Rofecoxib [Vioxx, MK-0966; 4-(4'-Methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone]: A Potent and Orally Active Cyclooxygenase-2 Inhibitor. Pharmacological and Biochemical Profiles


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ABSTRACT
The discoveries that cyclooxygenase (COX)-2 is an inducible form of COX involved in inflammation and that COX-1 is the major isoform responsible for the production of prostaglandins (PGs) in the gastrointestinal tract have provided a rationale for the development of specific COX-2 inhibitors as a new class of anti-inflammatory agents with improved gastrointestinal tolerability. In the present study, the preclinical pharmacological and biochemical profiles of rofecoxib [Vioxx, also known as MK-0966, 4-(4'-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone], an orally active COX-2 inhibitor, are described. Rofecoxib is a potent inhibitor of the COX-2-dependent production of PGE2 in human osteosarcoma cells (IC50 = 26 ± 10 nM) and Chinese hamster ovary cells expressing human COX-2 (IC50 = 18 ± 7 nM) with a 1000-fold selectivity for the inhibition of COX-2 compared with the inhibition of COX-1 activity (IC50 > 50 μM in U937 cells and IC50 > 15 μM in Chinese hamster ovary cells expressing human COX-1). Rofecoxib is a time-dependent inhibitor of purified human recombinant COX-2 (IC50 = 0.34 μM) but causes inhibition of purified human COX-1 in a non-time-dependent manner that could only be observed at a very low substrate concentration (IC50 = 26 μM at 0.1 μM arachidonic acid concentration). In an in vitro human whole blood assay, rofecoxib selectively inhibited lipopolysaccharide-induced, COX-2-derived PGE2 synthesis with an IC50 value of 0.53 ± 0.02 μM compared with an IC50 value of 18.8 ± 0.9 μM for the inhibition of COX-1-derived thromboxane B2 synthesis after blood coagulation. Using the ratio of the COX-1 IC50 values over the COX-2 IC50 values in the human whole blood assay, selectivity ratios for the inhibition of COX-2 of 36, 6.6, 2, 3, and 0.4 were obtained for rofecoxib, celecoxib, meloxicam, diclofenac, and indomethacin, respectively. In several in vivo rodent models, rofecoxib is a potent inhibitor of carrageenan-induced paw edema (ID50 = 1.5 mg/kg), carrageenan-induced paw hyperalgesia (ID50 = 1.0 mg/kg), lipopolysaccharide-induced pyresis (ID50 = 0.24 mg/kg), and adjuvant-induced arthritis (ID50 = 0.74 mg/kg/day). Rofecoxib also has a protective effect on adjuvant-induced destruction of cartilage and bone structures in rats. In a 51Cr excretion assay for detection of gastrointestinal integrity in either rats or squirrel monkeys, rofecoxib has no effect at doses up to 200 mg/kg/day for 5 days. Rofecoxib is a novel COX-2 inhibitor with a biochemical and pharmacological profile clearly distinct from that of current nonsteroidal anti-inflammatory drugs and represents a new therapeutic class of anti-inflammatory agents for the treatment of the symptoms of osteoarthritis and rheumatoid arthritis with improved gastrointestinal tolerability.

Cyclooxygenases (COXs) are bifunctional hemoproteins that catalyze the bisoxygenation of arachidonic acid to prostaglandin (PG)H2, which serves as the common precursor for the synthesis of PGs, prostacyclins, and thromboxanes (TBXs), collectively known as prostanoids. It is now well established that COXs exist as two isozymes that catalyze the same reaction but differ in terms of regulation of expression (see reviews in Vane and Botting, 1995; Smith and DeWitt, 1996). The constitutive isozyme COX-1 is responsible for the production of PGs involved in prostanoid-mediated physio-
logical functions such as gastric cytoprotection, maintenance of renal homeostasis, and maintenance of normal platelet functions. A second isoform, COX-2, has been identified and has been demonstrated to be highly expressed in response to inflammatory or mitogenic stimuli. Thus, it is proposed that COX-2 is responsible for the production of PGs associated with inflammatory conditions. It is well documented in the literature that the mechanism of action of nonsteroidal anti-inflammatory drugs (NSAIDs) involves inhibition of COX (Vane, 1971). The therapeutic actions of these compounds, such as their anti-inflammatory, analgesic, and antipyretic effects, can be explained by inhibition of PG formation. On the other hand, it is also well known that NSAIDs have mechanism-based side effects (Allison et al., 1992; Murray and Brater, 1993; Schafer, 1995) that limit the dose of NSAIDs in patients. The discovery of a second isoform of COX has provided the rationale for the development of specific COX-2 inhibitors as a novel class of anti-inflammatory compounds compared with current NSAIDs, which inhibit both COX-1 and COX-2 with similar potency (Meade et al., 1993; Brideau et al., 1996). It is hypothesized that a specific COX-2 inhibitor will achieve therapeutic efficacy in osteoarthritis and pain management while avoiding the serious side effects, in particular, gastrointestinal ulceration related to COX-1 inhibition observed with NSAIDs. This hypothesis is supported by data with a number of COX-2 selective inhibitors that have shown efficacy in animal models of pain, inflammation, pyresis, and superior gastrointestinal tolerability compared with NSAIDs (Futaki et al., 1993; Seibert et al., 1994; Chan et al., 1995; Riendeau et al., 1997b). Rofecoxib (Vioxx, also known as MK-0966) is a novel and highly selective COX-2 inhibitor that has been shown to be efficacious in the treatment of osteoarthritis, comparable to NSAIDs (Fig. 1) (Ehrich et al., 1998a,b; 1999) and is currently in the final stage of development. In the present study, the preclinical pharmacological and biochemical profiles of rofecoxib are described and are discussed with respect to clinical findings.

