IN VITRO EVALUATION OF THE EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON CYCLOOXYGENASE-1 AND -2 IN THE DOG

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by
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ABSTRACT

IN VITRO EVALUATION OF THE EFFECTS OF NONSTEROIDAL ANTI-INFLAMMATORY DRUGS ON CYCLOOXYGENASE-1 AND -2 IN THE DOG

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Selective cyclooxygenase (COX)-2 inhibitors are a new class of nonsteroidal anti-inflammatory drugs (NSAIDs) which may provide safer and more potent analgesia than currently available non-selective agents. Development of COX-2 inhibitors for veterinary use would be facilitated by assays to evaluate their biochemical effectiveness in the dog.

Canine COX-1 and COX-2 isoenzymes were found to be differentially sensitive to in vitro inhibition by NSAIDs using a canine monocyte/macrophage cell line which 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 mRNA expression using Northern analysis and RT-PCR.

Meloxicam and tolfenamic acid demonstrated preferential inhibition of COX-2; with meloxicam inhibiting COX-2 activity 12 times more effectively than COX-1 activity. Effects of carprofen and ketoprofen approached equipotency against both isoenzymes.
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DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in this thesis was performed by me.

Alexis Goth assisted in the performance of the Northern blot analysis.
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AA.................................................................Arachidonic acid
ADH............................................................Antidiuretic hormone
cAMP..........................................................Cyclic AMP
COX.............................................................Cyclooxygenase
DH82............................................................Canine DH82 monocyte/macrophage cells
DMPC-A.....................................................Autoclaved water containing 0.01% dimethylpyrocarbonate
DMEM..........................................................Dulbecco’s modified Eagle’s medium
DMSO...........................................................Dimethyl sulfoxide
DNA............................................................Deoxyribonucleic acid
E. coli.........................................................Escherichia coli
IL.................................................................Interleukin
kb.....................................................................Kilobase pairs
kDa.....................................................................Kilodaltons
LB.................................................................Luria-Bertani medium
LPS..............................................................Lipopolysaccharide endotoxin
mRNA..........................................................Messenger ribonucleic acid
MDCK.........................................................Madin-Darby canine kidney cells
MOPS...........................................................3-(N-Morpholino) Propane-Sulfonic Acid
NSAID........................................................Nonsteroidal anti-inflammatory drug
PG..............................................................Prostaglandin
PGHS........................................................Prostaglandin H Synthase
PGE2............................................................Prostaglandin E2
PGG2..........................................................Prostaglandin G2
PGH₂..........................................................Prostaglandin H₂
PGI₂..........................................................Prostacyclin
POX..........................................................Peroxidase
RNA..........................................................Ribonucleic acid
RT-PCR........................................................Reverse Transcriptase-Polymerase Chain Reaction
SDS..........................................................Sodium dodecyl sulphate
SSC..........................................................Sodium chloride/sodium citrate
SSPE..........................................................Sodium chloride/sodium phosphate
TXB₂..........................................................Thromboxane B₂
INTRODUCTION

Although 45 analgesic drugs are approved in Canada for humans, only eight are approved for dogs, and there are few clinical studies which describe their beneficial effects or potential toxicity. Nonsteroidal anti-inflammatory drugs (NSAIDs) are used daily for management of pain in veterinary practice; however, due to the immense interpatient variability among drugs in this family, every clinical practice situation presents a dilemma, since the risk of serious adverse effects may outweigh the benefits of using a particular NSAID as an anti-inflammatory analgesic.

Pain accompanies inflammation following injury and is partly mediated by prostaglandins (PGs). Phospholipase A\textsubscript{2} releases arachidonic acid (AA) from phospholipid membranes, which is then converted into PG’s by cyclooxygenase (COX) enzyme activity. There are two COX isoenzymes - COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and is considered a “housekeeping enzyme” responsible for producing PGs that play a role in sustaining normal cellular or tissue function. COX-2 is inducible through tissue damage, releasing proinflammatory PGs at the site of injury, causing swelling and pain (Smith et al., 1991; Pairet et al., 1995; Vane et al., 1995).

Currently available NSAIDs inhibit both COX isoenzymes to varying degrees. This non-selective COX inhibition reduces PG production and pain at the inflammatory site by suppressing COX-2, but may also cause gastric ulceration and compromised renal function due to reduction of homeostatic PGs in these organs through suppression of COX-1. Selective COX-2 inhibitors comprise a new class of therapeutic agents with anti-inflammatory effects like the conventional NSAIDs, but with fewer and less severe adverse effects (Vane et al., 1971; Cashman et al., 1996; Vane et al., 1998). These agents would provide veterinarians with greater options for analgesia following trauma or surgery, and for control of chronic inflammatory conditions such as osteoarthritis.
The purpose of this study was twofold; firstly, to establish an *in vitro* assay system utilizing canine cell lines specifically expressing COX-1 and COX-2, and secondly to utilize this system to differentiate the interactions of NSAIDs with these two isoforms in the dog.

Pilot studies to evaluate COX expression and PG synthesis by two canine cell lines were conducted. Northern blot analysis was employed to assess COX mRNA levels, and enzyme immunoassay used to measure prostaglandin production. These initial studies evaluated the suitability of these cell lines to serve as models of COX-1 and COX-2 activity. The resulting assay system was used to determine the differential suppressive activity of four NSAIDs which were recently approved for canine use in Canada and/or the United States.
Inflammation and Pain

Physiologic pain occurs when intense noxious stimuli such as heat, cold or pressure, activate high threshold sensory receptors, known as nociceptors. This unpleasant sensory experience serves a protective function which prevents further exposure by an individual to prolonged or repeated trauma (Woolf, 1991; Markenson, 1996).

Pathologic pain can result from inflammation. Following tissue damage, the subsequent inflammatory response and production of associated mediators, lowers the nociceptive threshold at the site of injury, and permits greater response to stimuli; a phenomenon known as primary hyperalgesia. If pain is present for a prolonged period of time, peripheral nociceptor stimulation increases the release of neuropeptides, such as substance P, and excitatory amino acids such as glutamate, thus altering sensory processing in the peripheral and central nervous system. Activation of N-methyl-D-aspartate (NMDA) receptors in the dorsal horn of the spinal cord follows, causing secondary hyperalgesia. Consequently, the animal experiences a reduction in the nociceptive threshold in the surrounding uninjured tissue, which is termed allodynia, and subsequent responses to even minimal stimuli are exaggerated (Rang et al., 1991; Woolf, 1991; Markenson, 1996).

Once tissue trauma occurs, the inflammatory cascade is set in motion and multiple inflammatory mediators are released. Several of these mediators are capable of directly stimulating peripheral nociceptors, while others sensitize the nociceptors and lower the stimulus threshold without actually initiating neuronal discharge. The inflammatory response is designed to isolate and remove the noxious stimulus, repair tissue damage and restore normal structure and function (Rang et al., 1991; Woolf, 1991; Cashman et al., 1996).

Acute inflammation is characterized by heat, redness, swelling, pain and loss of function. The underlying events responsible for these clinical signs include vasodilation, increased small vessel
permeability, accumulation of leukocytes and stimulation of nociceptors. If the inciting stimulus is not removed, inflammation may continue in a chronic form characterized by tissue destruction and local proliferation of cells and connective tissue. Acute pain subsides as healing progresses; however, chronic pain does not resolve and serves no useful biological function (Woolf, 1991; Cashman et al., 1996; Markenson, 1996).

**Cyclooxygenase**

Following tissue damage, arachidonic acid (AA), a 20 carbon polyunsaturated essential fatty acid is released from cellular membrane phospholipid as a free fatty acid by the enzyme phospholipase A2. The AA is then channeled into one of two pathways: the lipoxygenase or the prostaglandin H-synthase (PGHS)/cyclooxygenase (COX) pathways. Prostaglandin H-synthase is a membrane bound, bifunctional enzyme which catalyzes the conversion of AA to form PGs though its cyclooxygenase and peroxidase (POX) activity. Its cyclooxygenase activity (oxidative capacity) results in incorporation of two molecules of oxygen into AA and adds a 15-hydroperoxy group to form prostaglandin G2 (PGG2). The peroxidase activity reduces the 15-hydroperoxy group to form prostaglandin H2 (PGH2), which is subsequently converted to one, or several biologically active prostaglandins, PGD2, PGE2, PFG2, TXA2 or PGI2 (Vane et al., 1995; Smith et al., 1991; Meade et al., 1993).

Cyclooxygenase was first isolated and identified in 1976 (Hemler et al., 1976) and cloned in 1988 (DeWitt et al., 1988). It is now accepted that this original enzyme was actually COX-1, and a second distinct unique isoform, known as COX-2, was reported in 1991 (Xie et al., 1992). The two isoenzymes differ in their basal expression, tissue localization and induction during inflammation (Smith et al., 1991; Pairet et al., 1996).

Cyclooxygenase-1 is constitutively expressed in most tissues and is thought to be responsible for the majority of basal prostaglandin production in resting cells to regulate normal cellular activity. The enzyme concentration is very stable, with very small increases in expression of two
to four fold occurring in response to hormonal or growth factor stimulation (DeWitt et al., 1993; Smith et al., 1991; Pairet et al., 1996).

Cyclooxygenase-2 is induced following cellular exposure to pro-inflammatory cytokines and growth factors, suggesting a role for COX-2 in inflammation, and in growth and differentiation (Arias-Negrete et al., 1995; Xie et al., 1996; Yamamoto 1998). Cyclooxygenase-2 is normally absent from cells, and following induction, COX-2 protein levels rise rapidly and transiently 10 to 100 fold after exposure to a single stimulus (Xie et al., 1991; Herschman et al., 1993). This has been demonstrated in a variety of cell types following tissue trauma. The majority of stimuli known to induce COX-2 are associated with inflammation, and include bacterial lipopolysaccharide (LPS) endotoxin, cytokines such as interleukin (IL)-1, IL-2 and tumor necrosis factor alpha (Fu et al, 1990; Masferrer et al., 1994; Arias-Negrete et al., 1995). Conversely, anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 will decrease COX-2 induction, as will exposure to glucocorticoids (Masferrer et al., 1992; Vane et al., 1998).

**Enzyme Structure and Function**

Cyclooxygenase-1 and-2 are integral membrane proteins positioned in a monotopic arrangement, within one leaflet of the lipid bilayer. The x-ray crystallographic structures are remarkably similar, comprised of three independent folding units: an epidermal growth factor domain, a membrane binding domain, and a catalytic domain. Located within the catalytic domain, the two active sites for cyclooxygenase and peroxidase activities are adjacent, but distinct. Three alpha helices form the entrance to the long, hydrophobic channel comprising the COX active site. There are four amino acid residues lining the channel, Tyr-385, Arg-120, Glu-524 and Ser-530 (Picot et al., 1994). These residues are almost entirely conserved between COX-1 and COX-2. The overall structure of both isoforms is virtually identical; however, the COX-2 active site is wider, which may permit the access of larger chemical structures than those which are able to reach the COX-1 active site (Picot et al., 1994). These slight differences between the active sites of COX-1 and COX-2 likely results in the different affinities of certain
NSAIDs for each of the two isoenzymes (Laneuville et al., 1994; Smith et al., 1991; Picot et al., 1994).

The two COX isoforms are 60% homologous at the amino acid sequence level and approximately 90% homologous among most species (Yokoyama et al., 1989; Smith et al., 1990; Williams et al., 1996; Crofford et al., 1997). The enzymes both have a molecular weight of approximately 70 kDa, and have a similar affinity for AA and capacity to convert it to PGH$_2$ (Smith et al., 1991; Meade et al., 1993; Pairet et al., 1996). Despite the conservation of structure and function, each COX may perform as an independent enzyme system, occupying subcellular compartments in different concentrations. Cyclooxygenase-1 is located primarily on the endoplasmic reticulum, and only slightly detectable on the nuclear membrane, while COX-2 is much more concentrated on the nuclear membrane than on the endoplasmic reticulum (Morita et al., 1995). The major distinction seems to be how production of enzyme itself is controlled and the amount of enzyme that is manufactured (Smith et al., 1991; Meade et al., 1993; Williams et al., 1996; Yamomoto et al., 1998).

**Gene Sequence and Regulation**

Cyclooxygenase-1 and -2 are encoded by two distinct genes, each expressed via different sized mRNA transcripts (Funk et al., 1991; Ping et al., 1993; Kosaka et al., 1994). The human and mouse genes for COX-1 and COX-2 are located on chromosomes 9 and 1, respectively. Cyclooxygenase-1 is approximately 22 kb, containing 11 exons and 10 introns. Cyclooxygenase-2 is much smaller (approximately 8 kb) and contains 10 exons (Kosaka et al., 1994). For each COX gene, the intron/exon arrangement is identical, except that exons 1 and 2 of COX-1, containing the translational start site and signal peptide, are condensed into a single exon in COX-2 (Tazawa et al., 1994; Yang et al., 1996). The introns of COX-2 are smaller which accounts for the gene’s small size (Kosaka et al., 1994). The COX-2 promotor (63% conserved between species) contains a TATA box, while the COX-1 gene does not (Wang et al., 1993; Tazawa et al., 1994; Yang et al., 1996; Crofford et al., 1997). These features of COX-2 allow for
the rapid transcription and processing of mRNA associated with a significantly inducible gene. There is little similarity between the 5'-flanking regions of the two COX genes, and the 3'-untranslated region of COX-2 mRNA contains multiple instability sequences that mediate rapid degradation of the transcript (Kraemer et al., 1992; Kosaka et al., 1994; Williams et al., 1996; Crofford et al., 1997).

