Cyclooxygenase selectivity of nonsteroidal anti-inflammatory drugs in canine blood

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Objective—To evaluate cyclooxygenase (COX) selectivity of several nonsteroidal anti-inflammatory drugs (NSAID) in canine blood in vitro.

Animals—11 healthy adult male hound crosses.

Procedure—9 NSAID were studied at 5 concentrations. Thromboxane B_2 (TxB_2) was assayed as a measure of COX-1 activity in clotted blood. Prostaglandin E_2 (PGE_2) was assayed as a measure of COX-2 activity in heparinized, lipopolysaccharide (LPS)-stimulated blood. All assays were competitive ELISA tests. Cyclooxygenase selectivity was expressed as a ratio of the concentration of an NSAID that inhibited 50% of the activity (IC_{50}) of COX-1 to the IC_{50} of COX-2. A separate ratio of the concentration that inhibited 80% of COX activity (IC_{80}) was also determined. A ratio of < 1.0 indicated selectivity for COX-1, whereas a ratio of > 1.0 indicated COX-2 selectivity.

Results—Ketoprofen, aspirin, and etodolac were COX-1 selective. Piroxicam, meloxicam, and carprofen had COX-2 selectivity. The IC_{50} and IC_{80} values were similar for most NSAID.

Conclusions—This methodology provides repeatable data from individual dogs and is comparable to results of previous in vitro and ex vivo models. Findings are also consistent with those of canine studies performed in vivo, suggesting that this is a viable in vitro assessment of the COX selectivity of NSAID in dogs. (*Am J Vet Res* 2002;63:91–94)

Nonsteroidal anti-inflammatory drugs (NSAID) are commonly used in small animal medicine. Despite differences in chemical structures and other potential mechanisms of action,[1-10] these compounds share the effect of inhibiting the production of eicosanoids by targeting the **cyclooxygenase (COX)** pathway. Eicosanoids are products of the breakdown of arachidonic acid released from membrane phospholipids. The COX enzyme has 2 distinct isoforms: COX-1 and COX-2.[2,3,5,9-12] The COX-1 pathway produces eicosanoids that are protective and function in homeostatic roles. The enzyme is expressed constitutively in areas such as the gastrointestinal, renal, and central nervous systems. Cyclooxygenase-2 appears to be primarily an inducible enzyme in response to inflammatory processes. Nonsteroidal anti-inflammatory drugs that are COX-1 selective in their inhibitiory effects eliminate the protective COX-1 products. Likewise, a nonselective compound would eliminate both COX-1 products as well as the inflammatory products of the COX-2 pathway. This accounts for the common adverse effects associated with administration of COX-1 selective and nonselective NSAID, such as ulcers of the gastrointestinal tract. An NSAID that preferentially inhibits COX-2 activity and preserves COX-1 activity should be effective in decreasing signs associated with inflammation, while preventing the adverse effects associated with COX-1 inhibition.

Preliminary work has been performed to determine the COX activity of select compounds in dogs in vitro. In 1 study,[13] COX-1 and COX-2 activity of carprofen and other NSAID were assayed, using canine platelets and a macrophage cell line. A similar assay of COX activity has been developed in a canine monocyte/macrophage and kidney cell line.[14,15]

The latter 2 studies confirmed results that some newer NSAID are COX-2 selective, at least in vitro. A problem arises, however, when striving to compare these results (derived in vitro) with clinical use in dogs. It is difficult to determine which of the various assay techniques most appropriately reflects activity in vivo. A stronger link is needed to provide the means to extrapolate test tube results to clinical effects. On the basis of previous data,[16] a whole blood assay has been developed for use in vitro to compare the selectivity of NSAID in healthy humans.[17] This method, which does not rely on isolated cell lines, should more closely simulate physiologic activity in the body. One objective of the study reported here was to modify this whole blood assay to evaluate NSAID activity in dogs as an initial step in correlating in vitro, ex vivo, and in vivo studies. Additionally, questions have been raised as to whether the standard use of the **concentration that inhibits 50% of COX activity (IC_{50})** as a determination of the selectivity ratio is an accurate reflection of concentrations and activity in vivo.[3,14,18] It has been suggested that the **concentration that inhibits 80% of COX activity (IC_{80})** is a better approximation.[18] Our second objective, therefore, was to evaluate and compare the IC_{50} and IC_{80} selectivity ratios. Several NSAID that are commonly used in dogs were tested in vitro, and their selectivity was characterized.