**Experimental Procedures**

**In Vitro Biochemical and Pharmacological Assays**

Inhibition Studies with Recombinant Human COX-1 and COX-2. Micromolar preparations of recombinant human COX-1 and COX-2 were prepared from a vaccinia virus-COS-7 cell expression system (O’Neill et al., 1994) Recombinant human COX-1 and COX-2 were expressed in baculovirus-Sf9 cells, and enzymes were purified as described previously (Ouellet and Percival, 1995; Cromlish and Kennedy, 1996). Enzymatic activity was monitored continuously by either a fluorescence assay measuring the appearance of the oxidized form of the reducing agent cosubstrate homovanillic acid or by oxygen consumption (Ouellet and Percival, 1995). The HPLC assay for the assessment of inhibition of purified COX-1 by rofecoxib with 0.1 \( \mu \)M arachidonic acid substrate concentration, the determination of the stoichiometry of the complex between COX-2 and rofecoxib, the dissociation rate constant of the enzyme-inhibitor complex by recovery of enzymatic activity, and the recovery of intact rofecoxib from that complex were all performed as described previously (Riendeau et al., 1997b). The solvent system for the HPLC analysis of rofecoxib was 15:85 MeOH/aqueous potassium phosphate (1 g/liter), with elution by a linear gradient of 15 to 75% MeOH over 25 min with detection at 275 nm on a Nova Pak C18 column (Waters, Milford, MA).

Spectrophotometric Assay of Recombinant Human COX-2. Enzymatic activity of the purified COX-2 was measured using a chromogenic assay based on the oxidation of \( N,N,N',N' \)-tetramethyl-p-phenylenediamidine (TMPD) during the reduction of PGG2 to PGH2 (Copeland et al., 1994). The assay mixture (180 \( \mu l \)) contains 100 mM sodium phosphate, pH 6.5, 1 \( \mu \)M hematin, 1 mg/ml gelatin, 2 to 5 \( \mu \)g/ml of purified COX-2, and 4 \( \mu \)l of the test compound in dimethyl sulfoxide (DMSO). The assay was also performed in the presence of the detergent Genapol X-100 (CalBiochem, San Diego, CA) at a final concentration of 2 mM. The mixture was preincubated at room temperature (22°C) for 15 min before initiation of the enzymatic reaction by the addition of 20 \( \mu \)l of a solution of 1 mM arachidonic acid and 1 mM TMPD in assay buffer (without enzyme or hematin). For assays in the presence of Genapol, the arachidonic acid and TMPD solution was prepared in 50% aqueous ethanol. The enzyme activity was measured by estimation of the initial velocity of TMPD oxidation over the first 36 s of the reaction as followed from the increase in absorbancy at 610 nm. A low rate of nonenzymatic oxidation was observed in the absence of COX-2 and was subtracted before the calculation of the percentage of inhibition.

Whole-Cell Assays with Osteosarcoma Cells (COX-2) and U937 Cells (COX-1). The human osteosarcoma cell line has been shown to selectively express COX-2 by reverse transcription-polymerase chain reaction and immunoblot analysis, whereas undifferentiated human lymphoma U937 cells selectively express COX-1. The production of PGE2 by these cells after arachidonic acid challenge was used as an index of cellular COX-2 and COX-1 activity, respectively. Rofecoxib was preincubated for 5 to 15 min with the cells under serum-free conditions [Hanks’ balanced salt solution (HBSS)] before a 10-min stimulation with 10 \( \mu \)M arachidonic acid and measurement of PGE2 production as described previously (Wong et al., 1997). COX activity in each cell line is defined as the difference in PGE2 concentrations in samples incubated in the presence or absence of arachidonic acid.

Whole-Cell Assays with Transfected Chinese Hamster Ovary (CHO) Cells Expressing COX-1 and COX-2. Stably transfected CHO cells expressing human COX-1 and COX-2 were cultured and assayed for the production of PGE2 after stimulation by arachidonic acid as described previously (Kargman et al., 1996). Cells (0.3 \( \times \) 106 cells in 200 \( \mu l \)) were preincubated in HBSS containing 15 mM HEPES, pH 7.4, with 3 \( \mu l \) of the test drug or DMSO vehicle for 15 min at 37°C before challenge with arachidonic acid. Cells were challenged for 15 min with an arachidonic acid solution [10% ethanol (v/v) in HBSS] to yield final concentrations of 10 \( \mu \)M arachidonic acid in the CHO(COX-2) assay and of 0.5 \( \mu \)M arachidonic acid in the CHO(COX-1) assay. In the absence of addition of exogenous arachidonic acid, levels of PGE2 in samples from CHO(COX-1) were less than 30 pg PGE2/106 cells. In the presence of 0.5 \( \mu \)M exogenous arachidonic acid, levels of PGE2 in samples from CHO(COX-1) cells increased to 260 to 1500 pg PGE2/106 cells. After stimulation with 10 \( \mu \)M exogenous arachidonic acid, levels of PGE2 in samples from CHO(COX-2) cells increased from <120 to 700 to 1600 pg PGE2/106 cells. Compounds were typically tested at eight concentrations in duplicate using 3-fold serial dilutions in DMSO. COX activity in the absence of test compounds is determined as the difference in PGE2 levels of cells.

![Fig. 1. Structure of rofecoxib.](image-url)
challenged with arachidonic acid versus the PGE2 levels in cells mock-challenged with ethanol vehicle.

**Whole-Cell Assay for Rat COX-2 in Sf9 Insect Cells.** Rat COX-2 cellular activity was assayed using a procedure based on the arachidonic acid-dependent production of PGE2 by baculovirus-infected Sf9 cells expressing rat COX-2 (Cromlish and Kennedy, 1996). The assay for the production of PGE2 by arachidonic acid-stimulated cells was performed as described for the CHO(COX-2) cells using 10 μM arachidonic acid, a 10-min reaction time, and a total of 2 × 106 cells (infected plus noninfected cells) per well (final volume of 200 μl). The production of PGE2 by Sf9 (rat COX-2) cells increased 3- to 19-fold after stimulation with 10 μM arachidonic acid to values of 4.0 to 8.1 ng PGE2/106 total cells. Inhibitors, tested at eight concentrations using 3-fold serial dilutions of the highest inhibitor concentration in DMSO, were preincubated for 15 min before arachidonic acid challenge.