On Northern blot analysis, both COX isoforms exhibit multiple transcripts (Pairet et al., 1996; Yamamoto et al., 1998). The predominant COX-1 mRNA transcript is 2.8 kb in some cells, and 5.2 kb in others (Hla, 1996). COX-2 mRNA has been shown to be 4 or 4.5 kb in size (Xie et al., 1991; Arias-Negrete et al., 1995; Yamamoto et al., 1998).

Expression

Several studies have examined COX expression in normal tissue by Northern blot analysis, RT-PCR, immunohistochemical localization and in situ hybridization. The distribution of COX isoforms is now understood to be more complex than originally speculated. Nearly all tissues have been found to express COX-1 under basal conditions. Of greatest physiological significance, COX-1 has been demonstrated in endothelium, platelets, in the renal medullary collecting ducts and interstitium and throughout the gastrointestinal tract (O’Neill et al., 1993; Patrignani et al., 1994; Seibert et al., 1997).

Cyclooxygenase-2 is markedly expressed in the brain, prostate and renal cortex (O’Neill et al., 1993; Kargman et al., 1996). Localization of COX-2 in the epithelial cells of the cortical thick ascending loop of Henle and the macula densa of the juxtaglomerular apparatus has been demonstrated (Harris et al., 1994), while COX-1 has been found in arteries and arterioles, glomeruli and collecting ducts (O’Neill et al., 1993). Upregulation of COX-2 has been shown to occur in the macula densa following salt or volume depletion in healthy dogs and rodents (Harris et al., 1994; Yang et al., 1998; Khan et al., 1998). It is highly probable that COX-2 is critical for normal renal development, as severe congenital renal abnormalities have been reported in
knockout mice which have had the COX-2 gene deleted from their genome (Dinchuk et al., 1995).

In contrast, COX-2 is not present in substantial levels in the majority of tissues, and is generally not detected in untreated cells in culture (Kargman et al., 1996; O’Neill et al., 1993; Seibert et al., 1997). Monocytes, chondrocytes, synoviocytes and vascular smooth muscle cells express low levels of COX-1 transcript. Treatment with proinflammatory or mitogenic stimuli, including cytokines, tumour promoters and various growth factors, have been demonstrated to selectively and dramatically increase 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 PGE$_2$ levels (Fu et al., 1990; Lee et al., 1991; Hempel et al., 1994; Masferrer et al., 1994; Anderson et al., 1996). Although COX-2 enzyme activity can be inhibited by glucocorticoids, these drugs do not alter the basal COX-1 activity observed in vitro and in vivo (Raz et al., 1989; Lee et al., 1991; Masferrer et al., 1994; Barrios-Rodiles et al., 1996).

**Prostaglandins: Homeostasis, Inflammation and Pain**

Prostaglandin synthesis occurs following interaction of a homeostatic or inflammatory stimulus with the target cell surface receptor, leading to activation of the COX pathway. Prostaglandins are members of the biologically active eicosanoid family and are formed from PGH$_2$ though the action of a group of enzymes known as the prostaglandin D, E, F, I and TXA synthases (Smith et al., 1991; Dubois et al., 1998).

Prostaglandins can be described as local hormones which are formed in response to, and coordinate the effects of, other circulating hormones; thus acting in both autocrine and paracrine manners. Basal levels are synthesized in most organs, but not in all cell types comprising an organ. For example, in the kidney, only a third of the different cell types synthesize PGs; however, the PGs produced have a profound effect on renal salt and water excretion through
direct effects on renal tubules and by alteration of renal blood flow (Komhoff et al., 1997; Smith et al., 1997).

In the renal tubule, PGs are formed in response to antidiuretic hormone (ADH). Prostaglandin E\(_2\) (PGE\(_2\)) acts on tubular epithelium to attenuate cyclic AMP formation in renal cells by ADH, and ultimately inhibit ADH-induced water and sodium chloride reabsorption from the collecting tubules and the loop of Henle. Both PGE\(_2\) and prostacyclin (PGI\(_2\)) can stimulate renin secretion, which leads to release of aldosterone which promotes potassium secretion in the distal tubule and collecting ducts. Prostacyclin can also increase renal blood flow and glomerular filtration rate. Although PGs are not primarily responsible for homeostatic renal blood flow, PG production is crucial to preserving normal blood flow through the compromised kidney (Willoughby et al., 1976; Dubois et al., 1998; Breyer et al., 1998).

In the gastrointestinal tract, production of PGs aids in maintaining gastric mucosal integrity. Prostaglandin E\(_2\) and PGI\(_2\) are considered to be cytoprotective of gastrointestinal cells through stimulation of mucus secretion in the stomach and small intestine, and by inhibition of gastric acid secretion (Rask-Madsen, 1987; Somasundaram et al., 1995). They also act as vasodilators, locally enhancing blood flow through the gastric mucosa, and can promote healing of duodenal and gastric ulcers (Price et al., 1990; Somasundaram et al., 1995).

Prostaglandins play an important role in maintaining vascular homeostasis. Prostacyclin released from vascular endothelium is antithrombogenic and a potent vasodilator, and aids in maintaining a non-thrombotic barrier between the blood and vessel walls (Williams et al., 1977; Smith et al., 1991). Thromboxane A\(_2\) contributes to platelet aggregation in the majority of species, and promotes vasoconstriction, presumably to prevent blood loss accompanying injury (Johnson et al., 1991; Dubois et al., 1998).

Prostaglandins can also be synthesized in response to injury, and are considered to be inflammatory mediators which do not cause pain directly, but contribute to hyperalgesia through sensitization of nociceptor afferents to the effects of mechanical stimuli and other mediators
(Soloman et al., 1968; Vane 1976). They have also been demonstrated to facilitate central neuronal firing and augment neurotransmitter release from primary spinal sensory afferents, thus affecting spinal nociceptive processing (Rang et al., 1991; Woolf, 1991; Malmberg et al., 1992). Prostaglandin E_2 and PGI_2 are the predominant eicosanoids detected in acute inflammatory conditions and they act synergistically with other mediators to produce pain (Soloman et al., 1968; Vane 1976; Lees et al., 1991).

Prostaglandins are also responsible for many of the clinical characteristics of inflammation (Vane 1976). Prostaglandin E_2 released in response to tissue injury acts as a potent dilator of vascular smooth muscle, causing vasodilation and increased blood flow through tissues, which is responsible for the erythema observed in acute inflammation (Soloman et al., 1968; Williams et al., 1977). Increased circulation augments the extravasation of fluid caused by other inflammatory mediators such as bradykinin, which increase vascular permeability, leading to the development of edema (Willoughby et al., 1976; Williams et al., 1977). Prostaglandin E_2 is also a potent pyrogen, and its release may result in fever (Soloman et al., 1968; Willoughby et al., 1976).

Prostacyclin is commonly present in inflammatory lesions, but at much lower concentrations than is PGE_2. It produces similar vasodilatory effects to PGE_2; however, it is an even more potent hyperalgesic agent, also contributing to inflammatory pain (Soloman et al., 1968; Willoughby et al., 1976; Lees et al., 1991; Vane et al., 1995).

**Nonsteroidal Anti-inflammatory Drugs**

**Mechanism of Action**

Nonsteroidal anti-inflammatory drugs elicit a therapeutic effect through inhibition of prostaglandin synthesis by cyclooxygenase enzymes (Vane, 1971). Generally, NSAIDs bind to the COX active site of the PGHS complex to inhibit cyclooxygenase, but do not inhibit the peroxidase (POX) activity of the enzyme. Different mechanisms of inhibition by various
NSAIDs have been theorized. Time-dependent competitive binding of a drug to the enzyme complex may result in covalent (e.g. aspirin) or noncovalent (e.g. indomethacin) alteration of the PGHS structure, which can produce reversible or irreversible inactivation of COX, but not POX activity. A more rapid and reversible binding to the enzyme through competition of the NSAID with AA is also thought to occur (Cashman, 1996; Riendeau et al., 1997). Several of the new selective COX-2 inhibitors fall into this category (Riendeau et al., 1997).

It has been hypothesized that NSAIDs may display other mechanisms of action in addition to cyclooxygenase inhibition, including inhibition of neutrophil function, oxygen radical production and central action (Abramson et al., 1988; Cashman, 1996). These areas of research are under further investigation.

Currently available NSAIDs fall into two major classes, the carboxylic acids and enolic acids, and can be subdivided further on the basis of chemical structure; however, most NSAIDs share several common characteristics. The majority of NSAIDs are weak acids which are well absorbed following oral administration and are highly plasma protein bound (greater than 90%). The tissue distribution and half-life of the drugs varies, depending on the specific agent in question, and the species it is administered to; however, elimination of most NSAIDs is primarily via hepatic metabolism (Lees et al., 1991; Papich 1997).

Clinical use of NSAIDs in veterinary medicine has been a topic of great interest in recent years. These efficacious anti-inflammatory, antipyretic and analgesic agents have a longer duration of action than most other analgesic drugs, are convenient to administer and relatively inexpensive. Nonsteroidal anti-inflammatory drugs have been successfully employed for treatment of chronic conditions like osteoarthritis, and for control of traumatic or post-surgical pain (Lees et al., 1991; Mathews, 1996; Johnston et al., 1997; Papich 1997).
Toxicity

In both veterinary and human medicine, adverse effects caused by NSAIDs are considered to be the greatest limitation to their use. The most common unwanted side effects include gastritis and gastrointestinal ulceration and hemorrhage. Renal toxicity and platelet inhibition appear to be of greater concern in humans; however, under certain circumstances, they may occur in veterinary patients as well. As cyclooxygenase inhibition affects production of both regulatory prostaglandins and those responsible for the inflammatory process, it is generally accepted that the majority of adverse effects caused by NSAIDs result from inhibition of COX-1 (Fries et al., 1991; Lees et al., 1991; Vane et al., 1995; Papich 1997).

NSAIDs induce gastric damage through local and systemic effects. Since most NSAIDs are weak acids, they may damage the gastric mucosal barrier through concentration within mucosal cells. This alters cell membrane permeability and permits back diffusion of hydrogen ions, resulting in direct injury and consequent mucosal erosion and bleeding (Price et al., 1990; Somasundaram et al., 1995). Locally, NSAIDs may also cause cytotoxic damage to mucosal cells through production of free oxygen radicals (Price et al., 1990).

Non-selective NSAIDs can inhibit production of cytoprotective PGs in the gastrointestinal tract, resulting in decreased mucin quality and bicarbonate content of the gastric layer, which leads to increased mucosal vulnerability to acid-induced injury. As certain PGs have a vasodilatory effect, NSAIDs may also contribute to gastric damage by reducing blood flow in certain areas of the mucosa, and thus enhancing susceptibility to injury (Goddard et al., 1990; Price et al., 1990; Fries et al., 1991; Somasundaram et al., 1995).

Enterohepatic recycling of NSAIDs, whereby the drug is reabsorbed into the bloodstream after excretion into the intestine in the bile, is thought to magnify gastrointestinal effects. This process causes the duodenum to be repeatedly re-exposed to high concentrations of NSAID and may increase the risk of ulceration (Rask-Madsen, 1987; Goddard et al., 1990; Price et al., 1990).
As previously discussed, PGE₂ and PGI₂ contribute to autoregulation of blood flow in the kidney through their vasodilatory action. Under normovolemic conditions, short-term administration of NSAIDs to healthy animals has minimal effect on renal hemodynamics and function. However, dehydration, hemorrhage, heart failure, anesthesia, or pre-existing renal disease may compromise the hemodynamic balance, resulting in release of circulating vasoconstrictors to maintain vascular resistance and blood pressure, potentially at the expense of organ blood flow. Once this has occurred, the vasodilatory effect of PGs is critical in maintaining renal blood flow and glomerular filtration rate. Administration of an NSAID under these circumstances may result in ischemic injury that can progress to acute renal failure (Willoughby et al., 1976; Fries et al., 1991; Dubois et al., 1998; Breyer et al., 1998).

Post-traumatic hemorrhage resulting from impaired platelet function due to inhibition of TXA₂ has been reported in humans and animals (Vane et al., 1995; Papich 1997). However, a population of dogs has been identified that possess a recessive genetic trait which renders them insensitive to platelet aggregation in response to TXA₂. Theoretically, these dogs would not be at risk for coagulation problems resulting from NSAID induced platelet COX-1 inhibition (Johnson et al., 1991).

There is evidence to suggest that cartilage degeneration in arthritic animals may be exacerbated by chronic NSAID therapy (Johnston et al., 1997; Papich 1997). In an experimental model of arthritis in dogs, lesions were worse in arthritic joints of animals treated with aspirin when compared to joints not treated. This is presumed to be due to decreased synthesis of glycosaminoglycans in the inflamed joints (Palmoski et al., 1983).

