In vitro effects of nonsteroidal anti-inflammatory drugs on cyclooxygenase activity in dogs

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Objective—To establish an in vitro assay and determine the differential suppressive activity of non steroidal anti-inflammatory drugs (NSAID) on cyclooxygenase (COX)-1 and COX-2 isoenzymes in dogs.

Procedure—COX activity was evaluated in the presence and absence of 4 NSAID (meloxicam, tolfacinamic acid, carprofen, and ketoprofen), using a canine monocyte/macrophage cell line that constitutively expresses COX-1, but can be induced to express COX-2 when incubated with lipopolysaccharide. Inhibition of prostaglandin E2 (PGE2) synthesis by each NSAID was measured by enzyme immunoassay and attributed to specific COX-1 or COX-2 activity through assessment of COX messenger RNA expression by use of northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR). The COX selectivity of each drug was evaluated from dose-response curves by calculating a ratio (COX-1:COX-2) of inhibitory concentration values on the basis of concentrations that reduced PGE2 by 50% in each COX model.

Results—Meloxicam and tolfacinamic acid preferentially inhibited COX-2, with meloxicam inhibiting COX-2 activity 12 times more effectively than COX-1 activity. Carprofen was only 1.75 times more selective for COX-2 than for COX-1, and ketoprofen was slightly more selective for COX-1.

Conclusions—COX-1 and COX-2 were differentially sensitive to inhibition in vitro by NSAID. Meloxicam and tolfacinamic acid were selective for COX-2. Effects of carprofen and ketoprofen approached equipotency against both isoenzymes. Selective COX-2 inhibitors are a new class of drugs with anti-inflammatory effects similar to conventional NSAID but with fewer adverse effects. Development of these agents for veterinary use would be facilitated by the convenience of using a canine cell line as a model system to screen COX-1 and COX-2 inhibitor activities in vitro. (Am J Vet Res 2000;61:802–810)

Control of inflammation and pain is essential to veterinary care, but adequate pain management in dogs is hindered by the availability of only a few approved drugs for this purpose. Preferential cyclooxygenase (COX)-2 inhibitors are a new class of non steroidal anti-inflammatory drugs (NSAID) that may provide safer and more potent analgesia than currently available nonselective agents. However, little information is available on COX activity in dogs and COX inhibition by NSAID. Development of COX-2 inhibitors for veterinary use would be facilitated by assays to assess their biochemical effectiveness in dogs.

Pain accompanies inflammation following tissue injury and is partly mediated by release of prostaglandins (PG). Arachidonic acid (AA) is released from cellular membrane phospholipid as a free fatty acid by the enzyme phospholipase A2, and is channeled into 1 of 2 pathways: the lipoxygenase pathway or the prostaglandin H-synthase pathway (commonly referred to as the COX pathway). Cyclooxygenase was first isolated and identified in 1976 and cloned in 1988. It is now accepted that this original enzyme was actually COX-1, and another unique isoform, known as COX-2, was reported in 1991. The 2 isoenzymes have different basal expression, tissue localization, and induction during inflammation.

Cyclooxygenase-1 is constitutively expressed in most tissues and is thought to be responsible for most basal PG production, which regulates normal cellular activity. Tissue enzyme concentration of COX-1 is generally stable, with small increases in expression of 2- to 4-fold in response to stimulation by hormones or growth factors. Cyclooxygenase-2 is not substantially expressed in most tissues under normal conditions and is generally not detected in untreated cells in culture but is strongly induced following cellular exposure to pro-inflammatory cytokines, bacterial lipopolysaccharide (LPS), and tumor necrosis factor-α. Once induced, COX-2 protein concentrations increase rapidly and transiently 10- to 100-fold after exposure to a single stimulus, releasing proinflammatory PG at the site of injury, resulting in erythema, edema, and pain. Nonsteroidal anti-inflammatory drugs presently available inhibit both isoenzymes to various degrees; therefore, they reduce PG production and consequent pain and inflammation at the affected site through suppression of COX-2, but can also cause gastric ulceration and renal compromise because of inhibition of homeostatic PG in the stomach and kidneys.