**Human, Rat, and Dog Microsomal COX Assays.** Whole kidney (1–10 g of tissue) were suspended in 50 mM potassium phosphate buffer, pH 7.1, containing 0.1 M NaCl, 2 mM EDTA, and 1 mM phenylmethylsulfon fluoride (homogenization buffer). Samples were then homogenized for 2 min on ice using a hand-held tissue homogenizer (Biospec Products, Inc., Bartlesville, OK) at maximum setting, after which they were sonicated for 10 s using a microultrasonic cell disrupter (Kontes, Vineland, NJ). Tissue homogenates were then centrifuged at 100,000 g for 1 h at 4°C. The 100,000g microsomal pellet was resuspended in homogenization buffer and was sonicated (2 × 10 s) on ice. The resulting human, rat, and dog kidney microsomal suspensions diluted to protein concentrations of approximately 6, 10, and 12 mg/ml, respectively. Aliquots of microsomal preparations were stored at −80°C and thawed on ice immediately before assays.

Rofecoxib was preincubated at room temperature for 5 or 15 min with a microsomal preparation from rat, dog, or human kidneys. The preincubation buffer contained 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA, 0.5 mM phenol, 1 mM reduced glutathione, 1 mM hematin, and a protein concentration of 0.12 mg/ml. Arachidonic acid was then added to a final concentration of 2 μM, and the samples were further incubated at room temperature for 40 min. After the incubation period, the reaction was terminated by the addition of 25 μl of 1 N HCl with mixing. Samples were neutralized by the addition of 25 μl of 1 N NaOH before analysis of the amount PGE2 by radioimmunoassay. Assays were performed in duplicate or triplicate. Control reaction mixtures contained ethanolic vehicle instead of arachidonic acid. In the absence of the addition of arachidonic acid, levels of PGE2 in samples from human, dog, and rat kidney microsomes were approximately 1.5 ng/mg protein, 0.1 ng/mg protein, and 6.7 ng/mg protein, respectively. In the presence of arachidonic acid, levels of PGE2 in these preparations increased to approximately 4.2 ng/mg protein, 1.2 ng/mg protein, and 22 ng/mg protein, respectively. COX activity in the absence of test compounds is defined as the difference between PGE2 levels in samples incubated in the presence of arachidonic acid or ethanolic vehicle.

**Assay of U937 Microsomal COX-1 at Low Arachidonic Acid Concentration.** The activity of human COX-1 in a microsomal preparation from U937 cells was assayed at low arachidonic acid concentration (Riendeau et al., 1997a). Rofecoxib was preincubated with the microsomal preparation (protein concentration of 0.12 mg/ml) for 15 min at room temperature in 0.1 M Tris·HCl, pH 7.4, 10 mM EDTA, 0.5 mM phenol, 1 mM reduced glutathione, and 1 μM hematin. After preincubation, arachidonic acid was added to a final concentration of 0.1 μM, and the samples were further incubated for 40 min before quantification of PGE2 by radioimmunoassay. COX activity in the absence of rofecoxib was determined as the difference in PGE2 levels of microsomes incubated with arachidonic acid versus the PGE2 levels in microsomes incubated with ethanol vehicle.

**TBX B2 and 12-Hydroxyeicosatetraenoic Acid (12-HETE) Production by Calcium Ionophore-Activated Human Platelets.** Washed human platelets in HBSS buffered with 15 mM HEPES, pH 7.4, were preincubated at a final concentration of 4 × 107 cells/ml (0.2–0.25 ml) in the absence or presence of the inhibitor (from a 125-fold concentrated solution in DMSO) for 2 min before stimulation with 2 μM calcium ionophore A23187 for TBX B2 and 12-HETE production (Riendeau et al., 1994).

**Inhibition of Leukotriene B (LTB) Production by Human Polymericnuclear (PMN) Leukocytes.** Rofecoxib was preincubated with PMN leukocytes prepared from human blood from consenting volunteers (5 × 106 cells/ml) in HEPES (15 mM)-buffered HBSS, pH 7.4, for 2 min at 37°C. The cells were then challenged with 10 μM calcium ionophore A23187, and the reaction was terminated after 5 min by the addition of cold methanol. The production of LTB by human PMN leukocytes was determined by radioimmunoassay. The effect of rofecoxib was determined using a five-point titration over the concentration range of 0.31 to 25 μM. The percentage of inhibition was determined from the difference in LTB production by ionopore-challenged cells incubated in the presence of rofecoxib or with the DMSO vehicle.

**Leukocyte 15-Lipoxygenase Assay.** Rofecoxib was incubated at concentrations ranging from 0.7 to 20 μM with partially purified 15-lipoxygenase from human leukocytes in 0.05 M sodium phosphate, pH 6.3, 24 μg/ml phosphatidylcholine, and 20 μM arachidonic acid. After a 10-min incubation at room temperature, the reaction was quenched with acetonitrile and analyzed by reverse phase HPLC on a C18 column eluted with acetonitrile/water/trifluoroacetic acid (60:40:0.1) for the quantification of 15-hydroperoxyeicosatetraenoic acid.

**Human Whole Blood Assay.** The assay was done using identical procedures as reported previously (Brideau et al., 1996). Briefly, for the COX-2 assay, fresh heparinized human whole blood was incubated with lipopolysaccharide (LPS) from Escherichia coli at 100 μg/ml and with 2 μl of vehicle (DMSO) or a test compound for 24 h at 37°C. PGE2 levels in the plasma were measured using radioimmunoassay after proteolysis. For the COX-1 assay, an aliquot of fresh blood was mixed with either DMSO or a test compound and was allowed to clot for 1 h at 37°C. TBX B2 levels in the serum were measured using an enzyme immunoassay after proteolysis. The effects of rofecoxib, celecoxib, meloxicam, diclofenac, and indomethacin were examined under the same experimental conditions.

**In Vivo Assays**

All procedures used in the in vivo assays were approved by the Animal Care Committees or Institutional Animal Care and Use Committee at the Merck Frosst Centre for Therapeutic Research (Kirkland, Quebec, Canada), Merck, Sharp & Dohme Neuroscience Research Centre (Harlow, UK), and Merck Research Laboratories (Rahway, NJ) according to guidelines established by the Canadian Council on Animal Care, the British Home Office, and the U.S. Department of Agriculture and National Institutes of Health, respectively.