**Carprofen**

Carprofen (6-chloro-α-methylcarbazole-2-acetic acid) is a carbazole class, propionic acid-derivative. It is approved for use in dogs in Europe (Zenecarp®, Pfizer) and the United States (Rimadyl®, Pfizer), and is available in injectable, rectal suppository, paste and tablet forms. This drug is a racemic mixture and there is evidence that the S(+) isomer displays slightly more anti-
inflammatory activity than does the R(-) isomer. Carprofen is well absorbed following oral administration (90%), with peak plasma concentrations reached between one and three hours post administration. The mean elimination half-life is eight hours. This highly lipophilic drug is 99% plasma protein bound, with a volume of distribution of 0.18 L/kg. It is biotransformed in the liver and metabolites are excreted in the feces and urine (McKellar et al., 1994; Fox et al., 1997). There is evidence of enterohepatic recycling of the S(+) isomer (Priymenko et al., 1998).

In clinical studies, carprofen has been reported to provide effective analgesia following surgical procedures and has been used to manage pain associated with osteoarthritis in the dog (Holtsinger et al., 1992; Nolan et al., 1993; Lascelles et al., 1994; Vasseur et al., 1995; Welsh et al., 1997). The most commonly reported adverse effects are vomiting, diarrhea, anorexia and lethargy (Fox et al., 1997; Papich 1997). Hepatocellular toxicosis associated with administration of the drug has also been documented (MacPhail et al., 1998).

**Ketoprofen**

Ketoprofen, (2-(3-benzoyl phenyl) propionic acid, Anafen®, Merial) is a propionic acid derivative approved for use in dogs in Canada at a dose of 2 mg/kg intravenously, followed by 1 mg/kg per os every 24 hours for four days. In canine studies, it has been reported to be highly bioavailable when administered orally. It is 99% plasma protein bound and has a relatively short plasma half-life of three to four hours (Schmitt et al., 1989).

Ketoprofen is a racemic mixture which undergoes extensive chiral inversion in the dog, and there is evidence that the (S)-isomer is more active than is the (R)-enantiomer (Suesa, 1993; Aberg et al., 1995). It has been successfully employed for alleviation of postoperative pain, panosteitis and fever in dogs (Pibarot et al., 1997), and for postoperative analgesia following ovariohysterectomy in cats (Slingsby et al., 1998). It is commonly used in Canada and Europe; however, there are few published controlled studies concerning efficacy and toxicity in animal species (Mathews, 1996).
Meloxicam

Meloxicam ([4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-benzthiazine-3-carboxamide-1,1-dioxide], Metacam®, Boehringer-Ingelheim) belongs to the oxicam class of NSAIDs which are derived from enolic acid. The oral suspension is approved for canine use in Canada and Europe at a dose of 0.2 mg/kg once daily. Meloxicam is 97% plasma protein bound and the average elimination half-life following oral dosing in the dog is 24 hours. The bioavailability of the oral formulation is 100%. Peak plasma concentrations are attained at approximately 7.5 hours post administration, and steady state levels are reached after three to five days of treatment. This agent is hepatically metabolized and excreted mainly via the feces (Boehringer Ingelheim, unpublished data).

Meloxicam has been demonstrated to be effective in attenuating the local inflammatory response and associated lameness in both stifle and shoulder synovitis models in dogs, and decreasing the systemic inflammatory response in an endotoxin model in cats (Van Bree et al., 1994; Justus et al., 1995; Cross et al., 1997). It has also been used successfully to alleviate post-operative pain in dogs (personal communication, Mathews et al., University of Guelph, 1998).

Tolfenamic Acid

Tolfenamic acid (N-(2-methyl-3-chlorophenyl)-anthranilic acid, Tolfedine®, Vetoquinol) is an NSAID belonging to the fenamate group. An oral tablet formulation is approved for use in dogs in Canada at a dose of 4 mg/kg once daily for 3 to 5 days. This agent is 100% bioavailable when administered subcutaneously, reaching peak plasma concentrations in 1.4 hours. Tolfenamic acid is 99% protein bound and has an elimination half-life of approximately 6.5 hours, with a volume of distribution of 1.2 L/kg. It is cleared mainly through hepatic metabolism and appears to undergo enterohepatic recycling. (McKellar et al., 1991).

Tolfenamic acid is recommended by the manufacturer for short and long-term management of painful locomotor disorders in dogs. It has been reported to reduce ocular discharge and corneal edema in a post-surgical model of canine ocular inflammation (Roze et al., 1996).
Modelling of NSAID Inhibition of Cyclooxygenase

Assessment of currently available and potential new NSAIDs for their relative COX-1 and COX-2 inhibitory activity is a complex issue. Various in vitro models, including purified enzyme systems, intact cell systems and whole blood systems, have been utilized to characterize COX-1 and COX-2 inhibition by different NSAIDs (Mitchell et al., 1994; Tordjman et al., 1995; Brideau et al., 1996; Nakatsugi et al., 1996; Berg et al., 1997). The selectivity of each drug is evaluated by calculating the ratio of their inhibitory concentration 50 (IC\(_{50}\)) values, in that the relative activity against each isoenzyme is expressed as a ratio of COX-1 to COX-2 activity. The ratios are calculated based on the drug concentration which reduces each enzyme's prostaglandin biosynthesis by 50%. Preferential inhibition of COX-2 is indicated by a ratio greater than 1, and a greater inhibitory effect on COX-1 versus COX-2 is indicated by a ratio less than 1. If the enzyme activity is expressed as a ratio of COX-2 to COX-1 activity, which has also been reported in the literature, then the reverse is true: preferential inhibition of COX-2 is indicated by a ratio less than 1, and a greater inhibitory effect on COX-1 versus COX-2 is indicated by a ratio greater than 1.

The assessment of inhibitory potency of NSAIDs on COX isoenzymes is strongly influenced by variations in experimental conditions among different models; therefore, results from each model cannot be directly compared. In addition, although COX activity may be similar across species, it is generally agreed that drug studies must be conducted on the particular species in question to evaluate efficacy and toxicity accurately. However, the general trend of selectivity for COX-2 versus COX-1 within a range of compounds seems to be reproducible from one model to another (Pairet et al., 1998).
RATIONALE

Several *in vitro* assay models have been developed to assess the potential efficacy and toxicity of commonly used human NSAIDs by evaluating their ability to inhibit either COX-1 or COX-2 (Mitchell et al., 1994; Tordjman et al., 1995; Brideau et al., 1996; Nakatsugi et al., 1996; Berg et al., 1997). However, little information is available on inhibition of canine COX isoenzymes by NSAIDs, and assumptions have been made based on data from studies done in other species (Ricketts et al., 1998).

The objective of the pilot studies was to evaluate the *in vitro* cyclooxygenase mRNA expression and consequent prostaglandin synthesis, of two canine cell lines in an effort to identify cells which exclusively expressed COX-1 or COX-2. The effects of ethanol and DMSO on PGE2 production were also evaluated to ensure that use of either compound to dissolve NSAIDs for future COX inhibition studies would not alter results achieved.

Prostaglandin synthesis and cyclooxygenase expression by Madin-Darby canine kidney (MDCK) cells has been previously investigated (Lewis et al., 1981). Utilizing western blot analysis, Sciorra et al. (1996) reported that MDCK cells constitutively express COX-1, and that COX-2 is inducible following incubation with 12-O-Tetradecanoylphorbol-13-acetate. As the adverse renal effects of non-selective NSAIDs are thought to be due to reduced PG synthesis through COX-1 inhibition, the use of a canine kidney cell line as a source of COX-1 was potentially physiologically relevant.

It is well documented that human and rodent monocyte/macrophage cells express only COX-1 mRNA and synthesize very low levels of PGE2 in response to exogenous AA. Treatment with LPS has been demonstrated to selectively and dramatically increase 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 levels (Fu et al., 1990; Lee et al, 1992; Hempel et al., 1994; Masferrer et al., 1994; Arias-Negrete et al., 1995). These studies confirm
that monocytes/macrophages are the prototype of cells which contain one constitutively expressed isoform (COX-1) and one inducible isoform (COX-2). Therefore, for the purpose of our study, it was hypothesized that MDCK cells would potentially provide a source of COX-1, and canine DH82 monocyte/macrophages exposed to LPS would provide a source of COX-2.

The objective of the NSAID studies was to investigate the differential suppressive effects of four NSAIDs currently available for use in the dog on \textit{in vitro} PGE\textsubscript{2} production by the canine COX-1 and COX-2 expressing cells. The NSAIDs chosen for this study included anti-inflammatory agents currently available for use in dogs, which are reported to inhibit cyclooxygenase, and to be clinically efficacious and demonstrate favourable side effect profiles (McKellar et al., 1994; Van Bree et al., 1994; Mathews, 1996; Johnston et al., 1997; Papich, 1997).
MATERIALS AND METHODS

Cell Culture and Treatment

Pilot Studies - Assessment of Prostaglandin Synthesis and Cyclooxygenase Expression in Canine Cells

Madin-Darby canine kidney (MDCK) cells and DH82 canine monocyte/macrophage cells (American Type Culture Collection) were maintained in monolayers in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Life Technologies) containing 15% (DH82) and 10% (MDCK) fetal bovine serum and penicillin (50 units/mL)/streptomycin (50 units/mL) (Gibco BRL, Life Technologies). Each cell culture was regularly passaged using trypsin to disperse the monolayers (Gibco BRL, Life Technologies).

For each experiment, cells were seeded into 24 well plates at 1x10^5 cells/well in DMEM. After twenty four hours, the cells were incubated overnight in serum-free DMEM. Canine DH82 cells on one plate were incubated with lipopolysaccharide (LPS) (100 ng/mL) (Sigma Chemical Company) for a six hour period prior to any further treatment. The medium was removed and the 24 well plate divided into two treatments, of ten wells each (four wells were blank). The first set of treatments was applied for a 30 minute preincubation. Treatment I consisted of serum-free DMEM alone, and treatment II of serum-free DMEM and 0.1% ethanol (Commercial Alcohols Inc.). The medium was removed and a second 30 minute incubation conducted. Each treatment group was further subdivided and five of the wells from treatment I were treated with serum-free DMEM and the other five with serum-free DMEM and arachidonic acid (AA) (Sigma Chemical Company) to a final concentration of 30 uM. Half of the wells from treatment II were treated with serum-free DMEM and 0.1% ethanol, the other half with serum-free medium, 0.1% ethanol and AA to a final concentration of 30 uM. All incubations occurred at 37 °C and 5% CO₂. Following the second incubation, the medium was removed from each well, centrifuged and stored at -70 °C for prostaglandin analysis. Total mRNA was recovered and processed using
TRIzol® (Gibco BRL, Life Technologies) according to the manufacturer’s protocol. The contents of each set of treatment wells were pooled for maximum mRNA recovery. The COX-1 and COX-2 mRNA expression of the cells was assessed by Northern blot analysis. The experiments were repeated using dimethyl sulfoxide (DMSO) as a vehicle (Fisher Scientific). The effects of ethanol and DMSO on PGE₂ synthesis were evaluated to ensure that use of either compound to dissolve NSAIDs for future COX inhibition studies would not alter results achieved.

Nonsteroidal Anti-inflammatory Drug Studies

The DH82 canine monocyte/macrophage cells were seeded into 24 well plates as described above 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. No incubation was required for the COX-1 assay. The medium was removed and each 24 well plate divided into five treatments, consisting of five wells for each drug, and two wells each for positive and negative controls. The first set of treatments was applied for a 30 minute preincubation - treatment I: serum-free DMEM and carprofen; treatment II: serum-free DMEM and tolfenamic acid; treatment III: serum-free DMEM and meloxicam; treatment IV: serum-free DMEM and ketoprofen; treatment IV: serum-free DMEM and 0.1% ethanol (control). The medium was removed and a second 30 minute incubation, identical to the preincubation, but including the addition of AA to each well to a final concentration of 30 μM, was conducted. Two negative controls did not receive AA. All incubations occurred at 37 °C and 5% CO₂. Following the second incubation, medium was removed from each well, centrifuged and stored at -70 °C for prostaglandin analysis. Total mRNA was recovered and processed using TRIzol® (Gibco BRL, Life Technologies) according to the manufacturer’s protocol. The contents of each drug treatment were pooled for maximum mRNA recovery. The COX-1 and COX-2 mRNA expression of the cells was assessed by Northern blot analysis.
Preparation of Drugs and Reagents

Meloxicam (Metacam®) was supplied by Boehringer-Ingelheim. Tolfenamic acid (Tolfedine®, Vetoquinol), carprofen (Rimadyl®, Pfizer) and ketoprofen (Anafen®, Merial) were purchased from Sigma Chemical Company. Stock solutions of each drug were prepared in ethanol or DMSO and serum-free DMEM. The concentration of ethanol or DMSO in the wells did not exceed 0.1%. Each drug was evaluated at eight different concentrations. Concentration ranges were established for each drug from canine pharmacokinetic studies in the literature (McKellar et al., 1994; McKellar et al., 1994; Boehringer-Ingelheim, technical monograph, 1997; Merial, technical monograph, 1995). The concentration range spanned the mean maximum plasma levels of each drug after in vivo administration in dogs. All drugs were compared at an identical concentration on a single cell assay plate using a total of nine replicates for each concentration.