Assessment of NSAID for their relative effects on COX activity is complex. Several in vitro models, including purified enzyme systems, intact cell systems, and whole blood systems have been developed to characterize the efficacy and toxicity of NSAID commonly used in humans, through comparison of their relative inhibitory activities against COX-1 and COX-2.
Drug concentrations at which enzyme activity is inhibited by 50% (inhibitory concentration [IC₅₀]) are calculated and expressed as a ratio of COX-1 to COX-2. Preferential inhibition of COX-2 is indicated by a ratio > 1, and a greater inhibitory effect on COX-1 versus COX-2 is indicated by a ratio < 1. Assessment of inhibitory potency of NSAID on COX isoenzymes is strongly influenced by variations in experimental conditions among different models; therefore, results from each model cannot be directly compared. However, the general ranking order of selectivity for COX-2 versus COX-1 within a range of compounds appears to be reproducible from one model to another. In addition, although COX activity may be similar in different species, it is generally agreed that drug studies must be performed on the particular species in question to evaluate efficacy and toxicity accurately.

It is well documented that human and rodent monocyte/macrophage cells express COX-1 messenger RNA (mRNA) and synthesize low concentrations of prostaglandin E₂ (PGE₂) in response to exogenous AA. It has been reported that treatment with LPS selectively and substantially increases COX-2 mRNA expression, with no detectable effect on COX-1 mRNA. Increased expression of COX-2 mRNA is followed by increased COX-2 protein expression and tissue PGE₂ concentrations. These studies suggest that monocyte/macrophage cells may provide an ideal source of each COX isoenzyme in vitro, because these cells can express the constitutive isofoms (COX-1) and the inducible isofoms (COX-2).

The purpose of the study reported here was to establish an in vitro assay system, using a preexisting canine cell line that specifically expressed COX-1 and COX-2 under distinct conditions to differentiate the interaction of NSAID with these 2 isoforms in dogs. Cyclooxygenase expression and PGE₂ synthesis by the canine DH82 monocyte/macrophage cell line were evaluated. Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) were used to assess COX mRNA expression, and enzyme immunoassays were used to measure PGE₂ production. The NSAID chosen for this study included anti-inflammatory agents presently available for use in dogs, which are reported to be clinically efficacious COX inhibitors and have favorable adverse effect profiles.

Materials and Methods

Assessment of COX expression and PGE₂ synthesis by canine DH82 monocyte/macrophage cells (COX-1 and COX-2 models)—Cyclooxygenase expression was determined in intact canine DH82 monocyte/macrophage cells. Cells were maintained in monolayers in DMEM containing 15% fetal bovine serum and penicillin (50 U/ml)-streptomycin (50 U/ml). Passaging of cells was performed regularly with trypsin.

For each experiment, cells were seeded into 24 well plates at 1 × 10⁶ cells/well in DMEM. After 24 hours, cells were incubated overnight in serum-free DMEM. Canine DH82 cells on 1 plate were incubated with LPS (100 ng/ml) for a 6-hour period prior to any further treatment. Cells were preincubated for 30 minutes in serum-free DMEM alone or in serum-free DMEM and 0.1% ethanol or dimethyl sulfoxide (DMSO). The medium was removed and replaced with fresh serum-free DMEM alone or with serum-free DMEM with 0.1% ethanol or DMSO. Arachidonic acid was added to half of the treatment wells to a final concentration of 30 µM, and cells were incubated for 30 minutes. All incubations were performed at 37 C and 5% CO₂. Following the second incubation, medium was removed from each well, centrifuged, and stored at −70 C for PGE₂ analysis, and total cellular RNA was collected for northern blot analysis. The effects of ethanol and DMSO on PGE₂ synthesis were determined to ensure that the use of either compound as a vehicle to dissolve NSAID for future COX inhibition studies would not alter the results.

Inhibition of NSAID—The canine DH82 monocyte/macrophage cells were seeded into 24 well plates, as described, and incubated overnight in serum-free DMEM. Cells were incubated with LPS for 6 hours prior to any further treatment for the COX-2 assay. Incubation was not required for the COX-1 assay. Cells were preincubated with each compound in serum-free DMEM for 30 minutes. Medium was removed, and a serum-free 30 µM DMSO was added to each well. AA was added to a final concentration of 30 µM. Positive control wells contained serum-free DMEM and 0.1% vehicle. Positive control wells contained serum-free DMEM, 0.1% vehicle, and AA. All incubations were performed at 37 C and 5% CO₂. Following the second incubation, medium was removed from each well, centrifuged, and stored at −70 C for PGE₂ analysis, and total cellular RNA was collected for northern blot analysis.

