed by Roussel et al. (1998). Finally, a second variant (CYP2D15V2) was reported by Roussel et al. (1998) that was missing the 51 amino acids corresponding to exon 3 of human CYP2D6. The activities of CYP2D15WT2 and CYP2D15V1 for dextromethorphan demethylation were different.

The present paper describes a polymorphism in the metabolism of a xenobiotic by dogs. The cloning of cDNAs for several canine CYP proteins, CYP2B11, CYP2C21, CYP2D15, and CYP3A12, were also described. One CYP2D15 variant reported by Roussel et al. (1998) was cloned, as well as the original CYP2D15 described by Sakamoto.
et al. (1995). Also, three new variants of CYP3A12 were identified. These proteins were used as tools to understand the mechanism of polymorphism in the metabolism of celecoxib by dogs.

Materials and Methods

Chemicals. Celecoxib and radiolabeled celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl-5-14C]benzenesulfonamide were synthesized at Searle (Skokie, IL). The hydroxyl and carboxylic acid metabolites of celecoxib were also synthesized at Searle (Skokie, IL). The specific activity of the [14C]celecoxib was 141 µCi/mg.

HEPES, dimethyl sulfoxide (DMSO), monobasic sodium phosphate, dibasic sodium phosphate, NADP+, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, trichloroacetic acid, and MgCl2 were purchased from Sigma Chemical Co. (St. Louis, MO). Bufuralol and 1'-hydroxybufuralol were obtained from Gentest (Woburn, MA). All other reagents and solvents were of analytical grade.

Pharmacokinetics of Celecoxib. i.v. pharmacokinetics. Male (n = 19) and female (n = 19) purebred beagle dogs weighing between 7 and 14 kg were used (Hazleot Research Products, Inc., Cumberland, VA; HRP, Kalamazoo, MI). The animals were housed un restrained, individually in stainless steel cages and were allowed access to food (PMI Feeds, Inc., Richmond, IN) and water ad libitum except on the day of dose administration. Animals were fasted overnight before dosing and were given access to food approximately 4 h postdose. Dogs were administered celecoxib i.v. at a dose of 5 mg/kg. The dose was prepared in a vehicle of polyethylene glycol 400:saline (2:1, v/v) at a concentration of 5 mg/ml. The volume of the administered dose was 1 ml/kg. The animals were dosed at approximately 8:00 AM. Venous blood (approximately 3 ml) from the jugular vein was collected into chilled tubes containing 121) purebred beagle dogs were administered a single 5 mg/kg i.v. bolus injection of celecoxib as described above. Venous blood samples (approximately 3 ml) were collected from the jugular vein into chill tubes containing sodium heparin at approximately 6, 12, and 24 h postdose. Plasma was prepared as described above and analyzed for celecoxib concentrations. Dogs with a 24-h plasma celecoxib concentration below the limit of detection of the assay (0.010 µg celecoxib/ml plasma) were EMs of celecoxib and dogs with a 24-h plasma celecoxib concentration above the limit of detection of the assay were PMs of celecoxib.

Human Liver Microsomes. Human liver microsomes were purchased from Xenotech (Xenotech Reaction Phenotyping Kit; Xenotech, Kansas City, KS). The kit included 16 individual microsome samples characterized with respect to their specific activity toward isof orm-specific substrates and also a pool prepared from a subset of six samples.

Canine Hepatic Microsomes. Livers were obtained from purebred beagle dogs that had been characterized as PM or EM. The liver tissue was minced and homogenized for approximately 1 min with a Potter Elvehjem homogenizer on ice. The microsomes were prepared from the homogenates as described for the Sf/9 insect cells below. Canine liver microsomes were kept frozen at -80°C until use. Protein concentration in canine liver microsome preparations were determined using the method of Lowry using an assay kit obtained from Sigma Chemical Co. (St. Louis, MO). The canine microsomal CYP concentrations were determined using the dithionite difference spectrum method (Matsubara et al., 1996).