Prostaglandin E₂ and 6-keto-PGF₁α Assays

Prostaglandin E₂ (PGE₂) and prostacyclin metabolite (6-keto-PGF₁α) concentrations were assayed directly from culture medium using commercially available 96 well enzyme immunoassay kits (Cayman Chemical Company). This assay was based on the competition between free PG and a PG tracer linked to an acetylcholinesterase molecule for a limited number of PG-specific antiserum binding sites. The amount of PG tracer that was able to bind to the antiserum is inversely proportional to the concentration of free PG in each well. The antiserum-PG complex becomes bound to the monoclonal antibody coating the well surface. Ellman's Reagent (containing the substrate to acetylcholinesterase) is added to each well, and the absorbance of product of this reaction measured at a wavelength of 412 nm using a microplate autoreader (Bio-Tek Instruments Inc.).

The concentration of each sample was calculated using an analytical software package (Plate Reader Software 2.0, Cayman Chemical Company) which generated a standard curve from the ratio of a particular standard well (B) to that of a maximum binding well (B₀). The percent B/B₀
on the standard curve was located and its corresponding x-axis value identified. The final PG concentration for each sample was then calculated from this information. All PG samples were assayed in triplicate and the mean calculated from the separate values to achieve the final PG concentration of each sample.

Sub-cloning of Cyclooxygenase-1 and Cyclooxygenase-2 cDNA

Insert Purification

Two Eppendorf tubes, each containing eluted cDNAs for cyclooxygenase-1 and -2, were obtained from Dr. Kris Chadee (McGill University). The concentration in sterile water of hCOX-1 was 5.46 ug/uL and hCOX-2 was 3.2 ug/uL. Cyclooxygenase-1 cDNA (1 ug) was cut from its original vector using the Not I (Pharmacia Biotech Inc.) and Hind III (Promega) restriction enzymes. Concurrently, a 2.5 ug quantity of pBluescript II SK vector (Stratagene GmbH) was cut with the same enzymes. Cyclooxygenase-2 (1 ug) cDNA was cut from its original vector using the EcoRI (Pharmacia Biotech Inc.) and Apa I (Promega) restriction enzymes. Concurrently, a 2.5 ug quantity of pBluescript II SK vector was cut with the same enzymes. The products were then run on a 0.8% agarose gel (Gibco BRL, Life Technologies) for one hour at 80 volts. The desired DNA inserts were purified using a QIAEX II gel extraction kit (Qiagen Inc.) for DNA extraction from agarose

Ligation

The COX-1 and COX-2 cDNA were ligated into separate pBluescript KS vectors by adding the DNA to a reaction mixture containing T4 ligase (Boehringer Mannheim), linearized pBluescript KS, 10X ligation buffer (Boehringer Mannheim) and sterile water. The contents of the tube were then incubated at 14 ºC for 16 hours.
Heat Shock Transformation of Escherichia coli

The DNA was diluted to a concentration of 1 ug/ul and 10 uL added to an Eppendorf tube containing 200 uL of competent KL1 Blue Escherichia coli (E. coli) (previously stored at -70 °C and thawed). The mixture was incubated on ice for 30 minutes, then transferred to 37 °C for 2 minutes, then transferred back to ice for 2 minutes. An 800 uL quantity of SOC medium warmed to 37 °C was added to the mixture, and the solution placed in a shaking waterbath for 30 minutes at 37 °C. Plates of LB Agar plus ampicillin and tetracycline were warmed to 37 °C. Following shaking, 100 uL of solution was spread over the plate and incubated at 37 °C overnight. A single bacterial colony was picked by direct contact with a sterile plastic rod. A 50 mL suspension of LB broth with tetracycline and ampicillin was inoculated with the rod and the broth placed in a shaking water bath at 37 °C overnight.

Midiprep

The plasmid DNA was isolated from the E. coli using a Flexi-prep kit (Pharmacia Biotech Inc.). The DNA was resuspended in sterile water and stored at -20 °C.

Verification of plasmid and insert orientation

A restriction digest of 100 ug of plasmid DNA was performed using restriction enzymes Not I and Hind III for COX-1, and EcoRI and Apa I for COX-2. The fidelity of the cDNA fragments was determined using restriction mapping and confirmed by dye terminator cycle sequencing using the ABI Prism 377 DNA sequencer. The results were compared to the known sequence for pBluescript and human COX-1 and COX-2 to verify the identity of the inserts.

Preparation of DNA Templates

The remaining products from the restriction digest used to verify plasmid and insert orientation (as above) were run on a 0.8% agarose gel for 45 minutes at 80 volts. The inserts were then purified using electrophoresis. The final purified insert was resuspended in sterile double distilled water containing 0.01% dimethylpyrocarbonate (DMPC-A) and diluted to a concentration of 50 ng/ul to serve as template DNA.
Northern Blot Analysis

Isolation of Ribonucleic Acid (RNA)

The RNA from the cultured cells was isolated with 1 mL TRIzol® reagent (Gibco BRL, Life Technologies) per $5 \times 10^5$ cells and the cells lysed by repeated passage through a pipette. The lysate was then placed in a 1.5 mL microcentrifuge tube and incubated for 5 minutes at room temperature, followed by addition of 200 uL chloroform (Fisher Scientific). The tube was capped, shaken for 15 seconds and incubated at room temperature for three minutes. The mixture was then incubated at 4 °C for 30 minutes. The suspension was centrifuged at 12 000 x g at 4 °C for 15 minutes to separate the phases. The clear upper phase containing the RNA was transferred to a fresh microcentrifuge tube and 500 uL of cold isopropyl alcohol (2-propanol) (Fisher Scientific) was added. The sample was kept at -20 °C overnight to allow for RNA precipitation. The sample was then centrifuged at 12 000 x g for 10 minutes at 4 °C and the isopropyl alcohol decanted off. The resulting pellet was washed with 75% (v/v) ethanol at room temperature and the sample centrifuged at 7500 x g at 4 °C for 5 minutes. The ethanol was drawn off and the pellet dissolved in 10 to 20 uL sterile DMPC-A water (depending on the size of the pellet). The RNA concentration of the sample was determined by the absorbance at 260 nm using a UV-VIS 1201 spectrophotometer (Shimadzu).

Electrophoresis and Capillary Transfer of Gel

An electrophoresis gel was prepared by combining 0.8 g of electrophoresis-grade agarose (Gibco BRL, Life Technologies) with 62.5 mL DMPC-A water and boiling the mixture until the agarose became soluble. The mixture was cooled at room temperature for 10 minutes and then blended with 20 mL 5X MOPS buffer (Appendix I) and 17.5 mL 37% (v/v) formaldehyde (Fisher Scientific). The resulting solution was poured into a gel casting apparatus and allowed to polymerize for 45 minutes.

Samples of RNA were prepared by combining a total of 20 to 25 ug of RNA in a volume of
15 to 20 uL, with 2 uL 5X MOPS, 3.5 uL formaldehyde, 10 uL formamide, 2 uL loading buffer (Fisher Scientific) and 0.15 uL ethidium bromide (0.2 mg/mL) (Boehringer Mannheim). The samples were incubated in a 65 °C water bath for 15 minutes to denature the RNA and then immediately chilled on ice. They were then loaded onto the gel and run at 16 volts for 16 hours in 1X MOPS buffer. Following removal from the buffer, the gel was immersed in 0.05 N NaOH (Fisher Scientific) for 15 minutes to hydrolyze the RNA, and photographed under ultraviolet light. The gel was then rinsed with DMPC-A and the RNA transferred to a Hybond-N® nylon membrane (Amersham, Life Sciences) over a 48 hour period by capillary transfer. After RNA transfer to the membrane, the RNA was cross-linked to the membrane using a FB-UVXL-100 UV Crosslinker (Fisher Scientific). The membrane was stored in plastic wrap at room temperature to await probing.

Preparation of Radioactive Probes

The radioactive probe was prepared by combining 50 ng template DNA with DMPC-A water to a total volume of 45 uL, and boiling the sample for 5 minutes to denature the DNA. The sample was immediately chilled on ice to stabilize the now individual DNA strands. The DNA solution was added to the prepared Rediprime® random primer DNA labeling system (Amersham, Life Sciences), followed by addition of 5 uL (50 uCi) radioactive nucleotide ([³²P]dCTP (Amersham, Life Sciences). This mixture was incubated for 10 minutes at 37 °C and the reaction was halted by addition of 5 uL 0.2 M EDTA (pH = 8.0) (Appendix I). The radioactive probe was passed through a Quick Spin Column containing 1.5 mL Sephadex G-50 (Appendix I), followed by the addition of 300 uL TEN buffer (Appendix I), to remove the unincorporated radioactive nucleotide and increase the total volume of the probe preparation. The radioactivity of a 1 uL aliquot of the preparation was quantitated using a Delta 300 liquid scintillation system (Tracor Analytic).
Hybridization and Detection of RNA

A prehybridization solution consisting of 350 uL 10% SDS (Appendix I), 500 uL 50X Denhardt’s solution (Appendix I), 1.5 mL 20 X SSPE (Appendix I), 20 uL denatured salmon sperm DNA (10 mg/mL) (Boehringer-Mannheim), 2.5 mL formamide and 230 uL DMPC-A water was prepared and added in 5 mL aliquots to the hybridization tubes. The nylon membranes were rinsed in 5X SSPE, inserted into the tubes and incubated in a model 1400 rotating hybridization incubator (Robbins Scientific) at 42 °C for at least one hour.

A hybridization solution identical to the prehybridization solution was prepared. The radioactive probe was combined with 5.0 mL of this solution in a 50 mL tube, boiled for 10 minutes and then cooled on ice to room temperature. The prehybridization solution was decanted and replaced with hybridization solution. The tubes were then incubated in a rotating hybridization oven for 12 to 16 hours at 42 °C.

The hybridization solution was decanted, and two low stringency washes conducted at 42 °C for 15 minutes using 50 mL of a solution containing 2X SSC and 1% SDS (Appendix I). A high stringency wash followed using 50 mL of a solution containing 0.1X SSC and 0.1% SDS (Appendix I) at 50 °C for 15 minutes for COX-2, and 55 °C for 30 minutes for COX-1 and 7S (Balmain et al., 1982).

Phosphoimaging on a molecular imaging CS-screen using a GS-250 Molecular Imager® with Molecular Analyst Version 2.1 software (Bio-Rad Laboratories) was used for visualization of the radioactive blots.

Platelet Isolation and RNA Recovery

Single units of whole blood were collected from donor dogs by venipuncture into collection bags containing sodium citrate. The whole blood was centrifuged at 20 °C for 4 minutes at 1000 x g to prepare platelet rich plasma. The platelet-rich plasma was expressed into a collection bag
using a plasma extractor, and extraction was stopped when the red blood cell interface was 1 cm from the top of the bag. An aliquot was sent for a full platelet count and differential white cell count. Leukocyte and red cell contamination was consistently less than 1%. The platelet-rich plasma was then transferred into DMPC-A treated 10 mL siliconized glass tubes and centrifuged for 20 minutes at 2000 x g to produce a platelet pellet (Djaffar et al., 1991; Allyson et al., 1997). Total mRNA was recovered and processed using TRIzol® (Gibco BRL, Life Technologies) according to the manufacturer’s protocol.

Reverse Transcriptase - Polymerase Chain Reaction Analysis (RT-PCR)

Ribonucleic acid (1μg) obtained from canine platelets and DH82 canine monocyte/macrophage cells as previously described, was reverse-transcribed (RT) with 0.5 mL reverse transcriptase (200 U/μL) (Gibco BRL, Life Technologies) in the presence of 1.5 μL deoxynucleotides (dNTPs) (10 mM) (Pharmacia Biotech), 0.5 μL random primers (Promega), 1 μL each of Oligo (dT) primer (Promega), DNase I, 10X DNase I buffer (Gibco BRL, Life Technologies), 2 μL MDTT (0.1M) (Gibco BRL, Life Technologies), 1 μL 3’ (reverse) primer (50 pmol/μL), in DMPC-treated water. The reverse transcribed cDNA was diluted 5 times. In a thin walled PCR tube, 10 μL cDNA was amplified in a final volume of 100 μL containing 2 μL dNTPs (10mM), 5.0 μL magnesium chloride (MgCl₂) (50 mM), 0.2 μL Taq DNA polymerase, 10 μL 10X PCR buffer (Gibco BRL, Life Technologies), 2 μL (50 pmol/μL) each of sense and antisense gene primers and sterile double distilled water. The primers used for priming the COX-1 gene were designed by Sabine Schmoelzl based on the coding region which is homologous among species for which the COX-1 gene sequence is known. The primers sequences were as follows: sense, 5’ to 3’, GATGGCAGCAGAGTTGGGAG and antisense, 3’ to 5’ GACAGGTCTTGGTGTTGAGG. Amplification was performed using the Gene Amp PCR system 2400 (Perkin Elmer). The amplification conditions were 94 °C for 3 min for one cycle,
94 °C for 30 sec (denaturation), 55.5 °C for 30 sec and 72 °C for 1 min (primer annealing and extension) for 40 cycles, and 72 °C for 5 min. PCR bands were identified by size comparing 10 uL of total sample to a 100 bp DNA ladder (MBI Fermentas) following electrophoresis on a 2% agarose gel containing ethidium bromide (10 mg/mL) in 1X TAE buffer (Appendix I). The gel was viewed under ultraviolet light and photographed. The remaining 90 uL of sample was purified using a QIA quick PCR purification kit (Qiagen Inc.) and the identity of the COX-1 product determined using restriction mapping and confirmed by dye terminator cycle sequencing using the ABI Prism 377 DNA sequencer. The results were compared to the known sequences for COX-1 to verify the identity of the PCR product.