The following assays were done using identical procedures as described previously (Chan et al., 1995); these studies included carrageenan-induced rat paw edema assay, carrageenan-induced rat paw hyperalgesia assay, endotoxin-induced pyresis in rats, and 51Cr fecal excretion in rats and squirrel monkeys. Additional studies are described below.

**Rat Adjuvant-Induced Arthritis (AIA) Model.** AIA was induced in six groups of 10 rats (female Lewis, 144–172 g, 7 weeks old), each by an intradermal injection of 0.5 mg of Mycobacterium butyricum in light mineral oil in the left hind foot pad as described previously (Fletcher et al., 1998). Ten rats were not injected and served as nonadjuvant controls. Body weights, radiographs, and foot volumes of the noninjected (secondary) paws were determined on various days (0, 14, and 21). Rofecoxib (0.1, 0.3, 1.0, and 3.0 mg/kg/day p.o.; 0.05, 0.15, 0.5, and 1.5 mg/kg b.i.d.), indomethacin (1 mg/kg/day p.o.; 0.5 mg/kg b.i.d.), and appropriate vehicles were started on day 0 and continued throughout the experiment. Rats were euthanized by carbon dioxide inhalation on day 21. The thymus and spleen of all rats were removed and weighed.
were removed and weighed. To assess tibiotalar joint integrity, radiographic scores were assigned according to an adaptation of a previously described method (Clark et al., 1979) by a radiologist who was blinded to treatment. Two-factor (“treatment” and “time”) ANOVA with repeated measures on “time” were applied to the percent changes for body weight and foot volumes and to the rank-transformed radiographic total scores. A post hoc Dunnett’s test was conducted to compare the effect of treatments to vehicle. A one-way ANOVA was applied to the thymic and spleen weights, followed by the Dunnett’s test, to compare the effect of treatments to vehicle.

**Ex Vivo Whole Blood Assay in Anesthetized Dogs.** Fasted normal male laboratory beagles were anesthetized and bronchially intubated. After 15-min stabilization, a blood sample was collected into anticoagulant, and a bolus dose of either vehicle (80% PEG 200 in distilled water), diclofenac (0.1 mg/kg), or rofecoxib (0.05, 0.1, or 0.2 mg/kg) was administered i.v., followed by a continuous infusion of either vehicle, diclofenac (2.5 μg/kg/min), or rofecoxib (0.8–8 μg/kg/min). Additional blood samples were obtained 1 and 4 h after the beginning of the infusion and were incubated with LPS from *E. coli* (100 μg/mL) for 2 h at 37°C. Arachidonic acid dissolved in 30% ethanol in PBS, or the latter vehicle was then added (final arachidonic acid concentration of 100 μM) to 500-μL aliquots and incubated for 30 min. Prostanoid production was terminated by rapid centrifugation and methanolic extracts of plasma were prepared [plasma/MEOH 1:4 (v/v)]. The PGE₂ content of dilutions of these extracts was determined by a four-parameter logistic function using a nonlinear least-squares regression and methanolic extracts of plasma were prepared [plasma/MEOH 1:4 (v/v)]. The PGE₂ content of dilutions of these extracts was then measured by specific radioimmunoassay. Postdose inhibition of arachidonic acid-induced PGE₂ production was calculated by comparison with predose values.

**Statistics.**

Results are expressed as mean ± S.E.M. Unless otherwise specified, differences between vehicle control and treatment groups were tested using one-way ANOVA, followed by multiple comparison by the Dunnett’s test. A value of *P* < .05 was considered statistically significant. Dose-response curves for percent inhibition were fitted by a four-parameter logistic function using a nonlinear least-squares four-parameter equation.

**Materials.**

The following compounds were synthesized by the Medicinal Chemistry Department at Merck Frosst Centre for Therapeutic Research: rofecoxib, celecoxib (Penning et al., 1997), meloxicam. Sources of other compounds were: diclofenac, flurbiprofen, naproxen, LPS (Sigma-Aldrich, Oakville, Ontario, Canada); indomethacin (Merck, Sharp & Dohme Canada, Kirkland, Quebec, Canada).

**Results**

**In Vitro Studies.**

**Selective Inhibition of COX-2 by Rofecoxib in Intact Cell Assays.** Rofecoxib is a potent inhibitor of COX-2 in a variety of cell-based assays (IC₅₀ = 18–46 nM) and shows a 1000-fold selectivity for the inhibition of COX-2 compared with COX-1 (Table 1). Rofecoxib inhibited the arachidonic acid-dependent production of PGE₂ by osteosarcoma cells (COX-2) with an IC₅₀ value of 26 ± 10 nM (n = 5). No significant decrease in the potency of rofecoxib was observed in the presence of 1% human, dog, or rat serum. The IC₅₀ value for the inhibition of arachidonic acid-dependent production of PGE₂ by U937 cells (COX-1) was >50 μM (n = 4). Similarly, rofecoxib was a potent inhibitor of recombinant human COX-2 expressed in stably transfected CHO cells (IC₅₀ = 18 ± 7 nM, n = 6) and was >800-fold less potent as an inhibitor of human COX-1 in stably transfected CHO cells (IC₅₀ > 15 μM, n = 3). Rofecoxib was also found to be an inhibitor of rat COX-2 in a Sf9 whole-cell assay with a potency (46 ± 9 nM, n = 3) similar to that obtained for indomethacin (18 nM, n = 2). The data for rofecoxib and indomethacin are summarized in Table 1 and indicate that although indomethacin is a potent inhibitor of both COX-1 and COX-2 in cell-based assays, rofecoxib is a potent and selective inhibitor of human COX-2 in cell-based assays.

