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Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor

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1 DFU (5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone) was identified as a novel orally active and highly selective cyclo-oxygenase-2 (COX-2) inhibitor.

2 In CHO cells stably transfected with human COX isozymes, DFU inhibited the arachidonic acid-dependent production of prostaglandin E_2 (PGE_2) with at least a 1,000 fold selectivity for COX-2 ($IC_{50} = 41 \pm 14$ nM) over COX-1 ($IC_{50} > 50$ μM). Indomethacin was a potent inhibitor of both COX-1 ($IC_{50} = 18 \pm 3$ nM) and COX-2 ($IC_{50} = 26 \pm 6$ nM) under the same assay conditions. The large increase in selectivity of DFU over indomethacin was also observed in COX-1 mediated production of thromboxane B_2 (TXB_2) by Ca^{2+} ionophore-challenged human platelets ($IC_{50} > 50$ μM and 4.1 ± 1.7 nM, respectively).

3 DFU caused a time-dependent inhibition of purified recombinant human COX-2 with a K_i value of 140 ± 68 μM for the initial reversible binding to enzyme and a k_2 value of 0.11 ± 0.06 s^{-1} for the first order rate constant for formation of a tightly bound enzyme-inhibitor complex. Comparable values of 62 ± 26 μM and 0.06 ± 0.01 s^{-1}, respectively, were obtained for indomethacin. The enzyme-inhibitor complex was found to have a 1 : 1 stoichiometry and to dissociate only very slowly ($t_{1/2} = 1-3$ h) with recovery of intact inhibitor and active enzyme. The time-dependent inhibition by DFU was decreased by co-incubation with arachidonic acid under non-turnover conditions, consistent with reversible competitive inhibition at the COX active site.

4 Inhibition of purified recombinant human COX-1 by DFU was very weak and observed only at low concentrations of substrate ($IC_{50} = 63 \pm 5$ μM at 0.1 μM arachidonic acid). In contrast to COX-2, inhibition was time-independent and rapidly reversible. These data are consistent with a reversible competitive inhibition of COX-1.

5 DFU inhibited lipopolysaccharide (LPS)-induced PGE_2 production (COX-2) in a human whole blood assay with a potency ($IC_{50} = 0.28 \pm 0.04$ μM) similar to indomethacin ($IC_{50} = 0.68 \pm 0.17$ μM). In contrast, DFU was at least 500 times less potent ($IC_{50} > 97$ μM) than indomethacin at inhibiting coagulation-induced TXB_2 production (COX-1) ($IC_{50} = 0.19 \pm 0.02$ μM).

6 In a sensitive assay with U937 cell microsomes at a low arachidonic acid concentration (0.1 μM), DFU inhibited COX-1 with an IC_{50} value of 13 ± 2 μM as compared to 20 ± 1 nM for indomethacin. CGP 28238, etodolac and SC-58125 were about 10 times more potent inhibitors of COX-1 than DFU. The order of potency of various inhibitors was diclofenac > indomethacin ~ naproxen > nimesulide ~ meloxicam ~ piroxicam > NS-398 ~ SC-57666 > SC-58125 > CGP 28238 ~ etodolac > L-745,337 > DFU.

7 DFU inhibited dose-dependently both the carrageenan-induced rat paw oedema (ED_{50} of 1.1 mg kg^{-1} vs 2.0 mg kg^{-1} for indomethacin) and hyperalgesia (ED_{50} of 0.95 mg kg^{-1} vs 1.5 mg kg^{-1} for indomethacin). The compound was also effective at reversing LPS-induced pyrexia in rats ($ED_{50} = 0.76$ mg kg^{-1} vs 1.1 mg kg^{-1} for indomethacin).

8 In a sensitive model in which ^{51}Cr faecal excretion was used to assess the integrity of the gastrointestinal tract in rats, no significant effect was detected after oral administration of DFU (100 mg kg^{-1}, b.i.d.) for 5 days, whereas chromium leakage was observed with lower doses of diclofenac (3 mg kg^{-1}), meloxicam (3 mg kg^{-1}) or etodolac (10–30 mg kg^{-1}). A 5 day administration of DFU in squirrel monkeys (100 mg kg^{-1}) did not affect chromium leakage in contrast to diclofenac (1 mg kg^{-1}) or naproxen (5 mg kg^{-1}).