**Data Analysis**

Results from the preliminary studies were expressed as mean ± SD PGE$_2$ synthesis. Statistical differences between vehicle (ethanol or DMSO) control and treatment groups were tested using student’s t test. A p-value less than 0.05 was considered statistically significant.

Eight different concentrations were evaluated for each NSAID. Results of nine treatment wells (n=9), each containing a specific drug dissolved in a vehicle and AA from each experiment were calculated as mean ± SEM percent PGE$_2$ synthesis and compared to positive control wells containing vehicle and AA only. Data analysis was conducted using a statistical software package (PC-SAS 6.12 for Windows). Statistical interpretation was performed using analysis of variance (ANOVA). A logarithmic transformation of the data was performed to establish a normal distribution and constant variance to construct the ANOVA table. A quadratic regression was applied to describe the relationship of the qualitative variables of drug type and COX model to the quantitative variable of drug concentration. Comparisons between data sets were conducted by contrast using an F test. A p-value less than 0.05 was considered statistically significant.
RESULTS

Pilot Studies

Prostaglandin Synthesis by Madin-Darby Canine Kidney Cells

Prostaglandin E₂ synthesized by the Madin-Darby canine kidney cells was measured in serum-free culture medium by enzyme immunoassay. There was a significant difference between PGE₂ levels synthesized prior to (117.54 ± 36.03 pg/10⁵ cells) and following addition of AA (1137.10 ± 320.65 pg/10⁵ cells) (Figure 1). Comparison of means revealed no significant effect on PGE₂ synthesis by the presence of 0.1% ethanol or DMSO.

Prostaglandin Synthesis by DH82 Canine Monocyte/Macrophage Cells

Canine DH82 monocyte/macrophage cells were also capable of PGE₂ synthesis. Significant differences were detected between levels of PGE₂ synthesized by DH82 cells which were not exposed to LPS (24.81 ± 9.13 pg/10⁵ cells) and cells which were incubated with LPS for 6 hours (125.35 ± 31.65 pg/10⁵ cells), both in the presence and absence of AA (Figure 2). Comparison of means revealed no significant effect on PGE₂ synthesis by the presence of 0.1% ethanol or DMSO. The levels of 6-keto-PGF₁α consistently fell below the range of detection of the commercial EIA kit; thus accurate measurement was not possible.

Northern Blot Analysis

Madin-Darby canine kidney cells strongly expressed the 4.5 kb COX-2 transcript, in addition to the 2.8 kb COX-1 transcript (Figure 3). Cyclooxygenase-2 message was not detected in DH82 canine monocyte/macrophage cells prior to LPS exposure; however, following a six hour incubation with LPS, the 4.5 kb COX-2 mRNA transcript was strongly expressed. Cyclooxygenase-1 message was not detectable in DH82 cells by Northern analysis under any conditions (Figure 4).
Canine platelet mRNA present on the same membrane as non-LPS treated and LPS-treated DH82 cells expressed the 2.8 kb COX-1 transcript but COX-2 message was not detected (Figure 5). These findings confirm the ability of the human COX cDNA probes to detect canine COX-1 message, without cross-reacting with COX-2.

**Reverse -Transcriptase - Polymerase Chain Reaction Analysis**

The results of RT-PCR analysis of canine COX-1 are shown in Figure 6. DNA bands of 270 bp were identified following gel electrophoresis of the products obtained from RT-PCR of mRNA from canine platelets and DH82 monocyte/macrophage cells. Restriction mapping and dye terminator cycle sequencing revealed that the PCR product from the DH82 monocyte/macrophage cells was 98% homologous to the equivalent portion of the human COX-1 gene (Genbank M59979). The platelet product was 90% homologous to the equivalent portion of the human COX-1 gene. The sequence obtained from the DH82 monocyte/macrophages is shown below:

```
TTGTTCACCGGCGGGAGATGGTGAGTNGGANTTCTACCCCTGGACTGCTTCTTGA
AAAGTGCAATCCAAACTTCTATCTTTGGGAGATAGATAGATGGGGCTCCCTTT
TTCCCTCAAGGGTCCTANGGAATCCCATCTTCTTCTCCGGAGTACTGGAAGCGGAG
CACATTTTGGCGGGAGGTGGGCTTTACATTGTCAAGACGGCGCCACACTGAAAGC
GGTCTGCCCTCAACACCAAGACCTGTCA
```

Results achieved through Northern analysis and RT-PCR suggest that synthesis of PGE₂ by non-LPS treated DH82 cells was due to canine COX-1 activity, and PGE₂ synthesis by LPS-treated cells was primarily due to canine COX-2 activity (Figure 7).
FIGURE 1. *In vitro* PGE$_2$ synthesis by Madin-Darby canine kidney cells prior to and following addition of arachidonic acid (AA). Data shown represent mean ± SD (n=10 wells) from two independent experiments.

* The difference in PGE$_2$ synthesis between untreated cells, and cells exposed to AA was statistically significant by Student's paired t test (p<0.05).
FIGURE 2. *In vitro* PGE2 synthesis by canine DH82 monocyte/macrophage cells prior to and following a 6 hour incubation with LPS (100 ng/mL) in the presence and absence of arachidonic acid (AA). Data shown represent mean ± SD (n=10 wells) from two independent experiments. Statistical comparisons were performed using Student’s paired t test.

* p < 0.05 within non-LPS and LPS-treatment groups, differences in PGE2 synthesis by cells prior to and following addition of AA.

+ p < 0.05 between treatment groups, differences in PGE2 synthesis by non-LPS treated cells and LPS treated cells.
FIGURE 3: Northern blot analysis of total RNA (20 ug/lane) from Madin-Darby canine kidney cells (two identical lanes). The first and second panels show the results of probing the blot with human cyclooxygenase (COX)-1 and COX-2 cDNA probes, respectively. The lower panel shows the results of reprobing the blot with a 7S cDNA probe, which was used as a load control. Results shown are from a representative Northern blot, repeated three times.
FIGURE 4: Effect of lipopolysaccharide (LPS) on cyclooxygenase (COX)-2 mRNA expression in canine DH82 monocyte/macrophage cells. Northern blot analysis of total RNA (20 µg/lane for DH82 cells and 5 µg/lane for platelets). Panels one and two show the results of probing the blot with COX-1 and COX-2 human cDNA probes, respectively. The lower panel shows the results of reprobing the blot with a 7S cDNA probe, which was used as a load control. Results shown are from a representative Northern blot repeated three times.
FIGURE 5. Northern blot analysis of total RNA (20 ug/lane) from canine platelets and Madin-Darby canine kidney (MDCK) cells was performed using human cDNA probes. The first and second panels show the results of probing the blot with cyclooxygenase (COX)-1 and cyclooxygenase (COX)-2 cDNA probes, respectively. The lower panel shows the results of reprobing the blot with a 7S cDNA probe, which was used as a load control. Results shown are from a representative Northern blot repeated three times.
Figure 6. RT-PCR of canine cyclooxygenase (COX)-1. RNA obtained from canine platelets and DH82 monocyte/macrophage cells was reverse-transcribed into DNA and amplified by polymerase chain reaction using sense and anti-sense primers for human COX-1. Sterile water served as a negative control, and equine and rat genomic DNA served as positive controls.
Canine DH82 Monocyte/macrophage Cells

**COX-1 Model**

- AA
- Northern analysis
- RT-PCR
- COX-1 positive - RT-PCR
- COX-1 negative - Northern analysis
- COX-2 negative - Northern analysis

**COX-2 Model**

- LPS (6 hrs) + AA
- Northern analysis
- COX-1 negative - Northern analysis
- COX-2 positive - Northern analysis

EIA → PGE₂

**Figure 7.** *In vitro* canine cyclooxygenase (COX)-1 and COX-2 cell models. Northern analysis and RT-PCR of total RNA obtained from canine DH82 monocyte/macrophage cells prior to and following a 6 hour incubation with lipopolysaccharide (LPS), were used to assess COX expression. Prostaglandin E₂ (PGE₂) levels were measured in culture medium by enzyme immunoassay and attributed to specific COX-1 or COX-2 activity.
Nonsteroidal Anti-inflammatory Drug Studies

Northern Blot Analysis

Following NSAID incubation with non-LPS treated canine DH82 monocyte/macrophage cells in the COX-1 model, COX-1 was present as detected by RT-PCR. No COX-2 mRNA was detected using Northern analysis of mRNA. However, in the COX-2 model following NSAID incubation with DH82 cells which had been exposed to LPS for 6 hours, the 4.5 kb COX-2 mRNA transcript became strongly expressed. Cyclooxygenase-1 message was not detectable in DH82 cells by Northern analysis under any conditions (Figure 8).

Results achieved by Northern analysis and RT-PCR suggest that NSAID suppression of PGE\(_2\) synthesis in non-LPS treated DH82 cells was due to inhibition of COX-1, and suppression of PGE\(_2\) synthesis in LPS-treated cells was primarily due to inhibition of COX-2.

Effect of Nonsteroidal Anti-inflammatory Drug Treatment on Prostaglandin E\(_2\) Synthesis

Raw data of PGE\(_2\) production by non-LPS treated (COX-1) and LPS-treated (COX-2) canine DH82 monocyte/macrophage cells following nonsteroidal anti-inflammatory drug treatment are shown in Tables I and II. Data were converted to percent reduction in PGE\(_2\) production by each NSAID for each COX-1 and COX-2 model by expressing the PGE\(_2\) value from each drug treatment well (NSAID in vehicle and AA) as a percent of positive control well values (vehicle and AA) and subtracting that value from 100% (Tables III to IV). Statistical comparisons between drugs and between COX-1 and COX-2 models are shown in Figures 9-15.

Comparisons of the effects of each drug between COX models were evaluated. No significant differences in inhibition of PGE\(_2\) synthesis were detected between the COX-1 and COX-2 models for carprofen, nor for ketoprofen, except at 51.2 ug/mL. Meloxicam significantly inhibited PGE\(_2\) synthesis in the COX-2 model more than in the COX-1 model at all concentrations except the lowest (0.003125 ug/mL) and highest (51.2 ug/mL). Tolfenamic acid inhibited PGE\(_2\) synthesis significantly more in the COX-2 model than the COX-1 model, at all but the lowest concentrations (0.003125 ug/mL, 0.0125 ug/mL and 0.05 ug/mL).
Inhibition curves for carprofen, ketoprofen, meloxicam and tolfenamic acid are shown in Figures 9 to 13. All drugs significantly inhibited PGE2 synthesis 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; however, tolfenamic acid displayed no in vitro PGE2 inhibition at 0.003125 ug/mL or 0.0125 ug/mL in the COX-2 model.

In the COX-1 assay, no significant differences in inhibition of PGE2 synthesis were detected between carprofen and ketoprofen at each drug concentration. Carprofen and ketoprofen significantly inhibited PGE2 synthesis more than did tolfenamic acid at every drug concentration, and more than meloxicam at all but the lowest concentration evaluated (0.003125 ug/mL). Meloxicam significantly inhibited PGE2 synthesis more than did tolfenamic acid at the lowest (0.003125 ug/mL and 0.0125 ug/mL) and highest (12.8 ug/mL and 51.2 ug/mL) concentrations.

In the COX-2 model, no significant differences were detected between the inhibitory effects of carprofen and meloxicam, of carprofen and tolfenamic acid (except at 0.003125 ug/mL and 0.0125 ug/mL), or of ketoprofen and meloxicam. Ketoprofen displayed significantly greater inhibition of PGE2 synthesis than did carprofen at the lowest concentrations (0.003125 ug/mL and 0.0125 ug/mL). Ketoprofen and meloxicam were significantly more PGE2 suppressive than tolfenamic acid at concentrations between 0.003125 ug/mL and 0.2 ug/mL.

The COX selectivity of each drug was evaluated by calculating a ratio of the inhibitory concentration (IC50) values. The relative activity against each isoenzyme was expressed as a ratio of COX-1 to COX-2 activity (Table V). The ratios were calculated based on the drug concentration which reduced PGE2 by 50% in each COX assay.