Materials and Methods

Eleven healthy adult male mixed-breed dogs were used to test 9 drugs. Each drug was tested in 6 animals. No NSAID were given 2 weeks prior to performing the assays. The compounds assayed were aspirin,[a] etodolac,[a] ibuprofen,[a] meclofe-

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namic acid,[a] ketoprofen,[a] phenylbutazone,[a] piroxicam,[a] carprofen,[b] and meloxicam.[c] A 10,000 µ*M* stock solution of each drug was made in 90% **dimethyl sulfoxide (DMSO)** solution.[d] Serial dilutions were made, using 10 m*M* **phosphate-buffered saline solution (PBSS)**[a] so that the final 5 concentrations tested in the study were 100, 10, 1, 0.1, and 0.01 µ*M*. The control solution was 100 µl DMSO in 9.9 ml of PBSS.

Cyclooxygenase-1 whole blood assay—Cyclooxygenase-1 activity is represented by **thromboxane B_2 (TxB_2)** synthesis from platelets after coagulation of whole blood. The assay has been modified from previously described methodologies for use in dogs.[16,17] Briefly, 6 siliconized glass red-top tubes were prepared for each dog with 40 µl of each test concentration of a particular agent. Four milliliters of whole blood were allocated to each tube. The tubes were allowed to clot at 37 C for 1 hour, by which time TxB_2 concentrations reach a plateau. To stop further production, indomethacin solution was added at a final concentration of 30 µ*M*. Tubes were gently inverted 2 to 3 times and then centrifuged at 2,000 × g for 10 minutes at 21 C. One milliliter of serum from each tube was collected, and the sample was purified by extraction through an ethyl C2 minicolumn.[e] The sample was assayed for TxB_2 by use of an enzyme-linked immunoassay kit.[f]

Cyclooxygenase-2 whole blood assay—Cyclooxygenase-2 is represented by measurement of **prostaglandin E_2 (PGE_2)** from monocytes in whole blood after stimulation with **lipopolysaccharide (LPS)**. The assay had been modified from previously described methodologies.[16,17] Briefly, six 1.5-ml polypropylene microcentrifuge tubes were prepared for each dog with 5 µl of each test concentration. Five hundred microliters of heparinized blood were allocated to each microcentrifuge tube. The tubes were incubated for 15 minutes at 37 C. Fifty micrograms of LPS (*Escherichia coli* serotype 127:B8)[a] were added, and the tubes were inverted several times and incubated at 37 C for 24 hours, by which time COX activity is almost exclusively COX-2. After the incubation period, tubes were centrifuged at 12,000 × g for 5 minutes at 21 C. One hundred microliters of plasma were mixed with 900 µl of ethanol and the tubes were centrifuged again at 12,000 × g for 1 minute. The sample was then assayed for PGE_2 content, using an enzyme-linked immunoassay kit.[f]

Determination of COX selectivity—Cyclooxygenase activity was expressed as percentage inhibition of control values. Mean inhibitions for each NSAID were graphed (y axis) against the respective drug concentrations (x axis, logarithmic scale). The IC_{50} were derived, using a line of best-fit equation. The selectivity was then determined as a ratio of the IC_{50} of COX-1 to that of COX-2. A second ratio was determined, using IC_{80} values. An agent was deemed to be COX-1 selective if the ratio was < 1, and COX-2 selective if the ratio was > 1.