9 The results indicate that COX-1 inhibitory effects can be detected for all selective COX-2 inhibitors tested by use of a sensitive assay at low substrate concentration. The novel inhibitor DFU shows the lowest inhibitory potency against COX-1, a consistent high selectivity of inhibition of COX-2 over COX-1 (>300 fold) with enzyme, whole cell and whole blood assays, with no detectable loss of integrity of the gastrointestinal tract at doses >200 fold higher than efficacious doses in models of inflammation, pyresis and hyperalgesia. These results provide further evidence that prostanoids derived from COX-1 activity are not important in acute inflammatory responses and that a high therapeutic index of anti-inflammatory effect to gastropathy can be achieved with a selective COX-2 inhibitor.

Keywords: Prostaglandins; cyclo-oxygenase; COX-1; COX-2; nonsteroidal anti-inflammatory drugs; inflammation

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Introduction

Prostaglandin synthase catalyses the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) which serves as the common precursor for the synthesis of prostaglandins, prostacyclins and thromboxanes. PGH_2 synthesis by prostaglandin synthase occurs as a two-step reaction, the conversion of arachidonic acid to PGG_2 (cyclo-oxygenase activity) followed by the reduction of the hydroperoxide moiety of PGG_2 to yield PGH_2 (peroxidase activity). The cyclo-oxygenase activity of the enzyme is the site of action of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, ibuprofen and indomethacin which are widely used as anti-inflammatory, analgesic and antipyretic agents (Vane & Botting, 1995; Herschman, 1996; Griswold & Adams, 1996). However, the inhibition of prostanoid biosynthesis has also been associated with the side effects of NSAIDs such as irritation and ulcer formation in the upper gastrointestinal tract (Wallace, 1994) and impairment of kidney function (Murray & Brater, 1993). Gastric and intestinal ulceration and haemorrhage are major complications associated with the chronic use of NSAIDs and can lead to life-threatening situations (Allison *et al*., 1992).

It has been recognized recently that mammalian cells express two forms of cyclo-oxygenase (COX) activity. COX-1 is expressed in many normal tissues and is the major form present in platelets, kidney and gastrointestinal tract (Funk *et al*., 1991; Harris *et al*., 1994; Kargman *et al*., 1996a). COX-2 is induced in response to pro-inflammatory cytokines, lipopolysaccharide (LPS) and growth factors and subjected to repression by glucocorticosteroids. This second form is generally not detected in healthy tissues but is found in elevated levels in inflammatory exudates (Harada *et al*., 1996). These observations have led to the hypothesis that COX-1 is mainly associated with homeostasis and COX-2 with the oedematous, nociceptive and pyretic effects of inflammation.

Most classical NSAIDs, including indomethacin, flurbiprofen and ibuprofen, show little specificity of inhibition towards COX isoforms (Battistini *et al*., 1994). Studies with recently developed compounds, such as NS-398 (Futaki *et al*., 1993a,b; 1994), DuP 697 (Gans *et al*., 1990), L-745,337 (Chan *et al*., 1995), SC-58125 (Seibert *et al*., 1994) and CGP 28238 (Klein *et al*., 1994) have demonstrated that selective COX-2 inhibitors retain the anti-inflammatory effects characteristic of NSAIDs with a marked increased gastrointestinal tolerability as compared to less selective inhibitors. All these observations suggest that COX-1 inhibition is a major contributing factor in NSAID-related toxicity and has prompted the search for the identification of selective COX-2 inhibitors for clinical evaluation. We present here the detailed profile of DFU (Figure 1), a highly selective and orally active inhibitor of COX-2 with no sign of gastrointestinal ulceration at >200 times the dose for anti-inflammatory, analgesic and antipyretic effects.

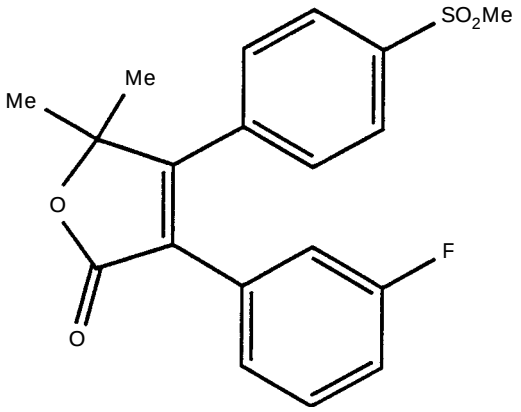

Figure 1 Structure of 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5H)-furanone (DFU).