Cloning of Canine CYP Proteins. Livers of beagle dogs were the sources of RNA for reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s suggested protocol. First strand synthesis was performed with 5 µg of total RNA from canine liver using Superscript II (Life Technologies, Gaithersburg, MD) and random hexamers (Promega, Madison, WI). Also, a canine liver Sf/9-stretch cDNA library from Clontech Laboratories, Inc. (Palo Alto, CA) served as a template for amplification. Polymerase chain reaction (PCR) was performed using Taq polymerase and buffers from Boehringer-Mannheim (Indianapolis, IN) with 2 µl of lysate from the cDNA library or 5 µl of first strand cDNA as template. Restriction and modification enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN), New England Biolabs (Beverly, MA), or Life Technologies (Gaithersburg, MD). Sequencing was done on ABI 373 or ABI Prism 377 automated sequencers (Perkin-Elmer Corporation, Foster City, CA) and sequence analysis was done with Sequencher software (Gene Codes Corporation, Ann Arbor, MI).

Primers for PCR amplification were synthesized by Life Technologies (Gaithersburg, MD) or Genosys Biotechnologies, Inc. (The Woodlands, TX), and contained restriction sites for directional cloning into pFastBac1 (Life Technologies, Gaithersburg, MD), the transfer vector for baculovirus-mediated expression in insect cells. Some forward primers included nucleotides “AAT” preceding the internal methionine (ATG) to enhance transcription from the baculovirus polyhedrin promoter; the preferred stop codon for insect cell expression (nucleotides TAA) was incorporated into the RT-PCR primers. GenBank accession numbers used as standards for canine cDNA clones were: CYP2B11, M33575; CYP2C21, A37222; CYP2D15, D17397; and CYP3A12, X54915.

PCR primers for the cloning of CYP2B11 were:

2B11A: GATCGGATCCATATGGAAGCTCAGGTCTTTC
2B11B: CACCATGCGGCAATGAAAGCC

Celecoxib Hepatic Microsomal Metabolism and Analysis. Celecoxib metabolism was examined in incubation mixtures consisting of dog liver microsomes (1.0 mg of protein/ml final concentration) or recombinant canine CYPs, an NADPH-generating system and substrate in 100 mM potassium phosphate buffer, pH 7.4 or 150 mM HEPES buffer, pH 7.4. The NADPH-generating system was composed of the following at the indicated final concentrations: MgCl_2 (7.5 mM), D-glucose 6-phosphate (7.5 mM), NADP (0.85 mM) and glucose 6-phosphate dehydrogenase (1.5 U/ml). The [14C]celecoxib solutions were prepared using DMSO as solvent, and solutions were stored at 20°C before use. Quinidine was dissolved in methanol (final concentration in the reaction medium, 1% v/v) and methanol was also added at the same final concentration to control incubations. Five concentrations of quinidine (0.3, 1, 3, 10, and 30 εM) were evaluated. These concentrations of quinidine are known to produce a significant inhibition of canine dextromethorphan O-demethylase activity (Kronbach, 1991).

Celecoxib samples from microsomal incubations were extracted using a Gilson Aspec XL automated solid-phase extraction system. Incubate was mixed with 2 volumes of 12 M urea and transferred to Varian Bond Elut C-18 solid phases.

Recombinant canine CYP enzymes were expressed in S9 insect cells using the bacmid methodology developed by Luckow et al. (1993) and commercially available from Life Technologies (Gaithersburg, MD). Transfections were performed in six-well tissue culture plates using CellFectin reagent (Life Technologies, Gaithersburg, MD), following the manufacturer’s protocol. After a 5-h incubation period, the transfection mixture was removed and the plates were replenished with 3 ml/well of IFP medium supplemented with 10% fetal bovine serum. After a 3-day incubation period, the cells were harvested, centrifuged, and the supernatants that contained recombinant virus (designated as P1) was removed and stored at 4°C. S9 cells were coincubated with virus particles that contained the CYP cDNA and a separate virus containing a human recombinant NADPH CYP-oxidoreductase generated from cell line Hep-G2 RNA by RT-PCR. Both viruses were added at a multiplicity of infection of 1 to 3. One day after infection, 0.9 mg/ml hemin chloride was added to the culture to supplement the endogenous level of heme in the insect cells; 3 days after infection, the cells were harvested by centrifugation and the washed cell pellets were frozen until processed for microsomes.