As tolfenamic acid did not achieve 50% PGE2 suppression in the COX-1 model, an IC50 could not be calculated; therefore, an additional set of IC25 ratios (the ratio of drug concentrations which reduced PGE2 synthesis by 25%) was calculated to allow for more complete comparison of all drugs (Table VI).
Over the dose range studied, using inhibitor concentration ratios, meloxicam and tolfenamic acid inhibited canine COX-2 more potently than COX-1. Carprofen and ketoprofen had a relatively equal inhibitory effect on COX-1 and COX-2 enzymes. The preference of meloxicam for canine COX-2 was more than 12 fold greater than for canine COX-1. Tolfenamic acid displayed a more than 8 fold preference using an IC$_{50}$ ratio, and carprofen a 1.75 fold preference for COX-2 over COX-1.
FIGURE 8: Cyclooxygenase (COX)-1 and COX-2 mRNA expression in canine DH82 monocyte/macrophage cells from NSAID studies: A) COX-1 Assay, no LPS treatment B) COX-2 Assay, LPS treated. A representative northern blot analysis (n=3) of total RNA (20 ug/lane) using human cDNA probes. Canine platelets (5 ug/lane) served as a control for COX-1, and LPS-treated DH82 monocyte/macrophages served as a control for COX-2. The first and second panels show the results of probing the blot with COX-1 and COX-2 cDNA probes, respectively. The lower panel shows the results of reprobing the blot with a 7S RNA cDNA probe, which was used as a load control.
<table>
<thead>
<tr>
<th>Drug Concentration (ug/mL)</th>
<th>Positive Control</th>
<th>Negative Control</th>
<th>Carprofen</th>
<th>Ketoprofen</th>
<th>Meloxicam</th>
<th>Tolfenamic Acid</th>
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<tr>
<td>0.003125</td>
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</tr>
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<td>± 26.94</td>
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</tr>
</tbody>
</table>

a - Treatment: wells treated with drug dissolved in vehicle and arachidonic acid

b - n: number of wells per concentration of each drug

c - Positive control: wells treated with arachidonic acid and vehicle only

d - Negative control: wells treated with vehicle only
TABLE II. Prostaglandin E2 production (pg/1x10^5 cells) by LPS treated (COX-2) canine DH82 monocyte/macrophage cells following nonsteroidal anti-inflammatory drug treatment^a (mean ± SD) (n = 9)^b.

<table>
<thead>
<tr>
<th>Drug Concentration (ug/mL)</th>
<th>Positive^c Control</th>
<th>Negative^d Control</th>
<th>Carprofen</th>
<th>Ketoprofen</th>
<th>Meloxicam</th>
<th>Tolfenamic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.003125</td>
<td>132.53 ± 4.75</td>
<td>47.17 ± 3.08</td>
<td>127.43 ± 28.51</td>
<td>113.61 ± 28.94</td>
<td>123.86 ± 17.22</td>
<td>151.52 ± 29.24</td>
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<tr>
<td>0.0125</td>
<td>181.93 ± 23.60</td>
<td>67.89 ± 6.15</td>
<td>161.91 ± 21.64</td>
<td>134.27 ± 15.19</td>
<td>148.68 ± 20.28</td>
<td>200.44 ± 35.15</td>
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<tr>
<td>0.05</td>
<td>338.17 ± 3.17</td>
<td>66.91 ± 0.69</td>
<td>260.24 ± 67.89</td>
<td>238.75 ± 58.64</td>
<td>238.75 ± 44.09</td>
<td>258.75 ± 72.81</td>
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<tr>
<td>0.20</td>
<td>246.53 ± 32.00</td>
<td>55.61 ± 5.96</td>
<td>152.56 ± 20.70</td>
<td>136.28 ± 11.39</td>
<td>145.02 ± 27.61</td>
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<td>0.80</td>
<td>267.32 ± 34.60</td>
<td>51.52 ± 2.89</td>
<td>128.15 ± 21.15</td>
<td>127.75 ± 23.45</td>
<td>139.89 ± 18.68</td>
<td>133.89 ± 24.87</td>
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<td>166.92 ± 34.73</td>
<td>161.87 ± 25.04</td>
<td>121.27 ± 25.31</td>
<td>169.86 ± 39.71</td>
</tr>
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</table>

^a Treatment: wells treated with drug dissolved in vehicle and arachidonic acid

^b n: number of wells per concentration of each drug

^c Positive control: wells treated with arachidonic acid and vehicle only

^d Negative control: wells treated with vehicle only
TABLE III: Percent reduction in prostaglandin E₂ production by nonsteroidal anti-inflammatory drugs\textsuperscript{a} in non-LPS treated (COX-1) canine DH82 monocyte/macrophage cells (mean ± SEM) (n = 9)\textsuperscript{b}.

<table>
<thead>
<tr>
<th>Drug Concentration (ug/mL)</th>
<th>Carprofen</th>
<th>Ketoprofen</th>
<th>Meloxicam</th>
<th>Tolfenamic Acid</th>
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<tbody>
<tr>
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<td>0.0125</td>
<td>22.50</td>
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<td>± 6.94</td>
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<td>46.44</td>
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<td>± 3.38</td>
<td>± 2.67</td>
<td>± 6.39</td>
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</table>

\textsuperscript{a} - Percent reduction in prostaglandin E₂ production by each drug
\quad = 100 - \left[ \text{drug treatment wells (drug + AA) / positive control (vehicle + AA)} \right]

\textsuperscript{b} - n: number of wells per concentration of each drug
TABLE IV: Percent reduction in prostaglandin E2 production by nonsteroidal anti-inflammatory drugs\textsuperscript{a} in LPS treated (COX-2) canine DH82 monocye/macrophage cells (mean ± SEM) (n = 9)\textsuperscript{b}.

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<th>Drug Concentration (\text{ug/mL})</th>
<th>Carprofen</th>
<th>Ketoprofen</th>
<th>Meloxicam</th>
<th>Tolfenamic Acid</th>
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<td>14.28 ± 7.28</td>
<td>6.54 ± 4.33</td>
<td>0 ± 7.35</td>
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<tr>
<td>0.0125</td>
<td>11.00 ± 3.97</td>
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<td>18.28 ± 3.71</td>
<td>0 ± 4.44</td>
</tr>
<tr>
<td>0.05</td>
<td>23.04 ± 6.69</td>
<td>29.91 ± 5.78</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>58.71 ± 2.13</td>
<td>57.42 ± 2.95</td>
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</tbody>
</table>

\textsuperscript{a} Percent reduction in prostaglandin E2 production by each drug:
\[
= 100 - \frac{\text{drug treatment wells (drug + AA)}}{\text{positive control (vehicle + AA)}}
\]

\textsuperscript{b} n: number of wells per concentration of each drug
Figure 9. Concentration-response curves of percent inhibition of PGE$_2$ by each NSAID in (A) COX-1 model (B) COX-2 model Data represent mean percent inhibition of PGE$_2$ as compared to cells treated with vehicle and AA only (n=9 drug treatment wells from two different culture plates). Curve fit was obtained through quadratic regression.
Inhibition of PGE2 synthesis by captopril between COX-1 and COX-2 models

Comparisons among data sets were conducted through quasilinear regression. Comparisons among data sets were conducted using an F test. There were no substantial differences in inhibition by captopril. Values are presented as mean ± SEM percent. Only (n = 9 cells from two different culture plates) curve fit was obtained.

Figure 10. Comparison of concentration-response curves for cyclooxygenase (COX)-1 and -2 inhibition by captopril. (A) Linear scale. (B) Semilogarithmic scale. Values are presented as mean ± SEM percent. (n = 9 cells from two different culture plates).
Figure 11. Comparison of concentration-response curves for cyclooxygenase (COX)-1 and -2 inhibition by ketoprofen. (A) linear scale  (B) semi-logarithmic scale. Values are presented as mean ± SEM percentage inhibition of PGE₂ synthesis compared to cells treated with vehicle and AA only (n=9 cells from two different culture plates). Curve fit was obtained through quadratic regression. Comparisons among data sets were conducted by contrast using an F test. * p < 0.05 Statistical differences for inhibition of PGE₂ synthesis between COX-1 and COX-2 models.
Figure 12. Comparison of concentration-response curves for cyclooxygenase (COX)-1 and -2 inhibition by meloxicam. (A) linear scale (B) semi-logarithmic scale. Values are presented as mean ± SEM percentage inhibition of PGE₂ synthesis compared to cells treated with vehicle and AA only (n=9 cells from two different culture plates). Curve fit was obtained through quadratic regression. Comparisons among data sets were conducted by contrast using an F test. * p < 0.05 Statistical differences for inhibition of PGE₂ synthesis between COX-1 and COX-2 models.
Figure 13. Comparison of concentration-response curves for cyclooxygenase (COX)-1 and -2 inhibition by tolfenamic acid. (A) linear scale (B) semi-logarithmic scale. Values are presented as mean ± SEM percentage inhibition of PGE₂ synthesis compared to cells treated with vehicle and AA only (n=9 cells from two different culture plates). Curve fit was obtained through quadratic regression. Comparisons among data sets were conducted by contrast using an F test. * p < 0.05 Statistical differences for inhibition of PGE₂ synthesis between COX-1 and COX-2 models.
FIGURE 14. Comparison of inhibition of PGE$_2$ by each NSAID in the COX-1 Model. Values are presented as mean ± SEM percentage inhibition of PGE$_2$ synthesis compared to cells treated with vehicle and AA only. Statistical comparisons were performed by contrast using an F test.

p < 0.05 No significant differences in inhibition of PGE$_2$ synthesis were detected between carprofen and ketoprofen.

* p < 0.05 Carprofen and ketoprofen significantly inhibited PGE$_2$ synthesis more than tolfenamic acid at all concentrations, and more than meloxicam at all concentrations except 0.003 125 ug/mL.

+ P < 0.05 Meloxicam significantly inhibited PGE$_2$ synthesis more than tolfenamic acid.
FIGURE 15. Comparison of inhibition of PGE$_2$ by each NSAID in the COX-2 Model. Values are presented as mean $\pm$ SEM percentage inhibition of PGE$_2$ synthesis compared to cells treated with vehicle and AA only. Statistical comparisons were performed by contrast using an F test.

$\text{p} < 0.05$ No significant differences in inhibition of PGE$_2$ synthesis were detected between carprofen and meloxicam or ketoprofen and meloxicam at any concentration, or between carprofen and tolfenamic acid except at 0.003 ug/mL and 0.0125 ug/mL).

$\ast \text{ p} < 0.05$ Ketoprofen significantly inhibited PGE$_2$ synthesis more than carprofen and tolfenamic acid.

$\ast \ast \text{p} < 0.05$ Meloxicam significantly inhibited PGE$_2$ synthesis more than tolfenamic acid.
TABLE V: Indices of canine COX selectivity of four nonsteroidal anti-inflammatory drugs. The concentration of each NSAID producing 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 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 higher than 1, and a greater inhibitory effect on COX-1 versus COX-2 is indicated by a ratio lower than 1.

<table>
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<tr>
<th>Drug</th>
<th>IC50 for COX-1 (ug/mL)</th>
<th>IC50 for COX-2 (ug/mL)</th>
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<td>Meloxicam</td>
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</tbody>
</table>

* An IC50 could not be calculated for tolfenamic acid, since in the COX-1 assay, PGE2 suppression by this drug did not reach 50%.
TABLE VI: Indices of canine COX selectivity of four nonsteroidal anti-inflammatory drugs. The concentration of each NSAID producing 25% inhibition (IC_{25}) of PGE_2 synthesis in non-LPS treated (COX-1) and LPS treated (COX-2) canine DH82 monocyte/macrophage cells was determined by quadratic regression, and 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 higher than 1, and a greater inhibitory effect on COX-1 versus COX-2 is indicated by a ratio lower than 1.

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<th>Drug</th>
<th>IC_{25} for COX-1 (ug/mL)</th>
<th>IC_{25} for COX-2 (ug/mL)</th>
<th>Ratio COX-1/COX-2 at IC_{25}</th>
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<td>Tolfenamic Acid</td>
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DISCUSSION

Pilot Studies

The purpose of the pilot studies was to evaluate COX expression and PG production by two canine cell lines in order to assess their suitability to serve as COX-1 and COX-2 assays for in vitro screening of canine NSAIDs. As the canine COX gene has yet to be sequenced, it was also necessary to confirm that canine COX expression was detectable by Northern analysis using human cDNA COX probes, and that the specificity of the probes was sufficient to discriminate between COX-1 and COX-2 transcripts.

Platelets are the only cells which have been consistently demonstrated to exclusively express COX-1 in all species previously studied, and thus provided an ideal control to test the homology of the COX probes to canine mRNA (Patrignani et al., 1994; Kawai et al., 1998). We also demonstrate this expression pattern in the dog following Northern analysis of mRNA from canine platelets, and in doing so confirm the ability of the human COX cDNA probes to detect COX-1 message, without cross-reacting with COX-2 under the conditions used here.

Our ability to detect only COX-1 by RT-PCR in untreated canine DH82 monocyte/macrophage cells, and demonstrate expression of COX-2 by Northern analysis following incubation of these cells with LPS provides further supportive evidence that expression and regulation of canine COX is similar to that of other species. These findings are consistent with previous studies which report dramatic increases in COX-2 mRNA expression following exposure to LPS, with no detectable effect on COX-1 mRNA in human and rodent monocytes/macrophages (Fu et al., 1990; Lee et al., 1992; Hempel et al., 1994; Masferrer et al., 1994; Arias-Negrete et al., 1995). However, COX-1 transcript was not identified in the canine DH82 cells using Northern analysis. The ability of the 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 level that it was below the level of detection of Northern 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 levels were too low to detect by Northern analysis. Reverse transcription and PCR of canine COX-2 was attempted, but amplification of COX-2 from either genomic DNA, or from reverse-transcribed canine DH82 monocyte/macrophage RNA was unsuccessful. 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 analysis. Our findings of low levels of PGE_2 synthesis by non-LPS treated DH82 cells corresponds to the expected low constitutive levels of COX-1 expression, and the four fold increase in PGE_2 synthesis following a six hour incubation with LPS corresponds with strongly induced COX-2 expression. If COX-2 is present in non-LPS treated DH82 cells, its expression is minimal and does not significantly contribute to PGE_2 synthesis.