Drugs—For NSAID enzyme inhibition studies, meloxicam, tolkenamic acid, carprofen, and ketoprofen were used. Stock solutions of each drug were prepared in ethanol or DMSO and serially diluted in serum-free DMEM for addition to cultures. Concentrations of ethanol or DMSO in wells did not exceed 0.1%. For each experiment, compounds were evaluated at identical concentrations on the same assay plate. Each drug was evaluated at 0.003125, 0.0125, 0.05, 0.2, 0.8, 3.2, 12.8, and 51.2 µg/ml, a range of concentrations that encompasses mean maximum plasma concentrations of each drug following in vivo administration in healthy dogs.

Eicosanoid enzyme immunoassay (EIA)—Prostaglandin E₂ was assessed by EIA according to manufacturer's protocol. The detection limit for PGE₂ was 7.8 pg/ml. Specificity of the EIA was 100%, and cross-reactivity with other eicosanoids was < 1%.

Northern blot analysis—The 1.8 kilobase (kb) fragments of COX-1 and COX-2 complementary DNA (cDNA) were subcloned into the NotI, HindIII, and EcoRI, Apal vector sites, respectively. Fidelity of cDNA fragments was verified by dye terminator cycle sequencing, using a DNA sequencer.

Total cellular RNA was isolated, using 1 ml of reagent per 5 × 10⁶ cells as described by the manufacturer. Contents of each set of treatment wells were pooled for maximum RNA recovery. Total RNA (20 µg) was subjected to electrophoresis in 0.8% agarose gels, transferred to nylon membranes, and prehybridized for 1 hour in 0.5% sodium dodecyl sulphate (SDS), 5% Denhardt solution, 0.1% sodium chloride/sodium phosphate buffer, 50% formamide, and denatured salmon sperm DNA (10 mg/ml). The human COX-1 and COX-2 probes were radiolabelled with [α-³²P]dCTP and a random primer labelling kit as per manufacturers' instructions. The ³²P-labelled cDNA were incubated for 16 hours, with membranes identical to the solution used for prehybridization. Membranes were then washed twice (15 minutes each) at 42 C in 2X sodium chloride/sodium citrate buffer.
Reverse transcription and PCR—Ribonucleic acid (1 µg), which was obtained from canine platelets and canine DH82 monocyte/macrophage cells as described, was reverse-transcribed with reverse transcriptase in the presence of 10 mM deoxynucleotides (dNTP), random primers, Oligo (dT) primer, DNase I, 10X DNase I buffer, 2 µl dNTP (0.1 M), and 3’ (antisense) primer (50 pmol/µl), in dimethylpyrocarbonate-treated water. Resultant cDNA was diluted 5 times. In a thin-walled PCR tube, 10 µl of cDNA was amplified in a final volume of 100 µl containing 2 µl dNTP (10 mM), 50 µl MgCl2 (50 mM), 0.2 µl Taq DNA polymerase, 2 µl (50 pmol/µl) each of sense and antisense primers, and sterile double distilled water. Primers used to amplify COX-1 cDNA were as follows: sense, 5’ to 3’, AGATGGCAGCAGAGTTGGGAG and antisense, 3’ to 5’ ACAGGTCTTGGTGTTGAGG. Amplification conditions were 94°C for 3 minutes for 1 cycle, 94°C for 30 seconds, and 72°C for 1 minute (primer annealing and extension) for 40 cycles, and 72°C for 5 minutes. Polymerase chain reaction products were identified following electrophoresis on a 2% agarose gel that contained ethidium bromide (10 µg/ml) in tris-acetate buffer. The remaining 90 µl of sample was purified by use of a PCR purification kit, and the identity of the COX-1 product confirmed by dye terminator cycle sequencing by use of a DNA sequencer.

Data analyses—Prostaglandin E2 synthesis by canine DH82 monocyte/macrophage cells was expressed as mean ± SD. Differences between vehicle (ethanol or DMSO) control and treatment groups were determined by use of a Student t-test.