Methods

Spectrophotometric assay of recombinant human COX-2

Recombinant human COX-1 (Cromlish and Kennedy, unpublished observations) and COX-2 were expressed in Sf-9 cells (Cromlish *et al*., 1994) and purified as previously described (Percival *et al*., 1994). Enzymatic activity was measured by use of a chromogenic assay based on the oxidation of N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2 (Copeland *et al*., 1994). The assay mixture (180 μl) contains 100 mM sodium phosphate, pH 6.5, 1 μM hematin, 1 mg ml^{-1} gelatin, 80–100 units of purified enzyme (one unit of enzyme is defined as the amount of enzyme required to produce an O.D. change of 0.001 min^{-1} at 610 nm) and 4 μl of the test compound in dimethylsulphoxide (DMSO). The mixture was preincubated at room temperature (22°C) for 15 min before initiation of the enzymatic reaction by the addition of 20 μl of a solution of 1 mM arachidonic acid and 1 mM TMPD in assay buffer (without enzyme or hematin). The enzyme activity was measured by estimation of the initial velocity of TMPD oxidation over the first 36 s of the reaction. A non-specific rate of oxidation was observed in the absence of enzyme (0.007–0.01 O.D. min^{-1}) and was subtracted before the calculation of the % inhibition. IC_{50} values were derived from the 4-parameter least squares non-linear regression analysis of the log-dose vs % inhibition plot. For measurement of COX-2 peroxidase activity, the reaction was initiated by the addition of 20 μl of a solution of 4 mM hydrogen peroxide and 0.5 mM TMPD in assay buffer.

Oxygen consumption assay for COX-2 activity

Cyclo-oxygenase activity was monitored continuously by oxygen consumption by a Clark-type polarographic oxygen probe. The oxygen chamber was filled with 0.60 ml of reaction buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 μM hematin, 1 mM phenol, 100 μM arachidonic acid, T = 30° or 37°C) and the reaction was initiated by addition of purified recombinant human COX-2. The enzyme activity was determined from the maximal (initial) velocity of oxygen consumption.

Determination of K_i *and* k_2 *values for the time-dependent inhibition of COX-2*

Purified COX-2 (2.3 μg) was preincubated with inhibitor for 0–15 min in 180 μl of the reaction buffer described above, before the initiation of the reaction with a mixture of arachidonic acid and TMPD. The cyclo-oxygenase activity was determined by the spectrophotometric method as described above. For experiments performed without preincubation of the inhibitor, the reaction was initiated by addition of the assay mixture containing the enzyme to the inhibitor and arachidonic acid/TMPD ethanolic solution. The rate constants (k_{obs}) for the time-dependent loss of activity at each inhibitor concentration were calculated by fitting of the data to a first order equation of the form $y = a + b.\exp(-k_{obs}t)$ by use of Sigmaplot software. Data were analysed in terms of the model developed by Rome and Lands (1975) for the time-dependent inhibition of ovine COX-1. In this model (Scheme 1), an initial reversible binding of enzyme and inhibitor (characterized by the dissociation constant K_i) is followed by a first order inactivation process (characterized by a first order rate constant k_2). The rate of reversal of this process (k_{-2}) is considered to be negligible.

$$E + I \overset{K_i}{\rightleftharpoons} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^* \qquad \text{(Scheme 1)}$$

This model predicts that the observed rate constant (k_{obs}) for the exponential loss of cyclo-oxygenase activity during the

preincubation of enzyme with inhibitor is given by

$$k_{obs} = \frac{k_2[I]}{K_i + [I]}$$

Determination of the stoichiometry of inhibitor binding

Aliquots of purified COX-2 (0.25 mg ml^{-1}, concentration of subunit of 3.4 μM) were incubated in buffer (100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol) in the presence of varying concentrations of inhibitors (0–8 μM) for 15 or 30 min. An aliquot (20 μl) was then removed for determination of the remaining cyclo-oxygenase activity by oxygen uptake as described above. Enzyme concentration was determined by amino acid concentration following acid hydrolysis (Percival *et al*., 1994).