**Inhibition Studies with Purified COX-2 and COX-1.** The kinetic mechanism of inhibition of COX-1 and COX-2 by rofecoxib was investigated using microsomal and purified recombinant human enzymes. Rofecoxib inhibits the COX activity of purified human COX-2 (spectrophotometric assay), with IC₅₀ values of 0.34 (n = 2) and 0.40 μM (n = 2) for the assays performed in either the absence or presence of the detergent Genapol X-100, respectively. The compound is approximately equipotent to indomethacin in this assay (Table 1). The level of inhibition of purified COX-2 by rofecoxib was dependent on the preincubation period of enzyme and drug before initiation of the reaction with arachidonic acid. In-

**TABLE 1**

Potency and selectivity of rofecoxib in in vitro assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>IC₅₀</th>
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<tr>
<td></td>
<td>Rofecoxib</td>
</tr>
<tr>
<td>PGE₂ production by osteosarcoma cells (COX-2)</td>
<td>26 ± 10 nM (n = 5)</td>
</tr>
<tr>
<td>PGE₂ production by U937 cells (COX-1)</td>
<td>&gt;50 μM (n = 4)</td>
</tr>
<tr>
<td>PGE₂ production by CHO [COX-2] cells</td>
<td>18 ± 7 nM (n = 6)</td>
</tr>
<tr>
<td>PGE₂ production by CHO [COX-1] cells</td>
<td>&gt;15 μM (n = 3)</td>
</tr>
<tr>
<td>PGE₂ production by LPS-induced human mononuclear cells (COX-2)</td>
<td>45 ± 7 nM (n = 11)</td>
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<tr>
<td>PGE₂ production by LPS-induced rat mononuclear cells (COX-2)</td>
<td>41 ± 10 nM (n = 10)</td>
</tr>
<tr>
<td>PGE₂ production by SF9 [rat COX-2] cells</td>
<td>46 ± 9 nM (n = 3)</td>
</tr>
<tr>
<td>PGE₂ production by human kidney microsomes</td>
<td>14 μM (n = 4)</td>
</tr>
<tr>
<td>PGE₂ production by rat kidney microsomes</td>
<td>&gt;30 μM (n = 3)</td>
</tr>
<tr>
<td>PGE₂ production by dog kidney microsomes</td>
<td>&gt;30 μM (n = 2)</td>
</tr>
<tr>
<td>PGE₂ production by U937 microsomes (low substrate)</td>
<td>2.0 ± 0.5 μM (n = 7)</td>
</tr>
<tr>
<td>Purified human COX-2</td>
<td>0.34 μM (n = 2)</td>
</tr>
<tr>
<td>Purified human COX-2 with detergent</td>
<td>0.40 μM (n = 2)</td>
</tr>
<tr>
<td>15-Lipoxygenase</td>
<td>26.3 ± 6.4 μM (n = 11)</td>
</tr>
<tr>
<td>LTB₄ production by human PMN leukocytes</td>
<td>&gt;20 nM (n = 2)</td>
</tr>
<tr>
<td>12-HETE and TXB₂ production by Ca²⁺ ionophore-challenged platelets</td>
<td>&gt;25 μM (n = 3)</td>
</tr>
<tr>
<td>12-HETE and TXB₂ production by Ca²⁺ ionophore-challenged platelets</td>
<td>&gt;20 nM (n = 5)</td>
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N.D., not determined.
creased concentrations of rofecoxib result in a faster rate of onset of inhibition (Fig. 2A). Analysis of the data in terms of a two-step mechanism of inhibition (Ouellet and Percival, 1995; Riendeau et al., 1997b) gave a value for the second order rate constant for the onset of inhibition of COX-2 by rofecoxib of 0.0036 ± 0.0024 μM⁻¹ s⁻¹. A similar time-dependent inhibition by rofecoxib was observed using microsomal COX-2, and an IC₅₀ value of 0.13 μM was obtained after a 15-min preincubation period. In contrast, the inhibition of COX-1 by rofecoxib is non-time-dependent. Microsomal COX-1 was inhibited approximately 15% by 10 μM rofecoxib in fluorescence assays performed without preincubation and using an initial arachidonic acid concentration of 20 μM. No increase in inhibition was observed with up to 15-min preincubation of enzyme and inhibitor. Higher levels of inhibition of purified COX-1 could only be observed in assays performed in the presence of very low concentrations of arachidonic acid (0.1 μM). Under these conditions, an IC₅₀ value of 26 ± 6 μM (n = 11) was obtained for rofecoxib.

Both active COX-2 and intact inhibitor could be recovered after time-dependent inhibition, demonstrating that the inhibition of COX-2 by rofecoxib was not due to the irreversible covalent inactivation of the enzyme. The recovery of active enzyme was demonstrated in an experiment in which purified COX-2 was inhibited with a small excess of rofecoxib and then dialyzed against a large excess of buffer. The results (Fig. 2B) show that the enzyme activity recovers after inhibition in a first order manner with a T₁/2 of 9 ± 2 h and that after a dialysis period of 23 h, close to 100% of the original activity has been recovered. In the second experiment, purified COX-2 was treated with 1 Eq of rofecoxib for 30 min. After this time, the enzyme was 91% inhibited compared with a vehicle-treated control. The enzyme-inhibitor complex was then denatured, and any released inhibitor was extracted from the protein by treatment with organic solvent. HPLC of the extract showed a single peak at the same retention time as that of authentic rofecoxib with quantitative recovery compared with an identically treated no-enzyme control. These results therefore are consistent with the noncovalent nature of the tight complex between COX-2 and rofecoxib. Time-dependent and reversible inhibition could also be demonstrated in CHO(COX-2) cells (data not shown).

The stoichiometry of the enzyme-inhibitor complex was determined by a titration in which aliquots of purified COX-2 (3.4 μM) were treated with 0 to 6 μM rofecoxib and the remaining COX activity was measured after a 30-min preincubation period. A linear decrease in enzyme activity with increasing inhibitor concentration was obtained (results not shown), with the maximal inhibition observed (94%) occurring at concentrations of rofecoxib of more than 3.2 μM. This result is therefore consistent with the formation of an enzyme-inhibitor complex having 1:1 stoichiometry.

Effect of Rofecoxib on Human, Dog, and Rat Microsomal COX Activities. Rofecoxib is a time-dependent inhibitor of human recombinant COX-2 in microsomal assays with an IC₅₀ value of 130 nM after a 15-min preincubation. In human, dog, and rat kidney microsome preparations (COX-1), rofecoxib was substantially less potent with IC₅₀ values of 14, >30, and >30 μM, respectively. An incomplete inhibition of PGE₂ production in human kidney microsomes by rofecoxib was observed at doses of rofecoxib exceeding the IC₅₀ value (maximum inhibition of 67–86%), probably due to the limited water solubility of the compound under the assay conditions. Data are summarized in Table 1 and compared with those obtained with indomethacin, which is a potent inhibitor of PGE₂ synthesis by the three different kidney microsomal preparations. Thus rofecoxib is a weak or totally ineffective inhibitor of PGE₂ production by microsomes from human, rat, or dog kidneys, at concentrations 30 to 100 times higher than those required for the inhibition by indomethacin, in agreement with the selectivity of inhibition of rofecoxib for COX-2.