Eight concentrations were evaluated for each NSAID in 2 experiments. Results of 9 treatment wells, each containing a specific drug dissolved in a vehicle and AA from each experiment were calculated as mean (± SEM) percentage PGE2 synthesis and compared with positive control wells containing vehicle and AA only. Data analyses were performed by use of a statistical software package. Statistical interpretation was performed by use of ANOVA. A quadratic regression was applied to describe the relationship of each drug and COX model to drug concentration. Comparisons among data sets were performed by contrast by use of an F test. Values of P < 0.05 were considered significant.

Results

Cyclooxygenase expression and PGE2 synthesis by canine DH82 monocyte/macrophage cells (COX-1 and COX-2 models)—Using northern blot analysis, COX-2 expression was not detected in canine DH82 monocyte/macrophage cells prior to LPS exposure (Fig 1). However, following a 6-hour incubation period with LPS, the 4.5 kb COX-2 mRNA transcript was strongly expressed. Cyclooxygenase-1 was not detectable in DH82 cells by northern blot analysis under any conditions but was detected by RT-PCR analysis of RNA obtained from canine DH82 monocyte/macrophage cells by use of primers designed on the basis of the human COX-1 gene sequence (Fig 2).

The DH82 cells that were not treated with LPS synthesized low amounts of PGE2 (24.81 ± 9.13 pg/10^5 cells). The LPS-induced gene expression was reflected by a significant increase in PGE2 synthesis by the DH82 cells (125.35 ± 31.65 pg/10^5 cells).

Significant differences were detected between amount of PGE2 synthesized by DH82 cells that were not exposed to LPS, and cells that were incubated with LPS for 6 hours, prior to and following addition of AA (Fig 1). Comparison of means revealed no significant effect on PGE2 synthesis by the presence of 0.1% ethanol or DMSO.

Canine platelet mRNA found on the same mem-
brane as DH82 cells that were and were not treated with LPS expressed the 2.8 kb COX-1 transcript, but COX-2 message was not detected (Fig 1). Platelets are the only cells that consistently demonstrate exclusive COX-1 expression in all species previously studied and thus provided an ideal control to test the homology of the COX probes to canine mRNA; therefore, these findings confirmed the ability of the human COX cDNA probes to detect canine COX-1 message, without cross-reacting with COX-2 under the conditions used in this study.1,6

Results achieved through northern blot analysis and RT-PCR indicated that canine DH82 monocytes/macrophage cells contain 1 constitutively expressed isoform (COX-1) and consequently synthesized low amounts of PGE2, and 1 inducible isoform (COX-2) with correspondingly increased amounts of PGE2 synthesis. Therefore, unstimulated DH82 cells were used as a COX-1 model, and LPS-treated DH82 cells were used as a COX-2 model to screen NSAID products indicated on left; bp = Base pairs.

Enzyme inhibition studies—In the COX-1 model, following NSAID incubation with non-LPS treated canine DH82 monocyte/macrophage cells, COX-1 was detected by RT-PCR, and COX-2 was not detected by northern blot analysis of total RNA. However, in the COX-2 model following NSAID incubation with DH82 cells that had been exposed to LPS for 6 hours, the 4.5 kb COX-2 mRNA transcript was strongly expressed. Cyclooxygenase-1 was not detected in DH82 cells by northern blot analysis under any conditions

Results obtained by northern blot analysis and RT-PCR suggested that NSAID suppression of PGE2 synthesis in non-LPS treated DH82 cells was caused by inhibition of COX-1, and suppression of PGE2 synthesis in LPS-treated cells was primarily caused by inhibition of COX-2.

All drugs significantly inhibited PGE2 production by DH82 monocyte/macrophage cells in the COX-1 (non-LPS treated cells) and COX-2 models (LPS-treated cells) in a dose-dependent manner (Fig 4); however, tolfenamic acid did not inhibit PGE2 in vitro at concentrations of 0.003125 µg/ml or 0.0125 µg/ml in the COX-2 model.