Recovery of inhibitor from the COX-2-inhibitor complex

Purified COX-2 (0.79 nmol) was treated with 1.0 mol equivalent of inhibitor and the mixture was incubated for 60 min at room temperature. The remaining activity at this time was 4% that of a vehicle-treated control. The sample was then divided in two and the protein denatured by treatment with four volumes of ethyl acetate/methanol/1 M citric acid (30 : 4 : 1). After extraction and centrifugation (10,000 $g \times 5$ min), the organic layer was removed and the extraction repeated. The two organic layers were combined and dried under N_2. The extract was dissolved in 100 μl of the high performance liquid chromatography (h.p.l.c.) solvent mixture consisting of water/acetonitrile/acetic acid (50 : 41 : 0.1) and 50 μl were injected onto a Novapak C-18 column (3.9 mm × 150 mm) and developed at 1 ml min^{-1}. The inhibitor was detected by absorption at 260 nm and eluted with a retention time of 6.6 min in this system. Control experiments for inhibitor recovery were performed with incubation of the inhibitor in the absence of enzyme and processing of the samples in an identical fashion before quantitation by h.p.l.c.

Determination of the dissociation rate constant of the enzyme-inhibitor complex

(i) Purified COX-2 (2.0 nmol, 2.0 ml) in 20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 2 μM hematin, 0.1% β-octylglucoside was treated with 2.0 nmol [^{14}C]-DFU (18 Ci mol^{-1}) and incubated at 20°C for 3 h. A control (0.7 ml) was removed and 13 nmol unlabelled DFU was added to the remaining 1.3 ml of the mixture containing COX-2 and [^{14}C]-DFU. At timed intervals, 0.1 ml (in duplicate) was transferred to a Microcon-30 micro concentration device (Amicon) and the free inhibitor separated from enzyme-bound inhibitor by centrifugation at 14,000 × *g* for 6 min at 4°C. Buffer (0.1 ml) was added to the retentate and the centrifugation repeated. The filtrate and retentate were then removed and mixed with 10 ml scintillation fluid and counted in a liquid scintillation counter. All of the added radioactivity (99%) was recovered in the sum of the retentate and filtrate solutions. (ii) An aliquot of purified COX-2 (1.0 nmol) was treated with 1.25 mol equivalents of inhibitor or with DMSO vehicle control and incubated at 20°C for 1 h. The enzyme-inhibitor mixture was then transferred to a Pierce Microdialyzer 100 apparatus and dialysed continuously against 2 l of buffer (20 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.1 mM phenol, 0.1% octylglucoside) for 5 h at 22°C during which aliquots were removed and frozen at −70°C until assayed for cyclo-oxygenase activity by oxygen uptake at 37°C as described above.

Competition of time-dependent inhibition of COX-2 by arachidonic acid

Purified COX-2 (3.6 μg) was diluted into preincubation buffer (0.03 ml, 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 mM phenol) containing 60 mM diethyldithiocarbamic acid to prevent substrate oxygenation (Lands *et al*., 1974) and either 10 μM inhibitor, or 10 μM inhibitor plus 5 μM arachidonic acid, or 10 μM inhibitor plus 30 μM arachidonic acid. After a preincubation period of 0–4 min, the total enzyme was assayed for enzymatic activity by oxygen consumption at 30°C as described above.

H.p.l.c. assay for oxygenation of radiolabelled arachidonic acid by COX-1

Purified recombinant human COX-1 (50 μl of 1 μg ml^{-1} in 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol, 1 μM hematin) was preincubated with 2 μl of the inhibitor solution (50 fold concentrated stock in DMSO, 0–2.5 mM) for 15 min. The reaction was then initiated by the addition of 5 μl of 1 μM [^{14}C]-arachidonic acid (0.005 μCi, NEN-DuPont) to obtain a final concentration of 0.1 μM. After a 7 min incubation at room temperature, the reaction was stopped by the addition of 5 μl 1 M HCl and 50 μl acetonitrile. Aliquots of 50 μl of each reaction mixture were analysed for substrate conversion by reverse phase h.p.l.c. onto a C-18 Nova-Pak column (3.9 × 150 mm) which was developed with acetonitrile/water/acetic acid (85 : 15 : 0.1) at 2 ml min^{-1}. Arachidonic acid metabolites and arachidonic acid eluted at 0.6–1 min and 2.2–2.5 min, respectively, and were quantitated by a Packard Flo-one Radiochromatography detector. Percentages of inhibition were calculated from the difference in conversion of arachidonic acid to prostaglandin metabolites between inhibitor-treated samples and controls exposed to the DMSO vehicle.