Inhibition of Microsomal COX-1 from U937 Cells at Low Arachidonic Acid Concentration. The effect of rofecoxib on COX-1 was determined using a sensitive assay based on the production of PGE₂ by U937 cell microsomes after incubation with a low, subsaturating concentration of arachidonic acid (0.1 μM). Potent nonselective inhibitors, such as indomethacin, show IC₅₀ values in the low nanomolar range in this assay (Table 1). Rofecoxib inhibited the production of PGE₂ by U937 cell microsomes in the assay at low arachidonic acid concentration with an IC₅₀ value of 2.0 ± 0.5 μM (n = 7).

Effect of Rofecoxib on TBX₂ and 12-HETE Synthesis by Human Platelets. The production of TBX₂ by Ca²⁺ ionophore-stimulated human platelets was used to further eval-
uate the potency of inhibitors at blocking COX-1-mediated prostaglandin production. In this assay, indomethacin and diclofenac were potent inhibitors (IC<sub>50</sub> = 2–4 nM) of TBX<sub>2</sub> production. Rofecoxib had no significant effect (<15% inhibition) on the production of TBX<sub>2</sub> and 12-HETE by calcium ionophore-challenged human platelets (IC<sub>50</sub> > 20 μM; n = 5 with two different platelet preparations).

**Human Whole Blood COX-1 and COX-2 Assay.** The results are summarized in Table 2. Rofecoxib is a potent inhibitor of human whole blood COX-2 activity with an IC<sub>50</sub> value of 0.53 ± 0.02 μM. Using the ratio of the COX-1 IC<sub>50</sub> value over the COX-2 IC<sub>50</sub> value, selectivity ratios for the inhibition of COX-2 of 36, 6.6, 2, 3, and 0.4 were obtained for rofecoxib, celecoxib, meloxicam, diclofenac, and indomethacin, respectively. Thus, rofecoxib has the highest selectivity among this panel of compounds when tested under the same experimental conditions.

**Other In Vitro Selectivity Studies.** In a series of selectivity studies, rofecoxib had no effect on LTB<sub>4</sub> biosynthesis by calcium ionophore-challenged PMN leukocytes (IC<sub>50</sub> > 25 μM, n = 3) and no effect on the activity of human leukocyte 15-lipoxygenase (IC<sub>50</sub> > 20 μM, n = 2; Table 1). Additionally, no detectable activities of rofecoxib were noted in a diverse array of receptor or enzyme assays performed by a contract laboratory (MDS Panlabs, Seattle, WA).

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>COX-1 IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Donor</th>
<th>COX-2 IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Donor</th>
<th>COX-1/COX-2 Ratio of IC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rofecoxib</td>
<td>18.8 ± 0.9</td>
<td>211</td>
<td>0.53 ± 0.02</td>
<td>614</td>
<td>35.5</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>6.3 ± 1.0</td>
<td>9</td>
<td>0.96 ± 0.24</td>
<td>12</td>
<td>6.6</td>
</tr>
<tr>
<td>Meloxicam</td>
<td>1.4 ± 0.4</td>
<td>6</td>
<td>0.70 ± 0.28</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>0.15 ± 0.04</td>
<td>10</td>
<td>0.05 ± 0.01</td>
<td>16</td>
<td>3.0</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.19 ± 0.02</td>
<td>36</td>
<td>0.44 ± 0.07</td>
<td>34</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**In Vivo Studies**

**Carrageenan-Induced Rat Paw Edema Assay.** The administration of rofecoxib 1 h before injection of carrageenan inhibited the edema response dose-dependently with an ID<sub>50</sub> value of 1.5 ± 0.1 mg/kg (Fig. 3A). In the control group, the paw volume increased by 1.1 ± 0.02 ml (n = 30) 3 h after injection of carrageenan. The potency of rofecoxib was comparable to that of indomethacin (ID<sub>50</sub> = 2.0 ± 0.2 mg/kg).

**Endotoxin-Induced Pyresis in Rats.** The injection of LPS (0.36 mg/kg i.p.) resulted in an increase of 2.26 ± 0.11°C (n = 15) in rectal temperature 7 h postinjection (compared with the saline-injected group). The administration of rofecoxib at the plateau of temperature elevation (5 h) reversed the LPS-induced pyrexia in a dose-dependent manner (ID<sub>50</sub> = 0.24 ± 0.07 mg/kg, Fig. 3B). Rofecoxib was about 5-fold more potent than indomethacin (ID<sub>50</sub> = 1.07 ± 0.16 mg/kg).

**Carrageenan-Induced Rat Paw Hyperalgesia.** Intraplantar injection of carrageenan (4.5 mg) induced marked paw edema and hyperalgesia to mechanical compression of the inflamed hind paw. Oral administration of rofecoxib (ID<sub>50</sub> = 1.0 mg/kg) or indomethacin (ID<sub>50</sub> = 1.5 mg/kg) 1 h before the test reversed the carrageenan-induced hyperalgesia dose-dependently (Fig. 3C).

**AIA in Rats.** Rofecoxib significantly reduced paw swelling, thymus weights, and radiographic total scores in rats with AIA. The efficacy of rofecoxib was similar to that produced by an effective dose of indomethacin (1 mg/kg/day). Rofecoxib reduced the secondary paw volume with an ID<sub>50</sub> value of 0.7 mg/kg/day (Fig. 4). Indomethacin (1 mg/kg/day) inhibited the paw swelling by 82% in this experiment. The total radiographic scores of the secondary paw in the vehicle-treated, adjuvant-injected animals were significantly greater than that in the control, nonadjuvant-injected animals. Animals that received rofecoxib at 1 and 3 mg/kg/day or indomethacin at 1 mg/kg/day showed a significant attenuation of radiographic changes at both days 14 and 21 (Fig. 5). At the end of the study, a general necropsy was performed, and abdominal, peritoneal, and thoracic cavities were normal in all rats.