In the COX-1 model, significant differences in inhibition of PGE2 synthesis were not detected between carprofen and ketoprofen at all concentrations tested. Carprofen and ketoprofen were more COX-1 suppressive than either meloxicam or tolfenamic acid, significantly inhibiting PGE2 synthesis more than tolfenamic acid at every drug concentration, and more than meloxicam at all but the lowest concentration evaluated (0.003125 µg/ml). Significant differences were not detected between tolfenamic acid and meloxicam in the COX-1 assay, except at 0.003125 µg/ml and 0.0125 µg/ml.

In the COX-2 model, all drugs had similar potency against COX-2. Significant differences were not detected between the inhibitory effects of carprofen and meloxicam, or carprofen and tolfenamic acid (except at 0.003125 µg/ml and 0.0125 µg/ml), or of ketoprofen and meloxicam (except at 0.2 µg/ml).

Meloxicam and ketoprofen were significantly more PGE2 suppressive than was tolfenamic acid at concentrations between 0.003125 µg/ml and 0.2 µg/ml. Ketoprofen significantly inhibited PGE2 synthesis more than carprofen at the lowest concentrations (0.003125 µg/ml and 0.0125 µg/ml).

Comparisons of the effects of each drug between COX models were evaluated (Fig 4). There were no significant differences in inhibition of PGE2 synthesis detected between COX-1 and COX-2 models for carprofen nor for ketoprofen except at the highest con-
Meloxicam inhibited PGE₂ synthesis in the COX-2 model significantly more than in the COX-1 model at all but the lowest (0.003125 µg/ml) and highest (51.2 µg/ml) concentrations. Tolfenamic acid inhibited PGE₂ synthesis significantly more in the COX-2 model than the COX-1 model at all but the lowest concentrations.

The COX selectivity of each drug was evaluated by calculating the IC₅₀ values, which were based on the drug concentration that reduced PGE₂ synthesis by 50% in each COX model. The relative activity against each isoenzyme was expressed as a ratio of COX-1 to COX-2 activity. Preferential inhibition of COX-2 is indicated by a ratio > 1, and a greater inhibitory effect on COX-1 versus COX-2 is indicated by a ratio < 1. Tolfenamic acid was not available.

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Table 1—Indices of canine COX selectivity of 4 nonsteroidal anti-inflammatory drugs (NSAID). Concentration of each NSAID inducing 50% inhibition (IC₅₀) of PGE₂ synthesis in non-LPS treated (COX-1) and LPS-treated (COX-2) canine DH82 monocyte/macrophage cells was determined by quadratic regression, and relative activity against each isoenzyme was expressed as a ratio of COX-1 to COX-2 activity. Preferential inhibition of COX-2 is indicated by a ratio > 1, and a greater inhibitory effect on COX-1 versus COX-2 is indicated by a ratio < 1. Tolfenamic acid was not available.

<table>
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<th>Drug</th>
<th>IC₅₀ for COX-1 (µg/ml)</th>
<th>IC₅₀ for COX-2 (µg/ml)</th>
<th>Ratio COX-1/COX-2</th>
<th>IC₅₀ for COX-1/COX-2</th>
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<tr>
<td>Tolfenamic</td>
<td>N/A</td>
<td>3.53</td>
<td>N/A</td>
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*For tolfenamic acid, only the IC₅₀ value for COX-2 was determined. Fifty percent inhibition of PGE₂ was not achieved in the COX-1 model; therefore, calculation of both the IC₅₀ for COX-1 and the COX activity ratio for tolfenamic acid was not possible.

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times more active against COX-2 than COX-1, and ketoprofen inhibited COX-1 slightly more potently than COX-2 (IC$_{50}$ ratio of 0.36).

**Discussion**

Our ability to detect only COX-1 in untreated canine DH82 monocyte/macrophage cells, and demonstrate expression of the 4.5 kb COX-2 transcript in LPS-treated DH82 cells following northern blot analysis and RT-PCR is consistent with previous reports in the literature that confirm substantial increases in COX-2 mRNA expression following exposure to LPS, with no detectable effect on COX-1 mRNA.

The ability of human COX cDNA probes to detect COX-2 but not COX-1 message in these cells may be explained by the possibility that under continuous culture conditions, COX-1 message is expressed at such a low constitutive concentration that it is below the level of detection of northern blot analysis.