Expression of CYP Genes in Insect Cells. Recombinant CYP cDNAs were inserted into the Baculovirus expression vector pFastBac, kindly provided by Drs. K. C. Luckow and T. Wimmer (University of Wisconsin, Madison, WI). The bacmid was produced by Transgenic Institute (Columbia Station, MD). The bacmid containing recombinant virus (designated as P1) was removed and stored at −80°C until use.

Carbon monoxide (CO) inhibition of CYP activity was determined in the same set of microsomal samples (A and B) used to assess NADPH requirements. Reactions were performed in duplicate (sample A and B) with 0.5 mg protein/ml in sodium phosphate buffer containing the NADPH regeneration system. Sample A was preincubated for 2 min at 37°C with no treatment. Sample B was preincubated 2 min by bubbling with a steady flow of CO. Celecoxib (10 εM) was then added to each vial, mixed, and incubated for 10 min at 37°C. Sample B continued to receive CO for the duration of the 10-min incubation.

Quinidine was tested for its inhibitory effects on celecoxib in dog and human liver microsomes using a substrate concentration of 20 εM celecoxib. Celecoxib was dissolved in methanol (final concentration in the reaction medium, 1% v/v) and methanol was also added at the same final concentration to control incubations. Five concentrations of quinidine (0.3, 1, 3, 10, and 30 εM) were evaluated. These concentrations of quinidine are known to produce a significant inhibition of canine dextrorhethorphan O-demethylase activity (Kronbach, 1991).

Celecoxib samples from microsomal incubations were extracted using a Gilson Aspec XL automated solid-phase extraction system. Incubate was mixed with 2 volumes of 12 M urea and transferred to Varian Bond Elut C-18 solid phases.

Celecoxib Hepatic Microsomal Metabolism and Analysis. Celecoxib metabolism was examined in incubation mixtures consisting of dog liver microsomes (1.0 mg of protein/ml final concentration) or recombinant canine CYPs, an NADPH-generating system and substrate in 100 mM potassium phosphate buffer, pH 7.4 or 150 mM HEPES buffer, pH 7.4. The NADPH-generating system was composed of the following at the indicated final concentrations: MgCl_2 (7.5 mM), D-glucose 6-phosphate (7.5 mM), NADP (0.85 mM) and glucose 6-phosphate dehydrogenase (1.5 U/ml). The [14C]celecoxib solutions were prepared using DMSO as solvent, and solutions were stored at −20°C before use. Final DMSO concentration in the reaction mixture was 0.1%. Incubations were carried out for the indicated times at 37°C in a water bath. After incubation, reactions were terminated by the addition of 1/20 the sample volume of 5% formic acid and stored at −70°C until analysis.

The NADPH requirement for metabolism of celecoxib was determined in hepatic microsomal fractions prepared from three male and three female dogs known to be either fast or slow metabolizers of celecoxib. Microsomes (1 mg protein/ml) and 10 εM [14C]celecoxib were incubated in sodium phosphate buffer with and without the NADPH generating system for 10 min at 37°C.

The CYP2C21 clone described by Uchida et al. (1990) did not encode the complete coding sequence but started with the codon for amino acid 27 (K). To complete coding sequence but started with the codon for amino acid 27, a reverse primer, 2C21NS-1, was designed for use with a primer based on sequence from the GT10 vector used in the library. The reverse primer for recovery of the 5' end of CYP2C21NS-1: CTCTGCTAGCTTGCTTAGG-GATGTC. Primers for amplification and expression of CYP2C21 were: 2C21-A: GATCTCTAGAATTTGATCTTCATGATGGT; 2C21-B: GATCTGTAAGTTAAGCTGGAACAAACACAGGATATAGGG. Primers for amplification and expression of CYP2D15 were 2D15-A: GATCGAATTCATTTCTTTGGAATTCTGGG; 2D15-B: GATCAAGCTTAGCGAGGCTCCACACAGAGC; 2D15-C: GCTTGGTGAGCTCCAGGACGC; 2D15-D: GCCGCCTTGAGTACGACG. Primers for amplification and expression of CYP3A12: 3A12-A: GATCGCGATCCAATAGGCTTATGCCAACGAC; 3A12-B: CCATTTCTTTGGAATTCTGGG; 3A12-C: GATAAAACAAAAACACCGAGTGG; 3A12-D: GATCTGCGATCGGCATCCACATCTCCC.