**Ex Vivo Whole Blood Assay in Anesthetized Dogs.** Infusion of vehicle had no significant effect on the ex vivo biosynthesis of LPS-stimulated dog whole blood over the 4-h period of the experiment. In contrast, diclofenac infused at 2.5 μg/kg/min reduced the ex vivo biosynthesis of PGE<sub>2</sub> in LPS-stimulated dog whole blood (4 h) to 12% of predose values (88 ± 2% inhibition; Fig. 6). Rofecoxib was also active...
against ex vivo PGE2 formation of LPS-stimulated dog whole blood, showing dose-dependent inhibition of 37, 64, and 75% after 4-h infusion with 0.8, 2.5, and 8 mg/kg/min, respectively.

**Gastrointestinal Studies in Rats.** Figure 7 shows that acute dosing of diclofenac or indomethacin at 10 mg/kg caused a significant increase in fecal 51Cr excretion in a 48-h period after the injection of 51Cr-labeled red blood cells in rats. In contrast, rofecoxib at 100 mg/kg was without effect. In chronic dosing studies, administration of diclofenac at 3 mg/kg b.i.d. for 5 days resulted in a significant increase in fecal 51Cr excretion. In chronic dosing studies with indomethacin at 3 mg/kg, one of five animals died of gastrointestinal side effects after 4 days of dosing. The remaining four animals showed overt clinical symptoms (loss of appetite, loss of body weight, constipation, jaundice), and 51Cr excretion experiments could not be performed. In contrast, oral dosing of rofecoxib at 100 mg/kg b.i.d. for 5 days had no effect on fecal 51Cr excretion. In a separate study, oral dosing of rofecoxib at 300 mg/kg/day for 2 weeks did not produce gastrointestinal lesions, whereas it has been reported previously that a single oral dose of indomethacin, flurbiprofen, or piroxicam at 3 to 10 mg/kg produced clear visible gastric lesions (Chan et al., 1995).

**Gastrointestinal Studies in Squirrel Monkey.** Figure 8 shows that chronic oral dosing of diclofenac (1 mg/kg b.i.d. for 4 days) or naproxen (5 mg/kg b.i.d. for 5 days) resulted in a significant enhancement in fecal 51Cr excretion. In comparison, rofecoxib at 100 mg/kg b.i.d. for 5 days administered in either 1% methocellulose or 5% Tween 80 vehicle had no significant effect compared with the vehicle control group. Neither rofecoxib nor diclofenac had any effect on 24-h fecal mass; however, naproxen at 5 mg/kg significantly increased 24-h fecal mass compared with the control group.

**Discussion**

**Potency and Selectivity of COX-2 Inhibition.** The present study demonstrates that rofecoxib is a highly selective inhibitor of COX-2 in a number of in vitro assays. The mechanism of inhibition of COX activity by rofecoxib is very similar to those reported with the COX-2-selective inhibitors DuP-697, NS-398, and DFU (Copeland et al., 1994; Ouellet and Percival, 1995; Riendeau et al., 1997b) and is consistent with the inhibition of COX-2 by rofecoxib occurring via a two-step time-dependent mechanism leading to the formation of a tightly bound inhibited complex. In contrast, the weak inhibition of COX-1 by rofecoxib is competitive and non-time-dependent. These results contrast with potent non-selective COX inhibitors such as indomethacin and flurbiprofen, which are time-dependent inhibitors of both isoforms.

Rofecoxib caused little or no inhibition of prostanooid synthesis (IC50 > 15–50 μM) in several COX-1-mediated in vitro assays. A lower IC50 value of 2 μM was obtained in the U937
microsomal assay in which the accumulation of PGE$_2$ is measured after incubation with a low, subsaturating concentration of the arachidonic acid substrate. This assay has been reported to give IC$_{50}$ values that correlate with those of other COX-1 assays, but it has been reported to be more sensitive to inhibition. For comparison, the IC$_{50}$ value of rofecoxib is similar to that of L-745,337 (IC$_{50}$ = 2.8 μM) but much higher that those of NS-398 (IC$_{50}$ = 0.3 μM), meloxicam (IC$_{50}$ = 0.14 μM), DuP 697 (IC$_{50}$ = 7 nM), or celecoxib (IC$_{50}$ = 52 nM; Riendeau et al., 1997a). These results indicate that rofecoxib shows a selectivity for the inhibition of COX-2 in vitro even under conditions of limiting arachidonic acid availability favoring the inhibition of COX-1 (i.e., IC$_{50}$ = 2 μM for rofecoxib against microsomal COX-1 at low arachidonic acid concentration versus 0.1–0.4 μM for COX-2 at saturating arachidonic acid).

The human whole blood COX-1 and COX-2 assays provide an additional and a more relevant measure of COX-2 inhibition selectivity under a pathophysiological environment rich in plasma protein and cells (Patrignani et al., 1994; Brideau et al., 1996). Other variations of the human whole blood/cell assays have been reported and have been used to show the efficacy of selective and specific COX-2 inhibitors (Grossman et al., 1995; Young et al., 1996). In the present study, using the ratio of the COX-1 IC$_{50}$ value over the COX-2 IC$_{50}$ value as an index for selectivity, the following COX-2 selectivity ratio was obtained: rofecoxib > celecoxib > meloxicam > diclofenac > indomethacin, showing that rofecoxib has the highest COX-2 selectivity when tested under the same experimental condition.

Rofecoxib inhibited dose-dependently the LPS-stimulated-, COX-2-derived PGE$_2$ synthesis in human whole blood in single or multiple oral dosing studies (Ehrich et al., 1996, 1999; Depre et al., 1998). COX-1-derived serum TBX$_2$ in clotting whole blood was not inhibited even at doses of up to 1000 mg. This is in contrast to indomethacin, which inhibited both TBX$_2$ generation and LPS-stimulated PGE$_2$ synthesis at therapeutic doses (Ehrich et al., 1999). The COX-2 specificity of rofecoxib was also demonstrated in a human gastric biopsy study in which COX-1-derived gastric PGE$_2$ or PGF$_{2α}$ was inhibited by indomethacin (10 μM) but not by rofecoxib (up to 3.3 μM; Cryer et al., 1996). Thus, at the clinical dose range of 12.5 to 25 mg (see below), rofecoxib inhibits only
COX-2, but not COX-1, activities in either human whole blood or gastric tissues, fulfilling the requirement for a COX-2-specific agent (Brooks et al., 1999) and in agreement with the results of the preclinical studies.