Reversibility of inhibition of COX-1

Purified COX-1 (25 μl of 30 μg ml^{-1} in 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol, 1 μM hematin) was incubated with either 1 μl of a solution of 2.5 mM inhibitor in DMSO or with vehicle control. After preincubation periods of 1 to 60 min at room temperature, 1.7 μl of the mixture was diluted into 50 μl of 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenol, 1 μM hematin containing 0.1 μM [^{14}C]-arachidonic acid (0.005 μCi). The reaction was stopped after 7 min and 80 μl was analysed by h.p.l.c. for the conversion of [^{14}C]-arachidonic acid as described above.

Intact cell assays with transfected CHO cells expressing COX-1 and COX-2

Stably transfected Chinese hamster ovarian (CHO) cells expressing human COX-1 and COX-2 were cultured and assayed for the production of PGE_2 following stimulation by arachidonic acid as previously described (Kargman *et al*., 1996b). Cells (0.3×10^6 cells in 200 μl) were preincubated in Hank's Balanced Salts Solution (HBSS) containing 15 mM HEPES, pH 7.4, with 3 μl 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 in HBSS) to yield final concentrations of 10 μM arachidonic acid in the CHO[COX-2] assay and of 0.5 μM arachidonic acid in the CHO[COX-1] assay. In the absence of exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-1] and CHO[COX-2] cells were <80 pg PGE_2 per 10^6 cells. In the presence of 0.5 μM exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-1] cells increased to 300–2300 pg PGE_2 per 10^6 cells, whereas in the presence of 10 μM exogenous arachidonic acid, levels of PGE_2 in samples from CHO[COX-2] cells increased to 500–1400 pg PGE_2 per 10^6 cells. Cyclo-oxygenase activity in the absence of test compounds is determined as the difference in PGE_2 levels of cells challenged with arachidonic acid versus the PGE_2 levels in cells mock-challenged with ethanol vehicle. Each experiment included a set of 8 positive and negative control samples (±arachidonic acid challenge) for cells preincubated in the absence of inhibitor. Compounds were typically tested at 8 concentrations in duplicate with 3 fold serial dilutions in

DMSO. Inhibition of PGE_2 synthesis by test compounds is calculated as a percentage of the activity in the presence of drug versus the activity in the positive control samples.

Reversibility of inhibition of PGE_2 production by CHO[COX-2] cells

CHO cells transfected with COX-2 were grown to confluency, harvested following incubation with 0.25% trypsin/0.1% EDTA (JRH Biosciences) and washed with Hanks balanced salt solution buffered with 15 mM HEPES (pH 6.9) and containing 50 μM cycloheximide. The cells (1.5×10^6 cells ml^{-1}, total volume of 8 ml) were preincubated with 0.15 μM DFU (from a 1,000 fold concentrated solution in DMSO) or vehicle control for 15 min. Three 0.5 ml aliquots were removed from each incubation mixture and assayed for arachidonic acid-dependent production of PGE_2. The remaining aliquots of 6.5 ml cells were spun at $300 \times g$ for 8 min at 19°C. The supernatant was removed and the cell pellet was then resuspended in 25 ml HHBSS containing cycloheximide, washed twice, and resuspended in 6.5 ml of the buffer. The cell suspension was divided into 0.5 ml aliquots and incubated at 37°C until assayed for the recovery of cellular COX-2 activity as described above.

TXB_2 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 4×10^7 cells ml^{-1} (0.2–0.25 ml) in the absence or presence of the inhibitor (from a 125 fold concentrated solution in DMSO) for 15 min before stimulation with 2 μM calcium ionophore A23187. After a further 10 min incubation at 37°C, cold methanol (50% by volume) was added to stop the reaction and TXB_2 levels were measured by enzyme immunoassay (Assay Designs, Inc.). Inhibitors were tested at 8 concentrations using 3 fold serial dilutions of the highest drug concentration. Human platelets released 13–20 ng of TXB_2 per 10^7 cells following challenge with the calcium ionophore (A23187) and 4–10 ng TXB_2 per 10^7 cells when stimulated with 1 μM arachidonic acid. Less than 5% of the production of TXB_2 was observed in the absence of ionophore challenge.