Expression of CYP Genes in Insect Cells. Recombinant CYP cDNAs were inserted into the Baculovirus expression vector pFastBac, kindly provided by Drs. K. C. Luckow and T. Wimmer (University of Wisconsin, Madison, WI). The bacmid was produced by Transgenic Institute (Columbia Station, MD). The bacmid containing recombinant virus (designated as P1) was removed and stored at −80°C until use.

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columns (Varian, Sunnyvale, CA) that were preconditioned with 2 column volumes of acetonitrile followed by 2 column volumes of water. After the addition of sample, the column was washed with 2 column volumes of water. The analyte was eluted with acetonitrile. The eluent was dried under nitrogen and dissolved in initial mobile phase composition for subsequent analysis by high performance liquid radiochromatography.

The HPLC system consisted of a model 1050 series pump and autoinjector (Hewlett-Packard, Naperville, IL) and a Flo-One/Beta Model A515 flow-through radioactivity detector (Packard Instruments, Meriden, CT). The column used was a Waters Symmetry C18 column, 4 mm, 4.6 × 50 mm (Waters Chromatography, Milford, MA), with a Brownlee Newguard RP-18, 3.2 mm particle size, 4.6 × 150 mm (Brownlee Labs, Inc., Santa Clara, CA). The mobile phase was conditioned with 2% acetonitrile in methanol. The extract was evaporated under nitrogen and the sample was dissolved into 200 μl HPLC mobile phase, acetonitrile/0.01 M potassium phosphate with testosterone concentrations of 0, 5, 10, 25, 50, 75, 100, 250, 500, 750, 1000, and 2000 μM for 15 min. An aliquot of the sample extract was injected onto a reversed phase HPLC C18 NOVA PAK column 15 cm × 3.9 mm, 4 M (Waters Associates, Milford, MA) using a 15 × 3.2 mm, 7 m RP-18 New Guard Cartridge (Brownlee Labs, Inc., Santa Clara, CA). The mobile phase, acetonitrile/0.01 M sodium phosphate buffer (pH 9) (50:50, v/v), was run at 1.0 ml/min. The quantifier was quantified by peak height ratioing to the internal standard using a fluorescence detector with excitation at 240 nm and emission at 380 nm. The analyte was compared against a standard curve (0.01 to 10 μg celecoxib/ml) prepared as described above.

Pharmacokinetic Calculations. The plasma celecoxib concentration-time curves after i.v. administration were analyzed using noncompartmental kinetics (Gibaldi and Perrier, 1982).

Statistics. The cluster method was used to group the plasma concentration data across all time points from the 38 dogs in Fig. 2. Clustering is a multivariate approach that can use any number of variables (Anderberg, 1973). The observations from each dog were treated as one multivariate variable with 13 components (plasma concentrations at 13 time points). The method starts with each dog as its own cluster. At each step the distance between each cluster is calculated and the two clusters that are the closest are combined. The combining of clusters continued until all dogs were in one final cluster. The appropriate number of clusters is then chosen based on how the distance changes as the number of clusters is reduced and the clustering tree is divided at that point. The statistical significance of the resultant cluster analysis was determined by the method of Engelman and Hartigan (1969).

An unequal-variance t test was used to determine statistical significance between celecoxib pharmacokinetic parameters and in vitro liver microsomal metabolic rate from EM and PM animals.

Results

In Vivo Pharmacokinetics. The mean (± S.D.) concentrations of celecoxib in plasma from male and female dogs after i.v. administration of 1 mg/kg were determined using noncompartmental kinetic analysis.