Although COX-2 expression was not detected in non-LPS stimulated canine DH82 monocyte/macrophage cells, it is possible that, as for COX-1, mRNA concentrations were too low to be detected by northern blot analysis. We attempted to evaluate possible COX-2 presence in non-LPS stimulated cells by use of RT-PCR, but were unable to amplify COX-2 from reverse-transcribed mRNA obtained from either unstimulated or LPS-stimulated canine DH82 cells. It is possible that the experimental conditions under which PCR was performed require further refinement to detect COX-2 activity in canine cells. However, as an immediate early response gene, COX-2 is rapidly induced, and profoundly expressed in response to LPS; once upregulated, COX-2 mRNA should be easily detectable by northern blot analysis. Our findings of low amounts of PGE$_2$ synthesis by non-LPS treated DH82 cells corresponds to the expected low constitutive amounts of COX-1 expression, and the 4-fold increase in PGE$_2$ synthesis following a 6-hour incubation period with LPS corresponds with strongly induced COX-2 expression. If COX-2 is present in non-LPS treated DH82 cells, its expression is expected to be minimal and would not substantially contribute to PGE$_2$ synthesis.

Previous studies of monocyte/macrophage cells in other species have also revealed a lack of COX-2 expression, and constitutive COX-1 expression in untreated monocyte/macrophage cells. Cyclooxygenase-2 mRNA was not detected by RT-PCR or northern blot analysis until cells were exposed to mitogenic or inflammatory stimuli, and the expression profile was similar to what we found in our study.

From our findings, it was suggested that low concentrations of PGE$_2$ synthesized by non-LPS treated canine DH82 cells were primarily the result of COX-1 enzyme activity, and increased PGE$_2$ synthesis by LPS-treated DH82 cells was predominantly in response to induced COX-2 enzyme activity. Thus, we were able to demonstrate the suitability of using canine DH82 monocytes/macrophage cells under different conditions as COX-1 and COX-2 models to screen NSAID in vitro.

A recent study reported NSAID COX inhibition profiles for dogs that were consistent with the results we observed. Canine platelets were used as a source of COX-1 in the previous study, and LPS-treated DH82 monocyte/macrophage cells were used as a source of COX-2. Results of general COX specificity in canine cells that had been reported were similar to results from our study. Ketoprofen was found to be marginally preferential for COX-1, and carprofen, meloxicam, and tolmetinic acid were found to be preferential for COX-2; however, the overall ranking order of COX-2 selectivity differed from the findings of our study. There are several model-specific differences that make comparison among assay systems difficult; however, time-dependent COX inhibition by NSAID may provide a partial explanation for the differences in COX-2 rankings between the 2 studies. In our study, equal incubation time was allotted to each NSAID for each COX model, whereas different NSAID incubation times between the COX-1 and COX-2 models were used in the other study, thereby potentially affecting rate of COX inhibition and calculated IC$_{50}$ ratios.

The ratio of the IC$_{50}$ of COX-1 and the IC$_{50}$ of COX-2 in a variable widely used throughout the literature as the gold standard of COX selectivity of various NSAID. In our study, significant differences in the individual effects of carprofen and ketoprofen on inhibition of PGE$_2$ synthesis between COX models were not detected; therefore, it would be expected that these 2 NSAID have relatively equal activity against both COX-1 and COX-2 enzymes. The calculated IC$_{50}$ ratios from our experiments reflect this, because a ratio of 1 designates equipotency against both enzymes; detected ratios of 1.75 for carprofen and 0.36 for ketoprofen do not deviate far from this standard.

For carprofen, this lack of specificity against either enzyme has been observed in other studies, and, in fact, it has been reported that carprofen is a relatively weak COX inhibitor, and as yet, its true mode of action is unclear. It has also been reported that ketoprofen is a relatively weak COX inhibitor, and as yet, its true mode of action is unclear.

Carprofen's equipotent activity (IC$_{50}$ ratio of 1) against COX-1 and COX-2 was also observed in another in vitro study, in which cultured bovine aortic endothelial cells were used as a source of COX-1 enzyme, and J774.2 macrophages stimulated with LPS were used as a source of COX-2 enzyme.

Ex vivo studies using models of inflammation have been unable to demonstrate significant COX inhibition by carprofen at clinical doses in the dog. Carprofen's equipotent inhibition of COX-1 and COX-2 enzymes in vitro may also occur in vivo, but because carprofen does not appear to inhibit canine COX at therapeutic doses, it is not possible to evaluate this effect at this time.