Previous studies of monocyte/macrophage cells in other species have also demonstrated absence of COX-2 expression, and constitutive COX-1 expression by untreated monocyte/macrophage cells. Cyclooxygenase-2 mRNA cannot be detected until the cells are exposed to mitogenic or inflammatory stimuli, and the expression profile is similar to what we demonstrate in our study (Fu et al., 1990; Arias-Negrete et al., 1995).

Detection of canine COX-1 by RT-PCR of RNA obtained from canine platelets and canine DH82 monocyte/macrophage cells using primers designed based on the human COX-1 sequence is a novel finding. The 98% sequence similarity between the 270 bp PCR products and the equivalent human portion of the gene suggests that there may be homology between other areas of the canine and human COX-1 gene. Cloning and sequencing of the canine gene is required to validate this theory. However, from these findings, we were able to infer that low levels of PGE_2 synthesized by non-LPS treated canine DH82 monocyte/macrophages were due to COX-1 enzyme activity, and elevated PGE_2 synthesis by LPS-treated DH82 cells was primarily in
response to induced COX-2 enzyme activity, as has been reported for human and rodent monocyte/macrophage cells (Fu et al., 1990; Lee et al., 1992; Hempel et al., 1994; Masferrer et al., 1994; Arias-Negrete et al., 1995).

The detection of COX-2 message in unstimulated Madin-Darby canine kidney cells by Northern analysis was unexpected, and conflicts with a previous report in the literature which reported constitutive expression of COX-1, and induction of COX-2 in MDCK cells following incubation with 12-0-Tetradecanoyl-phorbol-13-acetate. That study employed polyclonal COX-1 and COX-2 antibodies to perform immunodetection of COX protein (Sciorra et al., 1996). It is possible that the results achieved were due to cross-reactivity between COX-1 and COX-2 antibodies in that experiment.

Our findings concur with another study which examined both COX mRNA and protein levels in MDCK cells. Cyclooxygenase-1 and COX-2 mRNA were not detectable by Northern analysis using specific mouse cDNA probes, and the authors were also unable to detect either canine COX isoform by Western analysis using specific antibodies directed against mouse COX-1 or COX-2. These results may reflect the lack of species homology between the mouse and dog cDNA sequences for the specific probes used for Northern analysis, or difficulties in detection of low levels of COX expression. However, the study further investigated COX expression by semi-quantitative PCR, using primers taken from a consensus sequence of mouse, rat and human cDNAs. Cyclooxygenase-1 mRNA was still undetectable under various experimental conditions; however, COX-2 mRNA could be detected in very low levels in resting MDCK cells (Schaefers et al., 1995).

Several conclusions were drawn from results of the pilot studies. Firstly, COX-2 expression in canine cells is detectable by Northern analysis using human cDNA probes, and COX-1 expression is detectable by RT-PCR, using primers designed based on the human COX-1 gene sequence. These findings provide supportive evidence that there may be homology between human and canine COX.
Since Madin-Darby canine kidney cells were found to express COX-2, they were determined to be an unsuitable model for the canine COX-1 assay and were not investigated further. However, the DH82 canine monocytes/macrophage cells were shown to contain one constitutively expressed isoform (COX-1) and consequently synthesized low levels of PGE$_2$, and one inducible isoform (COX-2) with correspondingly elevated levels of PGE$_2$ synthesis. Thus, it was concluded that using unstimulated DH82 cells as a source of COX-1, and LPS-treated DH82 cells as a source of COX-2 would provide an excellent model system to screen canine NSAIDs for their in vitro activity against each COX enzyme.

**Nonsteroidal Anti-inflammatory Drug Studies**

The purpose of the nonsteroidal anti-inflammatory drug studies was to utilize the COX-1 and COX-2 assays which were developed in the pilot studies to determine the differential canine COX suppressive activity of four NSAIDs. The results obtained were compared with published in vivo canine NSAID data to assess the predictability of the in vitro assay.

During the initial design phase of this project, there were no existing reports of canine in vitro cell models used to screen NSAIDs for COX activity. However, a recent publication has examined COX inhibition in the dog, and reports canine NSAID COX profiles that are consistent with our in vitro model results (Ricketts et al., 1998). The study utilized canine platelets as a source of COX-1, and LPS-treated DH82 monocyte/macrophage cells as a source of COX-2. The general canine COX specificity reported was similar to our study; however, the overall ranking order of preferential COX-2 inhibitors differed from the findings of our study - ketoprofen was found to be COX-1 preferential, carprofen and tolfenamic acid were found to be COX-2 preferential, and meloxicam was deemed non-selective, although it too was slightly COX-2 preferential.

The ratio of the IC$_{50}$ of COX-1 and the IC$_{50}$ of COX-2 is a parameter widely utilized throughout the literature as the “gold standard” of COX selectivity of various NSAIDs. In our
study, neither carprofen nor ketoprofen display significant differences in their individual effects on inhibition of PGE₂ synthesis between COX models. Within the COX-1 model, the effects of carprofen and ketoprofen are also not significantly different from each other, nor are there differences between their effects for the majority of concentrations evaluated in the COX-2 model; thus, it would be expected that these two NSAIDs display relatively equal activity against both COX-1 and COX-2 enzymes. The calculated IC₅₀ ratios from our experiments reflect these statistical findings, since a ratio of one designates equipotency against both enzymes, and ratios of 1.75 for carprofen and 0.36 for ketoprofen do not deviate far from this standard. Although a ratio greater than one is literally interpreted as COX-2 preferential for carprofen, and less than one is COX-1 preferential for ketoprofen, the relative difference is so small that their effects are not significantly different in vitro.

For carprofen, this lack of specificity against either enzyme has been demonstrated in previous studies, and in fact carprofen has been reported to be a relatively weak COX inhibitor, and as yet, its true mode of action is unclear (Strub et al., 1982; Lees et al., 1994). Equipotent activity against COX-1 and COX-2 (an IC₅₀ value of 1) was also observed in another in vitro study conducted using cultured bovine aortic endothelial cells as a source of COX-1 enzyme, and J774.2 macrophages stimulated with LPS as a source of COX-2 activity (Vane et al., 1995). Ex vivo studies seem unable to demonstrate significant COX inhibition at clinical dosage regimens. In a canine tissue cage model of inflammation, carprofen did not significantly inhibit PG production in serum or inflammatory exudate (McKellar et al., 1994). However, in an equine model of carrageenan-induced inflammation, carprofen did significantly reduce edema, but only weakly inhibited PGE₂ production at the recommended dose (Lees et al., 1994). Inhibition of PGE₂ is a biochemical index of COX suppression by an NSAID; therefore, it can be interpreted that no reduction in PGE₂ levels indicates minimal efficacy of carprofen against COX isoenzymes, at least at dose ranges evaluated. The equal activity of carprofen against both
COX-1 and COX-2 enzymes in vitro demonstrated in our study may also occur in vivo, but since carprofen does not appear to inhibit COX at therapeutic doses in the dog or the horse, it is not possible to evaluate this effect at this time.

In clinical practice, carprofen displays effective analgesic properties, obviously a parameter that we are unable to evaluate in our in vitro model (Holtsinger et al., 1992; Nolan et al., 1993; Lascelles et al., 1994; Vasseur et al., 1995; Welsh et al., 1997). It has been suggested that analgesia may occur via a mechanism other than systemic COX inhibition, and among different species, higher than approved doses may be required to produce anti-inflammatory effects as compared to the dose needed to produce analgesia (Strub et al., 1982; Lees et al., 1994).

The tendency of ketoprofen to be preferential for COX-1 in vitro, which we report here, has also been demonstrated by other in vitro COX assays which have examined NSAID effects on whole blood or cultured cells (Brideau et al., 1996; Cryer et al., 1998; Ricketts et al., 1998). Despite the fact that there is minimal experimental literature regarding ketoprofen, evidence of COX inhibition does exist. In an equine tissue cage model, ketoprofen inhibited serum TXB₂ synthesis for up to 24 hours (interpreted as COX-1 suppression), in addition to PGE₂ synthesis in inflammatory exudate (interpreted as COX-2 suppression) (Landoni et al., 1995). It has also been reported to reduce LPS-stimulated synthesis of PGE₂ in equine synovial cells in culture, also considered COX-2 suppression (Landoni et al., 1996). The ability of ketoprofen to inhibit both COX-1 in a prolonged manner, in addition to COX-2 parameters in an animal model system reinforces the validity of our in vitro data.

Our findings of significant differences of the effect of meloxicam on PGE₂ inhibition between COX-1 and COX-2 models is also reflected by the calculated IC₅₀ value of 12.27, a much larger ratio than those calculated for carprofen or ketoprofen, and a more definitive indication of strong preferential affinity for COX-2 over COX-1. This finding is supported by several other reports in the literature. Meloxicam has been consistently shown to be COX-2 preferential in vitro (Engelhardt et al., 1995; Berg et al., 1997; Fenner et al., 1997; Kawai et al., 1998; Ricketts et al.,
and capable of COX inhibition \textit{in vivo}. In an equine tissue cage model, meloxicam was demonstrated to initially inhibit TXB$_2$ formation in both serum (interpreted as COX-1 suppression) and inflammatory exudate (interpreted as COX-2 suppression). However, after 8 hours, inhibition of serum TXB$_2$ no longer occurred, suggesting that inhibition of COX-1 by meloxicam is reversible. The authors reported that TXB$_2$ levels in exudate remained suppressed, reflecting prolonged COX-2 inhibition at the site of inflammation (Lees et al., 1991). Meloxicam also suppressed PGE$_2$ biosynthesis in inflamed tissue exudates from experimental models of pleuritis and peritonitis in rats and mice, also an indication of COX-2 inhibition (Engelhardt et al., 1996). Our data demonstrates that meloxicam is a preferential COX-2 inhibitor \textit{in vitro}, which is in agreement with findings of other \textit{in vitro} studies performed using intact cells from various species (Engelhardt et al., 1995; Berg et al., 1997; Fenner et al., 1997; Kawai et al., 1998; Ricketts et al., 1998). This finding is also supported by the ability of meloxicam to suppress COX-2 activity \textit{in vivo} continuously at the site of inflammation, with no prolonged effect on COX-1 (Lees et al., 1991).

Like meloxicam, tolfenamic acid displays significant differences in its inhibitory effect on PGE$_2$ synthesis between COX-1 and COX-2 models, and like meloxicam, its COX-2 preferential effect is demonstrated by its IC$_{25}$ ratio of 8.40 (greater than 1). The minimal degree of PGE$_2$ inhibition by tolfenamic acid in the COX-1 model (ie: 50% inhibition of PGE$_2$ was not achieved) and the plateau-like concentration-response effect which does not occur for the other NSAIDs, seems improbable. This effect has not been observed in previous COX assays which evaluated this NSAID (Vane et al., 1995; Ricketts et al., 1998). Tolfenamic acid has been reported to have an IC$_{50}$ value of 15.0 (Ricketts et al., 1998) which is similar to our IC$_{25}$ ratio of 8.4 and indicates preferential inhibition of COX-2. However, Vane et al. (1995) reported a COX-1 preferential IC$_{50}$ ratio of 0.06 (Table X). These conflicting reports of the COX specificity of tolfenamic acid may result from some chemical characteristic of the drug which exerts different effects under different model conditions, and is not a factor for the other NSAIDs examined in this study.
Failure of tolfenamic acid to achieve 50% inhibition of PGE$_2$ synthesis in the COX-1 model may reflect supersaturation of the COX active site secondary to the high free drug concentration in the serum-free culture medium, as compared to other studies in which protein binding and short incubation periods limited the amount of free drug available to act on COX enzyme.

Tolfenamic acid has been reported to inhibit both the lipoxygenase and cyclooxygenase pathways in vitro. However, although COX inhibition by tolfenamic acid has been demonstrated in a live animal model, there is no evidence of in vivo inhibition of the lipoxygenase pathway. We confirm COX inhibition in vitro, although lipoxygenase products were not measured. Clinical use of tolfenamic acid in a canine experimental ocular surgery model, resulted in a significant reduction in PGE$_2$ production (interpreted as COX-2 inhibition) in the aqueous humour (Roze et al., 1996). Tolfenamic acid has also been shown to almost completely inhibit ex vivo synthesis of TXB$_2$ (interpreted as COX-1 inhibition) in serum, in addition to suppressing PGE$_2$ formation (interpreted as COX-2 inhibition) in inflammatory exudate from tissue cage models using dogs and calves (McKellar et al., 1991; McKellar et al., 1994; Lees et al., 1998). The duration of TXB$_2$ inhibition in serum was short lived, 50% inhibition did not occur beyond 8 hours at clinical dosage regimens. An additional unusual finding of this study was that plasma concentrations of tolfenamic acid exceeded concentrations in exudate until 24 hours after administration, demonstrating slow penetration into fluids. Most NSAIDs, such as carprofen, and meloxicam penetrate into exudate relatively quickly (Lees et al., 1991; McKellar et al., 1994). These studies demonstrate the COX-2 suppressive effect of tolfenamic acid and support our in vitro findings of COX-2 preference for this NSAID. However, COX-1 inhibition exceeding 50% does seem to occur in vivo, and diminishes over time, a phenomenon we are unable to demonstrate in vitro. A possible explanation is that reversible inhibition of COX-1 occurs slowly in vivo secondary to the slow distribution of this NSAID from plasma to inflammatory site as compared to other NSAIDs, and that reversal of COX-1 occurs more rapidly at the high
concentrations of tolfenamic acid present in the in vitro system. Regardless, it appears that the in vitro model system does not entirely reflect the in vivo effects of tolfenamic acid in the dog.