<table>
<thead>
<tr>
<th>Population*</th>
<th>Sex/N</th>
<th>T1/2 (h)</th>
<th>Cl (l/min/kg)</th>
<th>V (l/kg)</th>
<th>Vdss (l/kg)</th>
<th>AUC0–t (μg/ml h)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM Male/11</td>
<td></td>
<td>1.77 ± 0.9</td>
<td>19.2 ± 7.7</td>
<td>2.63 ± 1.37</td>
<td>2.18 ± 0.65</td>
<td>4.95 ± 1.80</td>
</tr>
<tr>
<td>EM Female/8</td>
<td></td>
<td>1.66 ± 0.43</td>
<td>16.9 ± 4.2</td>
<td>3.22 ± 0.41</td>
<td>1.98 ± 0.16</td>
<td>5.20 ± 1.23</td>
</tr>
<tr>
<td>EM Male/19</td>
<td></td>
<td>1.72 ± 0.79</td>
<td>18.2 ± 6.4</td>
<td>2.50 ± 1.07</td>
<td>2.10 ± 0.50</td>
<td>5.05 ± 1.45</td>
</tr>
<tr>
<td>PM Male/8</td>
<td></td>
<td>4.69 ± 1.25</td>
<td>7.43 ± 1.23</td>
<td>2.95 ± 0.60</td>
<td>2.26 ± 0.27</td>
<td>11.5 ± 2.0</td>
</tr>
<tr>
<td>PM Female/11</td>
<td></td>
<td>5.54 ± 1.24</td>
<td>6.95 ± 1.55</td>
<td>3.27 ± 0.72</td>
<td>2.45 ± 0.32</td>
<td>12.5 ± 2.6</td>
</tr>
<tr>
<td>PM Male/19</td>
<td></td>
<td>5.18 ± 1.29</td>
<td>7.15 ± 1.41</td>
<td>3.13 ± 0.67</td>
<td>2.37 ± 0.31</td>
<td>12.1 ± 2.33</td>
</tr>
</tbody>
</table>

*Celecoxib T1/2, Cl, and AUC0–t were statistically different between EM and PM dogs (p < .001). Celecoxib Vdss and Vt were statistically different between EM and PM dogs (p < .01).
tion of celecoxib at 5 mg/kg are shown in Fig. 2 and the pharmacokinetic parameters are summarized in Table 1. The beagle population in this study separated distinctly into two groups as determined by cluster analysis of the plasma celecoxib concentration data ($p < .05$) (Engelman and Hartigan, 1969; Anderberg, 1973). One group of dogs eliminated celecoxib from plasma at a rapid rate and was designated EM. The other group of dogs eliminated celecoxib at a slower rate and was classified as PM. The mean plasma elimination half-life and clearance of celecoxib for dogs that eliminate celecoxib from plasma at a fast rate were 1.72 h and 18.2 ml/min/kg, respectively. The mean plasma elimination half-life and clearance of celecoxib for dogs that eliminate celecoxib from plasma at a slow rate were 5.18 h and 7.15 ml/min/kg, respectively. The mean apparent volume of distribution ($V_d$) of celecoxib for the dogs ranged from 2 to 3 liters/kg. The $V_d$ and apparent volume of distribution at steady state ($V_{dss}$) were slighter greater for the PM animals ($p < .01$).

A screening method, with i.v. dosing, was developed to distinguish between the two populations. Using this method, a total of 242 dogs were screened for population phenotype. 45.0% exhibited the EM phenotype, 53.3% exhibited the PM phenotype, and 1.65% of the dogs could not be adequately characterized (Fig. 3). There was an equal distribution of the two populations within each sex.

To validate the population phenotype screening method, beagle dogs characterized as EM or PM were administered oral doses of celecoxib and systemic exposure to drug determined (Fig. 4). Plasma concentrations of celecoxib were higher in the PM dogs as compared with EM animals given the same dose.

**Total CYP and Microsomal Protein Yield per Gram of Liver.** Animal body weights, total liver weights, yields of microsomal protein, and the total microsomal CYP content for dogs ($n = 9$ PM; $n = 6$ EM) that had been phenotyped for ability to metabolize celecoxib are listed in Table 2. There was no significant different in liver size or total liver CYP content between EM or PM dogs.

**Comparison of In Vitro Celecoxib Metabolism with In Vivo Clearance (Cl) Rates.** Microsomes from 20 dogs that were previously screened for population phenotype (10 males and 10 females, included 6 PM and 4 EM per sex) were incubated with three different celecoxib concentrations. The results of this experiment are summarized in Figs. 5 and 6. Consistent with the in vivo observations, hepatic microsomes from EM dogs generally metabolized this drug in vitro at a higher rate than microsomes from PM dogs (Fig. 6). Correlation analysis of in vitro metabolism rates and in vivo clearance rates ($n = 20$) was performed. In vitro celecoxib substrate concentrations of 2.6, 10, and 100 μM (approximately 1.0, 3.8, and 38 μg/ml)
gave correlation coefficients \( r \) of 0.944, 0.901, and 0.743, respectively (Fig. 5). The in vivo/in vitro correlation was maximal at the lowest substrate concentration of 1.0 \( \mu \text{g/ml} \), which is the concentration closest to the efficacious plasma level in vivo.