Carprofen is an effective analgesic, but it has been suggested that analgesic effects of carprofen may develop by an alternative mechanism than systemic COX inhibition, and, in some species, higher than approved doses may be required to induce anti-inflammatory effects, compared with the dose needed to relieve pain.

It has also been reported that ketoprofen is a potent analgesic for dogs and cats. Ketoprofen is typically preferential for COX-1 in vitro (as reported here); this has also been demonstrated by other studies in which the effects of NSAID on whole blood or cultured cells have been examined. In a model of
inflammation in horses, ketoprofen inhibited both serum thromboxane B₂ (TXB₂) synthesis (COX-1) and PGE₂ synthesis in exudate (COX-2) for up to 24 hours. The ability of ketoprofen to inhibit COX-1 and COX-2 in vivo reinforces the validity of our data collected in vitro.

Meloxicam has been reported to be an effective anti-inflammatory and analgesic in dogs and cats.²⁴⁻⁴¹ Our finding of the significantly greater effect of meloxicam on PGE₂ inhibition in the COX-2 model versus the COX-1 model was also reflected by the calculated IC₅₀ value of 12.27, a much higher ratio than values calculated for carprofen or ketoprofen. In concurrence with our findings, meloxicam has been consistently shown to be COX-2 preferential in vitro and is also capable of sustained COX-2 inhibition in vivo.⁴²⁻⁴⁶ In an equine tissue cage model, meloxicam initially inhibited TXB₂ formation in serum (COX-1) and exudate (COX-2).⁴⁶ However, there was no inhibition of serum TXB₂ after 8 hours, but concentrations in exudate remained suppressed. This indicates that inhibition of COX-1 is reversible, and COX-2 suppression is prolonged at sites of inflammation following administration of meloxicam.⁴⁰

Like meloxicam, tolfenamic acid preferentially inhibited COX-2 in our study. There are conflicting reports of the COX specificity of tolfenamic acid; one study recorded a COX-2 preferential IC₅₀ value of 15.0, which is in agreement with our findings, but another recorded a COX-1 preferential IC₅₀ value of 0.06.²⁵ Tolfenamic acid has been shown to almost completely inhibit ex vivo synthesis of TXB₂ (COX-1) in serum, and to suppress PGE₂ formation (COX-2) in exudate from tissue cages in dogs and calves.²⁶⁻⁶⁰ In dogs, the duration of TXB₂ inhibition in serum was short lived, because 50% inhibition was not detected beyond 8 hours at clinical dosage regimes. These studies demonstrate the COX-2 suppressive effect of tolfenamic acid and support our findings of the COX-2 preference of this NSAID.

Tolfenamic acid did not achieve 50% inhibition of PGE₂ in our COX-1 model; therefore, calculation of an IC₅₀ ratio for this drug was not possible.²⁵⁻⁵⁰ However, COX-1 inhibition exceeding 50% has been detected in vivo, diminishing over time. A possible explanation is that reversible inhibition of COX-1 develops slowly in vivo secondary to the relatively slow distribution of tolfenamic acid from plasma to the inflammatory site, compared with other NSAID and that reversal of COX-1 suppression develops more rapidly at the high free drug concentrations of tolfenamic acid present in the in vitro system.²⁵⁻⁶⁰ Regardless, it appears that the in vitro model system does not entirely reflect the in vivo effects of tolfenamic acid in dogs and requires further investigation.