Inhibition of COX-1 from U937 cells microsomes

U937 cells were resuspended in 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA, 2 μg ml^{-1} leupeptin, 2 μg ml^{-1} soybean trypsin inhibitor, 2 μg ml^{-1} aprotinin and 1 mM phenylmethylsulphonyl fluoride. The cell suspension was sonicated 4 times for 10 s (Cole Parmer Ultrasonic Homogenizer 4710, output control: 3.8; 70% duty cycle) and then centrifuged at $10{,}000 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $100{,}000 \times g$ for 1 h at 4°C. The remaining pellet was resuspended in 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA and protein concentration determined by use of the Bio-Rad Coomassie protein stain. Aliquots of microsomal preparations (7–20 mg of protein ml^{-1}) were stored at −80°C. Immediately before use, microsomal preparations were thawed, subjected to a brief sonication, and then diluted to a protein concentration of 125 μg ml^{-1} in 0.1 M Tris-HCl, 10 mM EDTA, pH 7.4 containing 0.5 mM phenol, 1 mM reduced glutathione, and 1 μM hematin. Compounds were tested at 8 concentrations in duplicate with 3 fold serial dilutions in DMSO of the highest drug concentration. A 5 μl sample of test compound or DMSO vehicle was added to 20 μl of 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA in a 96-well polypropylene minitube place (Beckman) and mixed with 200 μl of the microsomal suspension. After an incubation for 15 min at room temperature, 25 μl of a solution of 1 μM arachidonic acid (peroxide-free, Cayman Chemical Co.) in 0.1 M Tris-HCl, pH 7.4, 10 mM EDTA was then added to give a final concentration of arachidonic acid of 0.1 μM. The samples were mixed and incubated at room temperature for 40 min. Control samples contained ethanol vehicle instead of arachidonic acid. Following the incubation period, the reaction was terminated by the addition of 25 μl of 1 N HCl. Samples were neutralized by the addition of 25 μl of 1 N NaOH before analysis for PGE_2 levels by radioimmunoassay (NEN-Dupont or Amersham). Cyclo-oxygenase activity in the absence of test compounds is defined as the difference between PGE_2 levels in samples incubated in the presence of arachidonic acid versus ethanol vehicle.

COX-2 human whole blood assay (LPS-induced PGE_2 production)

The human COX-2 and COX-1 whole blood assays were performed with essentially the same procedures as described previously (Brideau *et al*., 1996), the subjects used had no apparent inflammatory conditions and had not taken any NSAIDs for at least 7 days before the blood collection. Aliquots of 500 μl of blood were incubated with either 2 μl vehicle (DMSO) or 2 μl of a test compound at final concentrations ranging from 40 nM to 30 μM for 15 min before the addition of 10 μl LPS in PBS (100 μg ml^{-1} final concentration) for 24 h at 37°C for induction of COX-2. At the end of the incubation, the blood was centrifuged at $12{,}000 \times g$ for 5 min to obtain plasma. Following protein precipitation with absolute methanol (1 : 4), the supernatant was obtained and was assayed for PGE_2 by radioimmunoassay (Amersham).

COX-1 human whole blood assay (coagulation-induced TXB_2 production)

Fresh blood was collected into vacutainers containing no anticoagulants. Aliquots of 500 μl were immediately transferred to siliconized microcentrifuge tubes preloaded with 2 μl of either DMSO or a test compound at final concentrations ranging from 1 to 97 μM. The tubes were vortexed and incubated at 37°C for 1 h to allow the blood to clot. At the end of the incubation, the serum was obtained by centrifugation ($12{,}000 \times g$ for 5 min), proteins were precipitated with absolute methanol (1 : 4), and the supernatant was assayed for TXB_2 by use of an enzyme immunoassay kit (Cayman Chemical).

In vivo *experiments*

All *in vivo* experimental procedures were approved by the Animal Care Committee at the Merck Frosst Centre for Therapeutic Research (Canada) and at the Merck, Sharp and Dohme Neuroscience Centre (UK) according to guidelines established by the Canadian Council on Animal Care and the British Home Office, respectively. These procedures were essentially the same as those described previously (Chan *et al*., 1995). They are briefly described below. In all cases, the test compound was administered orally in 1% methocellulose suspension.