### NADPH Requirement and Carbon Monoxide Inhibition.

Inhibition of CYP enzyme activity by carbon monoxide and the requirement for NADPH as a source of electrons are general, nonselective tests for determining CYP-mediated metabolism. Figure 7 illustrates that carbon monoxide significantly inhibited celecoxib metabolism by dog liver microsomes and that NADPH was required for metabolism to occur. These results strongly support the active participation of CYP enzyme(s) as the major or only route of celecoxib metabolism by dog liver.

### Cloning and Expression of Canine CYP Proteins.

Canine CYP2B11, CYP2C21, CYP2D15, and CYP3A12 were cloned from canine liver cDNA by PCR and expressed in Sf9 insect cells. The sequence obtained for CYP2C21 differed from the original sequence described by Blaisdell et al. (1998) with the new report of initial three amino acid residues “MDL”. A previously unreported isoform or variant of CYP3A12 is also described (Table 3) as well as three unique variants of CYP2D15 species that were not reported by Sakamoto et al. (1995) or Roussel et al. (1998) (Table 4).

Five different CYP2D15 clones were obtained. Approximately half of the CYP2D15 clones had an in-frame deletion of exon 3 and were designated CYP2D15 \( ^d \). A similar species was reported by Roussel et al. (1998) and was termed CYP2D15 V2. Sequence analysis of CYP2D15 cDNA delta and full-length clones revealed three point mutations, which resulted in altered amino acids at three positions: S186G, I250F, and I307V. The CYP2D15 full-length clone with all three amino acid changes was the same as CYP2D15 WT2 reported by Roussel et al. (1998). The splice variant (CYP2D15V2) reported by Roussel et al. (1998) had these three point mutations (S186G, I250, and I307) by only one amino acid. CYP2D15 clones that encoded the same predicted amino acid sequence as Sakamoto et al. (1995) were designated CYP2D15 wt. Two additional novel variants were constructed: CYP2D15*2 combined the S186G mutation with the wt I250 and I307; CYP2D15*3 combined the wt S186 with I250F and I307V (Table 4). All five CYP2D15 species were expressed in the insect cells.

The metabolism of bufuralol to 1'-hydroxybufuralol has been shown to be catalyzed relatively selectively by the polymorphic
human enzyme CYP2D6 and has been used to monitor metabolism in vitro. Others have demonstrated that canine CYP2D15 readily metabolized the 1'-hydroxylation of bufuralol (Rousell et al., 1998). The apparent enzyme constants for the generation of 1'-hydroxybufuralol were obtained after incubation of CYP2D15 wt, CYP2D15*2, CYP2D15*3, CYP2D15WT2, and CYP2D15 δ. CYP2D15 wt, CYP2D15*2, CYP2D15*3, and CYP2D15WT2 exhibited similar $K_m$ values of 5.6, 6.1, 2.7, and 12.4 μM, respectively. The apparent $K_m$ value for CYP2D15 δ was markedly higher than the $K_m$ values of other CYP2D15 isoforms at 39.0 μM. The apparent $V_{max}$ values for CYP2D15 wt, CYP2D15*2, CYP2D15*3, and CYP2D15WT2 were all quite similar at 0.57, 0.79, 0.61, and 0.36 nmol/min/mg CYP protein. The apparent $V_{max}$ for CYP2D15 δ was substantially lower at approximately 0.05 nmol/min/mg CYP protein.

A CYP3A12 isoform or variant was amplified by RT-PCR from the liver of an individual beagle dog and differed in nucleotide sequence from the GenBank X54915 at five bases, predicting amino acid sequence differences at five positions, including one amino acid change in exon 10 and four amino acid changes in exon 11. This CYP3A12 species was termed CYP3A12*2 (Table 3). Total RNA isolated from the liver of a second beagle dog was used as a PCR template for CYP3A12. This reaction yielded fragments that encoded the amino acid sequence identical with the GenBank entry except for two silent changes: T165C and G963A. This isolate was designated CYP3A12*3.