**Anti-inflammatory, Analgesic, and Antipyretic Effects.** In established rodent models of acute and chronic inflammation, pain, and fever, rofecoxib was as effective as conventional NSAIDs with ID₅₀ values ranging from 0.7 to 1.5 mg/kg. In a separate study using a nonhuman primate model of pyresis (Chan et al., 1997), the antipyretic effectiveness of rofecoxib was also demonstrated (Schwartz et al., 1999). It should be mentioned that the plasma levels of rofecoxib at the effective dose range of 1 to 3 mg/kg (<1 μM, Wang et al., unpublished observation) are below the level required to inhibit COX-1. Thus, in accord with previous data reported for other selective COX-2 inhibitors (Chan et al., 1995; Riendeau et al., 1997b), inhibition of COX-2 alone is sufficient to achieve anti-inflammatory, analgesic, and antipyretic effects in preclinical models.

In a chronic, and more severe, inflammatory model such as the AIA in rats, rofecoxib was effective in reducing paw swelling and thymus weight with efficacy comparable to that of indomethacin. In particular, rofecoxib had a protective effect on adjuvant-induced destruction of cartilage and bone structures, as measured by a composite radiographic score, suggesting that rofecoxib has a beneficial effect on prevention of loss of function. A similar observation has been reported with a COX-2 inhibitor, SC-58125, in this model (Anderson et al., 1996).

In a dental pain analgesia study, rofecoxib at single doses of 50 or 500 mg demonstrated full analgesic activity, equivalent to that observed with ibuprofen (400 mg) (Ehrich et al., 1999). As well, rofecoxib (12.5 or 25 mg) significantly reduced oral temperature with efficacy comparable to that of ibuprofen (400 mg) in patients with fever (Schwartz et al., 1999). In a 6-week study in patients with knee and hip osteoarthritis, significant efficacy was observed with rofecoxib (up to 50 mg) on the basis of each of three primary endpoints (Ehrich et al., 1998a) and health-related quality of life assessment (Ehrich et al., 1998b). A 6-month extension study was initiated, and clinical improvement was sustained in the rofecoxib treatment groups throughout the study period, with efficacy comparable to that of diclofenac (Ehrich et al., 1998a). In contrast to NSAIDs, however, rofecoxib did not inhibit human whole blood COX-1 activities even at doses more than 10-fold higher (i.e., 375 mg) than the efficacious doses of 12.5 to 25 mg (Deppe et al., 1998). These studies clearly demonstrate that clinical efficacy can be achieved with rofecoxib at doses that inhibit only COX-2, consistent with preclinical results and in accord with the COX-2 hypothesis.

**Gastrointestinal Tolerability.** It has been shown unequivocally and consistently in experimental models that COX-2 inhibitors do not cause gastric lesions even at above effective anti-inflammatory doses (Putaki et al., 1993; Seibert et al., 1994; Chan et al., 1995; Riendeau et al., 1997b), in stark contrast to NSAIDs, which induce gastrointestinal lesions after a single acute dose. Rofecoxib did not produce any gastric or intestinal lesions after 300 mg/kg/day in a 2-week oral study in rats. This is echoed by a gastrointestinal integrity study using ⁵¹Cr-labeled red blood cells as permeability markers. Rofecoxib had no effect at 200 mg/kg/day for 5 days whereas a 2- to 3-fold increase in fecal ⁵¹Cr excretion was observed with indomethacin at a single dose of 3 mg/kg. Similar gastrointestinal sparing effects were observed with rofecoxib in squirrel monkeys (Chan et al., 1995; Riendeau et al., 1997b). Using the ED₅₀ values in the rat paw edema assay and the ulcerogenic dose in the rodent study as measures for efficacy and ulcerogenicity, therapeutic index values of more than 200 and 0.7 were obtained for rofecoxib and indomethacin, respectively.

In a 7-day endoscopy study of the stomach and duodenum in human subjects, the effect of rofecoxib at a dose (250 mg) of more than 10-fold the clinically effective dose for the treatment of osteoarthritis was statistically indistinguishable from the placebo control (Lanza et al., 1997), compared with aspirin or ibuprofen, which produced significant mucosal damage at their clinical doses. This is supported by two gastrointestinal integrity studies using well-documented ⁵¹Cr permeability markers similar to those used in the preclinical models mentioned above. Rofecoxib at 25 or 50 mg, a dose two to four times the clinical dose, had no effect compared with placebo on the urinary recovery of ⁵¹Cr-EDTA in a 7-day study in human subjects (Bjarnason et al., 1998). Similarly, rofecoxib did not affect ⁵¹Cr-labeled fecal blood loss compared with placebo in a 4-week study (Hunt et al., 1998). In these studies, indomethacin or ibuprofen produced statistically significant effect compared with both placebo and rofecoxib. More importantly, rofecoxib was well tolerated in patients with osteoarthritis in a 6-month efficacy study, with no report of clinically important gastrointestinal complications such as upper gastrointestinal bleeding or ulceration (Ehrich et al., 1998a). This contrasts strongly with current NSAIDs, in which case the major cause of withdrawal is gastrointestinal complications.

**Conclusion.** The present study has clearly demonstrated that rofecoxib has equivalent anti-inflammatory, analgesic, and antipyretic activities compared with current NSAIDs in preclinical animal models, while having a substantially different gastrointestinal side effect profile. This is consistent with the clinical data available for rofecoxib when compared with standard NSAIDs. In both preclinical and clinical studies, highly selective COX-2 inhibitors, such as rofecoxib, are distinct from current NSAIDs with respect to their pharmacological profiles. Thus, highly selective COX-2 inhibitors can be classified as a new class of therapeutic agent for the treatment of acute and chronic inflammatory conditions, with a well-defined molecular and biochemical target.

**Acknowledgments**

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**References**


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