Carrageenan-induced rat paw oedema Male Sprague-Dawley rats (150–200 g) were fasted for 16–18 h before the oral administration of either the vehicle (1% methocel) or a test compound. One hour later, the paw volume was measured by a water displacement plethysmometer (Ugo-Basile, Italy). The animals were then injected intraplantarly with 50 μl of a 1% carrageenan solution in saline (i.e. 500 μg carrageenan per paw). Three hours later, the paw volume was measured again and the increases in paw volume from time zero were calculated. Paw oedema was compared with the vehicle-control group and the % inhibition calculated taking the values in the control group as 0%. A dose that gave 50% inhibition (ID_{50}) was calculated. All treatment groups were coded to eliminate bias from the observer.

Carrageenan-induced rat paw hyperalgesia Hyperalgesia to mechanical compression of the hind paw of male Sprague-

Dawley rats (90–110 g) was induced by intraplantar injection of carrageenan (4.5 mg per paw) 3 h previously. A test compound was given orally 2 h after carrageenan. The vocalization response to compression of the carrageenan-injected paw was measured 1 h later by an algesiometer (Ugo-Basile, Italy).

Endotoxin-induced pyretic responses in rats Male Sprague-Dawley rats (150–200 g) were deprived of food for 16–18 h before use. At approximately 9 h 30 min, the animals were placed temporarily in plexiglass restrainers and their baseline rectal temperatures were recorded with a flexible temperature probe (YSI series 400) connected to a digital thermometer (Model 08502, Cole Parmer). At time zero, the rats were injected intraperitoneally with either saline or LPS (360 mg kg^{-1}) and the rectal temperatures were measured again at 5, 6 and 7 h following LPS injection. A test compound was administered orally 5 h after LPS injection (when the rectal temperature increased by approximately 2°C) to determine whether the compound could reverse the pyretic responses. % reversal of the pyrexia was calculated by use of the rectal temperature obtained at 7 h in the control group as the reference (zero reversal) point. Complete reversal of pyrexia to the pre-LPS baseline value is taken as 100%.

^{51}Cr faecal excretion in rats Male Sprague-Dawley rats (150–200 g) were dosed orally with a test compound either once (acute dosing) or twice daily for 5 days (chronic dosing). A plasma sample was obtained 1 h after the morning dose on day 4 for measurement of drug concentration. Immediately after the administration of the last dose on day 5, the rats were injected via a tail vein with 0.5 ml of ^{51}Cr-labelled red blood cells from a donor rat after incubation with sodium ^{51}chromate. The rats were placed individually in metabolism cages with food and water *ad libitum*. Faeces were collected for a 48 h period and ^{51}Cr faecal excretion was calculated as a % of total injected dose (20 μCi per animal).

^{51}Cr faecal excretion in squirrel monkeys Squirrel monkeys (*Saimiri sciureus*; 0.8–1.4 kg) were dosed orally with a test compound twice daily for 1–5 days. One hour after administration of the last dose, $^{51}CrCl_3$ in sterile saline (1 ml kg^{-1}, 4–5 μCi per animal) was injected via a saphenous vein and plasma samples were obtained for measurement of drug concentration. The monkeys were then housed individually in metabolism cages. Faeces were collected for a 24 h period and ^{51}Cr faecal excretion was calculated as a % of total injected dose.

Determination of plasma levels of DFU

Plasma levels of DFU were determined by reverse phase h.p.l.c. on a 4 μm Nova Pak C18 column (3.9 × 150 mm, Waters, Milford, MA) following extraction with an equal volume of acetonitrile. The solvent system was absolute methanol/10 mM phosphate buffer, pH 7.0 (45 : 55) at a flow rate of 1 ml min^{-1}, samples were monitored at 275 nm.