The metabolism of testosterone to 6β-hydroxytestosterone has been demonstrated to be catalyzed selectively by CYP3A4. The kinetic constants for recombinant CYP3A12 and CYP3A12*2 were determined for the 6β-hydroxylation of testosterone. The apparent $K_m$ for CYP3A12 was 72.5 μM whereas CYP3A12*2 exhibited an apparent $K_m$ of 66.8 μM. The apparent $V_{max}$ value for CYP3A12 was 3.49 nmol/min/mg and for CYP3A12*2 the apparent value was 2.88 nmol/min/mg.

### Quinidine Inhibition of Celecoxib Metabolism in Dog Liver Microsomes

The effect of quinidine, a potent CYP2D inhibitor, is shown in Fig. 9. In the presence of increasing quinidine concentrations, a concentration-dependent inhibition of celecoxib hydroxylase activity was observed in the EM and PM dog liver microsomes tested. Quinidine caused greater than 50% inhibition of celecoxib hydroxylase activity at concentrations as low as 3 μM. In the same experiment, celecoxib hydroxylase activity in pooled human liver microsomes was not meaningfully affected (Fig. 9) at concentrations approximately 100 times higher than the reported $K_i$ value for human microsomal CYP2D6 inhibition (≈0.4 μM) (Newton et al., 1995). The inhibition of celecoxib metabolism by quinidine suggests CYP2D involvement in the metabolism of celecoxib by dog.

### Metabolism of Bufuralol and Celecoxib in EM and PM Dogs

The metabolism of bufuralol and celecoxib in hepatic microsomes from 20 dogs (10 males and 10 females, including 6 PM and 4 EM per sex) were evaluated. Bufuralol hydroxylase activity correlated poorly ($r = 0.382$) with celecoxib metabolism by dog liver microsomes (EM and PM dogs combined; Fig. 10). The correlation was not improved if EM or PM dogs were grouped separately.
Discussion

The present study describes for the first time a polymorphism in the metabolism of a xenobiotic by dog. There are at least two distinct populations of dogs that eliminate celecoxib from plasma at either a fast or a slow rate (designated PM for poor metabolizers and EM for extensive metabolizers). The two phenotypes are about evenly distributed within a population of 242 beagle dogs, with 45.0% of the dogs of the EM phenotype and 53.3% of the PM phenotype. There was also an equal distribution of the two populations within each sex. The difference between the two populations was shown to be due to a difference in the rate of metabolism of celecoxib by liver CYPs. Liver was shown to be the principal site of metabolism by the high correlation ($r > 0.90$) between in vivo clearance and in vitro hepatic metabolism rate of celecoxib. The involvement of CYP in dog liver microsomal metabolism of celecoxib was confirmed by its inhibition by carbon monoxide and the requirement for NADPH. The liver weights or the total amount of liver microsomal CYP did not differ between the two populations, suggesting that the polymorphism is likely due to the differential expression of an individual CYP.