If the COX selectivity of each NSAID evaluated in our in vitro canine cell assay were extrapolated to the in vivo situation, it would be expected that administration of preferential COX-2 inhibitors, such as meloxicam and tolfenamic acid, to dogs would provide effective relief of symptoms associated with pain and inflammation, and cause fewer and less severe adverse effects than nonselective inhibitors, such as carprofen and ketoprofen. These conclusions appear to be supported by clinical studies which have examined adverse affects associated with use of canine NSAIDs. Results achieved seem to reflect the anticipated physiologic effects associated with COX-1 or COX-2 preference, as demonstrated by our model system. A study conducted by Forsyth et al. (1996), reported that 5 of 6 dogs treated with ketoprofen at the recommended dosage displayed endoscopic evidence of hemorrhage or gastric erosions, although the dogs appeared clinically normal. In a more recent study by the same author which also investigated gastric ulceration post-NSAID administration in the dog, there was a trend towards fewer dogs developing lesions after carprofen and meloxicam administration, compared to ketoprofen (Forsythe et al., 1998). These adverse gastrointestinal findings may be consistent with 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 in vitro screening using our canine cell model. It would be expected that the gastrointestinal effects of carprofen would be similar to ketoprofen, based on the nonspecific in vitro COX inhibition displayed by these two agents. However, the lack of detectable in vivo COX-1 or COX-2 inhibition following therapeutic doses of carprofen in dogs may explain why its gastrointestinal safety profile is similar to that of a COX-2 inhibitor like meloxicam (Lees et al., 1994; McKellar et al., 1994).

It was surprising that in our study total inhibition of PGE₂, as compared to untreated positive controls, was not achieved by any NSAID tested. However, similar findings were reported by
Oshima et al. (1984), who examined the effect of constant IV infusion of acetylsalicylic acid on canine cyclooxygenase activity. The study reported sixty percent platelet COX inhibition after one day of treatment; however, 95% inhibition was not reached until after three days of treatment. The authors offered no explanation for their findings. In our system, we can only speculate that the effects of supersaturation on enzyme equilibrium must be overcome in order to achieve total COX inhibition, which is suggested by results from the canine aspirin study. The correlation of our data with this in vivo study reinforces the scientific validity of the predictability of this model.

Predictive Value of the in vitro Canine COX Model

Several issues should be addressed when evaluating the predictive value of any model. It is desirable to utilize whole cell systems containing the intact COX enzyme of the species under investigation. Protein binding, time-dependent inhibition and source of arachidonic acid are factors which should all be taken into consideration (Laneuville et al., 1994; Chulada et al., 1997; Rienneau et al., 1997; Lora et al, 1998; Pairet et al., 1998). Therefore, in designing our in vitro cell assay system, characteristics of the ideal model were incorporated as closely as possible. The resulting predictive value of the model system is excellent, as evidenced by the correlation of the data with other in vitro studies and documented clinical experience with the efficacy and toxicity of the NSAIDs utilized in this study.

It has been demonstrated that inhibition of COX-2 by time-dependent competitive NSAIDs depends on length of preincubation, and that certain NSAIDs may require less time to suppress one COX isoenzyme over the other (Laneuville et al., 1994; Lora et al., 1998). Instantaneous NSAID inhibition assays display poor correlation with clinical results; however, inhibition assays using preincubation with the drugs tested have been shown to more closely resemble the data from in vivo studies (Lora et al., 1998). Inhibitor time-dependency was addressed in this study by preincubating each drug with the DH82 cells for 30 min prior to the 30 minute incubation with both drug and AA.
Another important characteristic of NSAIDs is their high degree of protein binding. This property has a profound effect on the distribution and actual unbound concentration of drug available at a site of inflammation. Effect of protein binding is difficult to mimic in vitro, and the degree of protein binding can vary between different agents (ie: 97% for meloxicam, 99% for carprofen, ketoprofen and tolfenamic acid). To avoid this dilemma, a protein-free (serum-free) system was utilized; therefore, all drugs are acting in free and unbound form.

We added only exogenous AA at a single concentration, which is a technique employed in several previous studies (Mitchell et al., 1994; Berg et al., 1997; Kawai et al., 1998). It has been demonstrated that NSAIDs can inhibit PGE_2 production from exogenous AA mediated by both COX-1 and COX-2. However, PG synthesis can also be assessed from endogenously released AA. The results of an assay may vary depending on the source of AA and the concentration utilized (Chulada et al., 1997; Riendeau et al., 1997). Further investigation of the phenomenon in this model is warranted.

An in vitro screen using only the monocyte/macrophage cells minimizes comparative differences between two assay systems, preserves membrane characteristics and maintains the same cellular source of COX enzyme. It has been suggested that since the ability of a drug to penetrate a cell membrane is crucial to determine its relative potency, and as membranes differ between cells from different tissues, using different cellular sources of enzyme may bias the results of a study (Smith et al., 1998).

Inhibitory concentration ratios for COX-1/COX-2 reported from previous studies are shown in Table VII. These findings demonstrate the variability in results achieved by different model systems, and support the concept that, while it may be possible to predict the general COX preferential tendency of a particular NSAID, it is perhaps unwarranted to interpret the overall “superiority” of one NSAID to another based on in vitro data alone. Many of these studies do not take into consideration all factors which are capable of influencing the outcome of an in vitro study. For example, certain studies include serum in their test medium, affecting the amount of
free drug available to act on the COX active site. Other studies do not preincubate cells with NSAID, skewing the results in favour of rapid inhibitors versus time-dependent inhibitors. Ricketts et al. (1998) incubated the COX-2 model with NSAID for a three fold longer period of time than that allotted to the COX-1 model, potentially biasing the study in favour of COX-2 inhibition, and minimizing time-dependent effects in the COX-1 model. These model differences among studies contribute to the discrepancies in reported ratios, and make comparisons between assay systems very difficult.

Finally, one must take note that in the majority of ex vivo or in vitro studies, TXB₂ or PGE₂ are measured in serum or culture medium from untreated cells as biochemical indexes 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 (Lees et al., 1991; McKellar et al., 1991; McKellar et al., 1994; Ricketts et al., 1998). Rarely are other indices beyond PG measurement evaluated when examining COX inhibition by NSAID. The ability of our study to measure PGE₂ synthesis and attribute it to canine COX-1 or COX-2 gene expression through Northern analysis or RT-PCR is novel, and provides a more complete evaluation of canine COX inhibition than measurement of PG synthesis alone. Analysis of canine COX enzyme protein via Western blotting is an additional method of assessing COX enzyme activity, and requires further investigation.
TABLE VII: Reported inhibitory concentration (IC$_{50}$) ratios for each nonsteroidal anti-inflammatory drug. Preferential inhibition of COX-2 is indicated by a ratio higher than 1, and a greater inhibitory effect on COX-1 versus COX-2 is indicated by a ratio lower than 1.

<table>
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<td>Vane et al., 1995</td>
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SUMMARY AND CONCLUSIONS

It would be advantageous to establish an in vitro assay for canine COX inhibition by NSAIDs 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, as only NSAIDs that significantly inhibit COX-2 would be further evaluated in vivo. In vitro assessment would increase our understanding of these agents prior to approval, and also permit evaluation of existing and newly approved drugs, potentially enabling veterinarians to use NSAIDs in a more informed manner.

The ability of NSAIDs to preferentially inhibit COX-2 over COX-1 in vivo is dependent on a variety of factors, including drug pharmacokinetics, plasma levels, protein binding and tissue distribution. Thus, true predictability of drug efficacy and toxicity is not possible from an in vitro assay. However, comparison of differential inhibition of COX enzyme by each drug relative to others evaluated in the same model system is possible.

The ideal in vitro screening assay is rapid, reproducible, and possesses good predictive value. It is important to bear in mind that selectivity ratios should only be considered trends which are not necessarily predictive of the level of COX-1 or COX-2 inhibition at a given dose in vivo (Pairet et al., 1998). Care must be taken to not over-extrapolate in vitro model findings to the in vivo situation. Cellular systems are capable of demonstrating basic molecular events and assist us in understanding mechanisms of action.

In this study, we have described a simple and convenient assay system to screen canine COX-1 and COX-2 inhibitor activities in intact cells in vitro. We have utilized a convenient source of COX-1 found in an untreated permanent culture of DH82 canine monocyte/macrophage cells, and induced COX-2 in the same cell type through incubation with lipopolysaccharide endotoxin. This assay offers the advantage of assessing COX NSAID inhibition within a similar test protocol, and
circumvents the need of time-consuming purification of either platelets or peripheral blood monocytes.


APPENDIX I

Materials and Solutions used in Northern Blot analysis

**Dimethylpyrocarbonate (DMPC)-A Water**

Dimethylpyrocarbonate 4 mL was added to 1.996 litres of double distilled water (DMPC water). A ten-fold dilution was prepared using double distilled water and sterilized by autoclave.

**Luria-Bertani (LB) Medium**

Bacto-tryptone 10 g, bacto-yeast extract 5 g and NaCl 10 g was dissolved in 950 mL of double distilled water. The pH was adjusted to 7.0 with 5 N NaOH and the volume adjusted to one litre with double distilled water. The solution was autoclaved for 20 minutes for sterilization.

**SOC Medium**

Bacto-tryptone 20 g, bacto-yeast extract 5 g and NaCl 0.5 g was dissolved to 950 mL of double distilled water. A 10 mL volume of a 250 mM solution of KCl (1.86 g of KCl dissolved in 100 mL of double distilled water) was added and the pH adjusted to 7.0 with 5 N NaOH. The volume was adjusted to 1 liter with double distilled water and the solution autoclaved for 20 minutes for sterilization. Following autoclaving, 20 mL of sterile 1 M glucose solution was added, followed by 5 mL of sterile 2 M MgCl₂ (19 g MgCl₂ dissolved in 100 mL of double distilled water).

**Sodium dodecyl sulphate (10%)**

Sodium dodecyl sulfate 100 g electrophoresis-grade was dissolved in 900 mL DMPC-A water and heated to 68 °C to assist dissolution. The pH was adjusted to 7.2 with concentrated hydrochloric acid and the volume adjusted to one litre with DMPC-A water.

**Sodium chloride/sodium citrate buffer (SSC) Solution (20X stock)**

Sodium chloride 175.3 g and 88.2 g of sodium citrate were dissolved in 800 mL of DMPC-A water. The pH was adjusted to 7.0 with 10 M sodium hydroxide and the volume adjusted to one litre with DMPC-A water.
Sodium chloride/sodium phosphate buffer (SSPE) Solution (20X stock)

Sodium chloride 175.3 g, 27.6 g sodium hydrogen phosphate and 7.4 g of EDTA were dissolved in 800 mL of DMPC-A water. The pH was adjusted to 7.4 with 10 N sodium hydroxide and the volume adjusted to one litre with DMPC-A water.

3-(N-Morpholino) Propane-Sulfonic acid (MOPS) buffer (5X stock)

MOPS 41.2 g, 10.89 g of Sodium acetate and 3.72 g of EDTA were dissolved in 800 mL of DMPC-A water. The pH was adjusted to 7.0 with 2 N sodium hydroxide and the volume adjusted to one litre with DMPC-A water.

Denhardt’s solution (5X stock)

Five grams each of Ficoll 400, polyvinylpyrrolidone and bovine serum albumin were added to DMPC-A water to a total volume of 500 mL. The solution was sterilized by filtration, aliquotted and stored at -20 °C.

TEN buffer

Sodium chloride 584 mg, 158 mg of Tris HCl and 200 uL of 0.5 M EDTA (pH 8.0) was dissolved in 80 mL of DMPC-A water. The pH was adjusted to 8.0 and the volume adjusted to 100 mL.

Sephadex G-50

Three grams of Sephadex G-50 (medium grade) was allowed to swell overnight in DMPC-A water. The mixture was washed once with DMPC-A water and equilibrated with TEN buffer (pH 8.0). The preparation was sterilized by autoclave and stored at 4 °C.

Tris-acetate (TAE) buffer

Tris base 242 g, 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.0) was dissolved in double distilled water, and adjusted to a 1 liter volume to form 50X stock solution. A 1X solution was made through 50 fold dilution of this 50X stock.
### APPENDIX II

#### Sources of Supplies and Materials

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