Table 1—Cyclooxygenase selectivity of several nonsteroidal antiinflammatory drugs (NSAID) expressed as a ratio of cycloooxygenase (COX)-1 inhibitory concentration at 50% (IC_{50}) to COX-2 IC_{50} (and inhibitory concentration at 80% [IC_{80}]). A ratio of < 1 indicates COX-1 selectivity, whereas a ratio of > 1 indicates COX-2 selectivity

	Cox selectivity	
NSAID	**IC_{50}**	**IC_{80}**
Ketoprofen	0.17	0.26
Aspirin	0.388	0.087
Etodolac	0.53	1.08
Ibuprofen	0.74	1.01
Piroxicam	2.03	1.34
Meloxicam	2.72	3.72
Meclofenamic acid	5.06	2.87
Phenylbutazone	9.74	21
Carprofen	16.8	101.2

Results

Data were summarized in order of the COX selectivity of the agents tested (**Table 1**). Four drugs had ratios of < 1, indicating a greater tendency to inhibit COX-1 activity. They were, therefore, considered to be COX-1 selective. Five drugs had a ratio exceeding 1, indicating that they were COX-2 selective (COX-1 sparing).

The data also revealed that the IC_{50} and IC_{80} ratios yielded similar COX selectivity and sparing. Most of the NSAID tested had the same selectivity at the higher concentrations. Etodolac and ibuprofen were COX-1 selective with IC_{50} ratios but shifted to a more neutral selectivity with the IC_{80} ratios. Piroxicam shifted from COX-2 selectivity to near neutrality at the higher concentration. Carprofen was COX-2 selective at IC_{50} and was the only NSAID that had a considerable increase in COX-2 selectivity at IC_{80} (IC_{50} = 16.8; IC_{80} = 101.2).

Discussion

It is difficult to draw comparisons between in vitro studies in the literature. First, in vitro techniques vary, sometimes greatly. One review indicates that the same NSAID can have a different selectivity profile by using different cell types or assay techniques.[3] Although PGE_2 and TXB_2 are usually the standard outcome measurements of COX-2 and COX-1 activity, respectively, there are various protocols for eliciting their production. Lipopolysaccharide from *E coli* is often the stimulant for COX-2 activity, but it has been added either before or after stimulation with test drug, and incubation times vary from 6[14,19] to 24 hours.[13,16,17] In 1 study,[18] interleukin-1β was used to stimulate COX-2 activity in a human airway epithelial cell line, and aspirin was used to inhibit COX-1 activity in human heparinized blood. Several described techniques involve the use of arachidonic acid[14,19] or calcium ionophores[13,18] to promote

Table 2—Cyclooxygenase selectivity, compared with previous studies performed in vitro in dogs and humans. Etodolac is COX-1 selective in dogs, but is COX-2 selective in humans. All other NSAID evaluated had similar selectivity. Only evaluated IC_{50} ratios were evaluated in these other studies; therefore, IC_{80} values could not be compared

NSAID	**IC_{50}**	**Ricketts*[20]**	**Kay Mugford*[21]**	**Cryer†[24]**	**Brideau†[23]**
Ketoprofen	0.17	0.232	0.36	0.125	0.019
Aspirin	0.388	< 0.343	N/A	0.32	N/A
Etodolac	0.53	0.517	N/A	7.92	N/A
Ibuprofen	0.74	N/A	N/A	0.6	0.158
Piroxicam	2.03	N/A	N/A	1.27	0.085
Meloxicam	2.72	2.9	12.27	N/A	N/A
Meclofenamic acid	5.06	15.4	N/A	12.1	N/A
Phenylbutazone	9.74	> 2.64	N/A	N/A	1.0
Carprofen	16.8	129	1.75	N/A	N/A

*Assays performed in vitro with canine cells and cell lines. †Assays performed in vitro and ex vivo with human whole blood and cells.
N/A = Not assayed.

COX activity. The purpose of our study was to verify the efficacy and application of a whole blood assay of COX selectivity in dogs in vitro. Whole blood should more accurately reflect physiologic NSAID activity than pure cell lines or isolated cell types because of the presence of plasma proteins and other potential cofactors. Our data (**Table 2**) indicate that similarities in methodology result in similar selectivity profiles.