If the COX selectivity of each NSAID evaluated in our canine cell assay were extrapolated to an in vivo situation, it would be expected that administration of preferential COX-2 inhibitors (eg, meloxicam and tolfenamic acid) to dogs would provide effective relief of pain and inflammation and cause fewer and less severe adverse effects than the nonselective inhibitor ketoprofen. These conclusions appear to be supported by clinical studies that have examined adverse effects associated with use of NSAID in dogs. Results appeared to reflect the anticipated physiologic effects associated with COX-1 or COX-2 preference, as reflected by the system we used. Forsyth et al reported that 5 of 6 dogs treated with ketoprofen at the recommended dosage had endoscopic evidence of hemorrhage or gastric erosions, although the dogs appeared clinically normal. In a more recent study by the same author in which gastric ulceration was evaluated in dogs after administration of NSAID, fewer dogs developed gastric lesions after administration of carprofen and meloxicam, compared with ketoprofen, although the difference was not significant.⁴⁷ These adverse findings may be consistent with the nonselective and even slightly preferential COX-1 inhibition by ketoprofen at clinical dosage regimens, and provide supportive evidence of a more desirable safety profile expected by preferential COX-2 inhibitors such as meloxicam, as predicted by the in vitro screening technique we used. On the basis of the nonspecific in vitro COX inhibition observed in these 2 agents, it would be expected that the effects of carprofen on the gastrointestinal tract would be similar to those for ketoprofen. However, the lack of detectable COX-1 or COX-2 inhibition in vivo following therapeutic doses of carprofen in dogs may explain why its safety profile is similar to that of a COX-2 inhibitor such as meloxicam.⁴⁸⁻⁵⁰,⁵¹

Several issues should be addressed when evaluating the predictive value of any model. It is desirable to use whole cell systems containing the intact COX enzyme of the species under investigation. Protein binding, time-dependent inhibition, and source of AA factors that should all be considered.²¹⁻⁵⁰ In designing our in vitro cell assay system, characteristics of the ideal model were implemented as closely as possible. Effect of protein binding is difficult to mimic in vitro; therefore, a protein-free (serum-free) system was used in our study and all drugs were acting in a free and unbound form. Results from NSAID inhibition assays that allow for time-dependent inhibition by preincubating cells with the drugs being tested correlate more closely with clinical data.²³ Inhibitor time-dependency was also addressed in our study by preincubating each drug with DH82 cells for 30 minutes prior to the 30-minute incubation period with drug and a single concentration of exogenous AA, which is a technique used in other studies.²¹⁻⁵⁰ The resulting predictive value of the model system correlates well with data from other in vitro studies and is supported by findings of clinical studies that examined efficacy and toxicity of the NSAID evaluated in our study.²³⁻⁴³,⁴⁵⁻⁶⁰,⁶¹

Most in vitro or ex vivo studies measure TXB₂ or PGE₂ concentrations in serum or culture medium from untreated cells as indices of COX-1 activity; however, TXB₂ and PGE₂ are also measured in inflammatory exudate or culture medium from stimulated cells as indices of COX-2 activity.²³⁻⁴³ Evaluation of COX inhibition by NSAID based solely on measurement of TXB₂ or PGE₂ concentrations may not accurately reflect specific COX-1 and COX-2 inhibition, because these PG can be a product of either COX-
isoenzyme. Our ability to measure PGE2, synthesis, and attribute it to canine COX-1 or COX-2 gene expression through northern blot analysis or RT-PCR is novel and may provide a more complete evaluation of COX inhibition than measurement of PG synthesis alone.

The ability of NSAID to preferentially inhibit COX-2 over COX-1 in vivo is dependent on various factors that cannot be achieved with an in vitro model, including individual pharmacokinetic differences, protein binding, and tissue distribution. Therefore, true prediction of in vivo drug efficacy and toxicity is not possible from an in vitro assay.12 However, comparison of differential inhibition of COX enzyme by each drug relative to others evaluated in the same model system may be possible.

It would be advantageous to establish an in vitro assay for canine COX inhibition by NSAID to permit rapid screening of potential COX-2 selective inhibitors in the early stages of clinical development. Such an assay would minimize experimental animal usage and decrease the time required for new drug approval, because only NSAID that significantly inhibit COX-2 would be further evaluated in vivo. Preferential COX-2 inhibitors would provide veterinarians with greater options for analgesia in animals following trauma or surgery and for control of chronic inflammatory conditions such as osteoarthritis.

In this study, we have described a simple and convenient assay system to screen COX-1 and COX-2 inhibitor activities in intact canine cells in vitro. This assay offers the advantage of assessing canine COX inhibition with a similar test protocol and circumvents the need for purification of either platelet or peripheral blood monocytes. We have used a convenient source of COX-1 found in an untreated permanent culture of canine DHB2 monocyte/macrophage cells and induced COX-2 in the same cell type through incubation with LPs.

References