To determine which canine CYPs contribute to celecoxib metabolism in the dog, a series of recombinant canine CYPs were examined for the ability to metabolize celecoxib including CYP2B11, CYP2C21, CYP2D15, CYP3A12, and one variant allele designated CYP3A12*2 and four variant alleles of CYP2D15 designated CYP2D15*2, CYP2D15*3,
CYP2D15WT2, and CYP2D15 δ. The CYP2D15WT2 was described previously by Roussel et al. (1998). Isoforms in the CYP2D subfamily exhibited high activity for the oxidative metabolism of celecoxib, whereas low activities were observed for CYP2B11, CYP2C21, and CYP3A12. The CYP2D15 δ, which has exon 3 deleted, had little activity toward celecoxib. Metabolism of bufuralol to 1'-hydroxybufuralol was measured to confirm that the recombinant CYP2D15 proteins in the isolated microsomes were metabolically active. Bufuralol is a substrate for CYP2D, although there is overlapping specificity with other isoforms at high substrate concentration (Kronbach, 1991). Bufuralol was readily metabolized by four CYP2D15 isoforms and to a lesser extent by CYP2B11, CYP2C21, and CYP3A12. CYP2D15 δ had very low bufuralol hydroxylase activity. Quinidine, a potent CYP2D inhibitor, was evaluated for its ability to limit dog liver microsomal celecoxib metabolism. The metabolism of celecoxib in liver microsomes prepared from both EM and PM dogs was significantly inhibited by quinidine, further supporting CYP2D involvement. Bufuralol hydroxylase activity was highly correlated with celecoxib metabolism in preparations of the recombinant proteins (r = 0.961). Collectively, these results suggest CYP2D15 is an important CYP involved in canine celecoxib metabolism. However, there was a poor correlation between bufuralol and celecoxib metabolism in liver microsomes prepared from EM and PM animals, suggesting that other CYP isoforms may contribute to the polymorphism. Although the possibility that canine CYPs other than CYP2D contributed to the bufuralol hydroxylase activity cannot be discounted. A polymorphism of CYP2C41 was recently described and the possibility that this isoform may contribute to celecoxib metabolism in PM and EM animals has not been examined (Blaiddel et al., 1998). Likewise, the contribution of the recently described CYP3A26 to metabolize celecoxib has not been studied (Fraser et al., 1997). The fact that there is an almost equal distribution of PM and EM dogs in the population suggests that the explanation for these phenotypes will not be the distribution of a single minor variant of a single canine CYP. The observed complexity of the canine CYP system, with the presence of several variants of CYP2D15 and CYP3A12, may yet explain the differences in the rate of celecoxib metabolism in the populations of EM and PM dogs.

Although the present data support the existence of at least two populations of dogs that metabolize celecoxib at different rates, the existence of additional populations within the already described PM and EM phenotypes cannot be ruled out. CYP2C9 was reported previously to be the cytochrome predominantly responsible for metabolism of celecoxib in humans (Karim et al., 1997, 1998). Quinidine, a specific inhibitor of CYP2D6 (Guengerich et al., 1986a; Newton et al., 1995), had no effect on human microsomal metabolism of celecoxib, supporting that, unlike the dog, the CYP2D family is not involved in human celecoxib metabolism. There are examples in the literature that demonstrate that the activity of canine CYPs toward substrates differs significantly from what is observed for the corresponding human CYP isoforms. For example, Sharer et al. (1995) found that dog microsomes show significantly lower coumarin 7-hydroxylase activity and no detectable tolbutamide 4-hydroxylase activity when compared with human liver microsomes. In contrast, the enzymatic activity of canine liver microsomes for midazolam and erythromycin was significantly higher than that of human microsomes. Recently, Chauret et al. (1997) confirmed extremely low tolbutamide 4-hydroxylase activity for dog microsomes; tolbutamide hydroxylase (CYP2C9) is generally quite active in human liver microsomes and celecoxib metabolism is highly correlated with tolbutamide 4-hydroxylase in human microsomes (Karim et al., 1997). The results for S-mephentoin 4-hydroxylase (CYP2C19) activity are equivocal. Stereoselective differences in metabolism between canine and human CYP enzymes for S-mephentoin 4-hydroxylation are noted by Yasumori et al. (1993). Both Sharer et al. (1995) and Chauret et al. (1997) report comparable activities for the hydroxylation of the S-mephentoin in canines and humans.

The canine is an important species that is used in the pharmaceutical industry to support new drug development. Dogs are often used for evaluating pharmacological efficacy of potential drug candidates, and dogs are the primary nonrodent species of choice for nonclinical safety and toxicology evaluations. In addition, dogs are frequently used in allometric scaling to project clinical dose. The results presented in this paper show that there is a polymorphism in canine liver metabolism of celecoxib. These data imply that the potential of obtaining markedly different systemic exposure of certain drugs after dose administration to dogs may result from such a polymorphism. The distribution of EM or PM animals into dose groups of safety assessment studies could potentially impact the interpretation of data obtained if one phenotype was preferentially randomized into a particular group. Furthermore, predictions of human clearance arrived at through allometric scaling may vary markedly depending on whether clearance data from EM or PM dogs are used in the calculations. Finally, the discovery of this polymorphism in dog xenobiotic metabolism would impact the development of drugs for veterinary use. Additional investigation into the molecular basis for this polymorphism is warranted.

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