A second difficulty encountered when drawing comparisons is species variation. The selectivity of etodolac demonstrates the importance of the target species (Table 2). In human studies[17,18] performed in vitro and ex vivo, etodolac is COX-2 selective. Certainly the safety profile of this NSAID suggests a protective effect in the gastrointestinal system, consistent with COX-2 selectivity.[20] In dogs, similar safety profiles have been observed clinically[21,22]; however, results from this and another study[13] performed in vitro revealed that etodolac is actually COX-1 selective in dogs. This suggests that there is species variability in the structure or activity of the COX enzyme. Although there is no variability in the selectivity of the remaining NSAID evaluated in this study, certainly there is the possibility that other NSAID may differ in activity. Testing agents on target species, therefore, is critical in obtaining the correct activity profile.

Although there are different factors contributing to the varying results of in vitro studies, use of similar technique[16,17] or the same target species[13,14] may yield consistent results for a particular NSAID (Table 2). However, differences are seen; this was evident in a study[16] in which differing results for piroxicam were observed. This is possibly attributable to disparities in techniques (though the species may be the same as with another study[17]) or differences in species (though the technique may be similar to that used in our study).

We also compared COX selectivity determined by the IC_{50} ratio versus that of the IC_{80} ratio. It has been suggested that use of the standard in vitro concentrations that cause 50% inhibition does not accurately approximate plasma concentrations achieved with therapeutic dosing,[3,14] and it has been suggested that at higher concentrations, NSAID lose their selectivity. In 1 study,[18] IC_{50} ratios were compared with IC_{80} ratios, stating that inhibition curves are not always parallel; therefore, potency may not be equivalent at different concentrations. In that study, it was concluded that the IC_{80} values may more closely resemble steady-state plasma concentrations. As our results revealed, selectivity of most NSAID remained the same at IC_{80} (Table 1). Exceptions were etodolac, ibuprofen, and piroxicam, all of which became less selective with ratios close to 1. The clinical significance of this is debatable, as etodolac and ibuprofen are COX-1 selective and, therefore, loss of selectivity may be beneficial. With this assay, piroxicam is not strongly COX-2 selective at a concentration inhibiting 50% of activity and becomes even less so at the IC_{80}. Carprofen had a considerable increase of COX-2 selectivity with the IC_{80} ratio, but it is unknown whether this change would be mirrored in the clinical setting.

The most important question to be answered by this, and all other in vitro assays, is how well do these results correlate with activity in vivo? Historically, in vivo selectivity of NSAID in dogs was an assumption based on human literature, clinical efficacy, and frequency of adverse effects.[21-25] Recent in vivo and ex vivo findings provide a more objective means of assessing selectivity.[g] Evaluation of PGE_2 and TxB_2 in gastric biopsy specimens, synovial fluid, and blood revealed that aspirin and meloxicam were COX-1 and COX-2 selective, respectively. These results correlate well with the data presented here, as aspirin and meloxicam had the same selectivity profile in vitro. Certainly, previous studies of clinical behavior and safety profiles have correctly suggested the activity of these 2 compounds, but results of our study provide a confirmed link between in vitro and in vivo studies, suggesting that it is a viable means of predicting or confirming COX selectivity of NSAID in dogs.

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[a]Sigma Chemical Co, St Louis, Mo.
[b]Carprofen, Pfizer, Inc, Groton, Conn.
[c]Meloxicam, Boehringer Ingelheim Vetmedica Inc, Ingelheim, Germany.
[d]Dimethyl sulfoxide, Fort Dodge Animal Health, Fort Dodge, Iowa.
[e]Ethyl C2 minicolumn, Amersham Life Science, Buckinghamshire, England.
[f]Enzyme-linked immunoassay kit, Cayman Chemical Co, Ann Arbor, Mich.
[g]Jones CJ, Streppa HK, Budsberg SC. In vivo effect of a COX-2 selective and nonselective nonsteroidal anti-inflammatory drug (NSAID) on gastric mucosal and synovial fluid prostaglandin synthesis in dogs (abstr). *J Vet Intern Med* 2001;15:273.

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