Materials

Diclofenac, ketoprofen, nimesulide, piroxicam, naproxen, etodolac, ibuprofen and LPS were obtained from Sigma Chemicals. Indomethacin and flurbiprofen were purchased from Cayman Chemicals. Sulindac sulfide, NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl)methanesulphonamide), DuP 697 (5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulphonyl) phenyl] thiophene, CGP 28238 (6-(2,4-difluorophenoxy)-5-methylsulphonamido-1-indanone), meloxicam, 6-MNA (6-methoxy-2-naphthaleneacetic acid), SC-58125 (5-(4-fluorophenyl)-1-[(4-methylsulfonyl)phenyl]-3-trifluoromethylpyrazole) (Seibert *et al*., 1994) SC-57666 (1-[2-(4-fluorophenyl)cyclopenten-1-yl]-4-(methylsulphonyl)benzene) (Reitz *et al*., 1994), L-745,337 (6-(2,4-difluorophenylthio)-5-methanesulphonamido-1-indanone) (Chan *et al*., 1995) and DFU and [^{14}C]-DFU were synthesized in the Department of Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research, Canada.

Statistical and data analysis

Results are expressed as mean ± s.e.mean. Differences between vehicle control and treatment groups were tested by one-way analysis of variance (ANOVA) followed by Dunnett's test. A P value less than 0.05 was considered statistically significant.

Results

Selective inhibition of COX-2 by DFU in intact cell assays

The potency of DFU as an inhibitor of cellular cyclo-oxygenase activity was evaluated with arachidonic acid-stimulated production of PGE_2 by CHO cells expressing human COX-1 and COX-2. Figure 2 shows a comparison of the dose-dependent inhibition of PGE_2 production by cells that were pretreated with various amounts of DFU, indomethacin, DuP 697 and piroxicam. DFU was a potent inhibitor of PGE_2 production by CHO[COX-2] cells with an IC_{50} value (41 ± 14 nM) similar to that of indomethacin (26 ± 6 nM) and piroxicam (35 ± 7 nM). When tested for the inhibition of PGE_2 production by CHO[COX-1] cells, indomethacin was found to be non-selective (IC_{50} = 18 ± 3 nM) whereas piroxicam was about a 100 fold less potent at inhibiting COX-1 (IC_{50} = 3.5 ± 1.0 μM). DuP 697 was a more potent inhibitor of COX-2 (IC_{50} = 2.1 ± 0.9 nM) and exhibited only a 30 fold se-

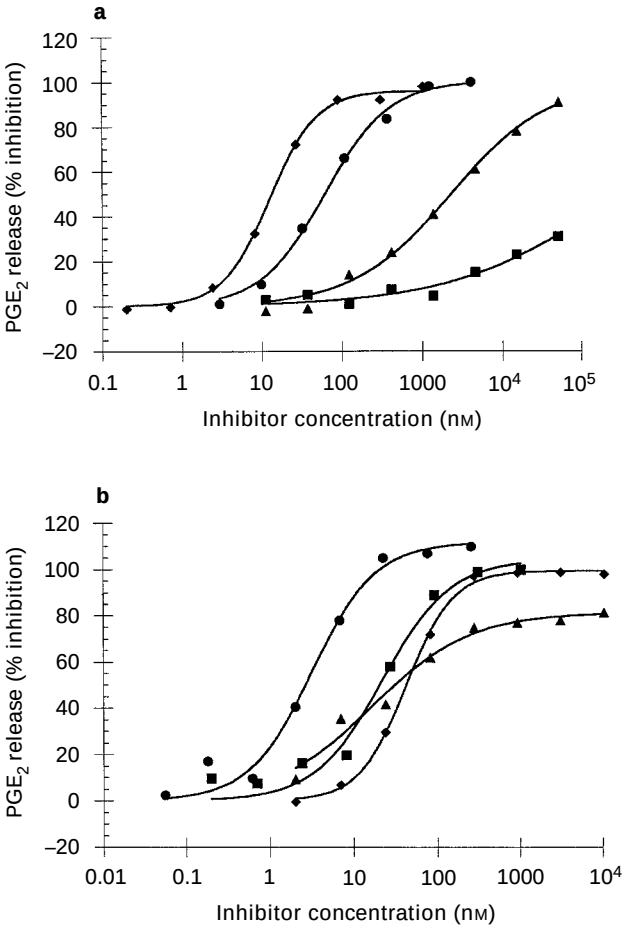

Figure 2 Effect of DFU, indomethacin, piroxicam and DuP 697 on the production of PGE_2 by arachidonic acid stimulated CHO cells stably transfected with human (a) COX-1 or (b) COX-2. Cells were preincubated for 15 min with indomethacin (◆), DuP 697 (●), piroxicam (▲) or DFU (■) before stimulation with (a) 0.5 μM (COX-1) or (b) 10 μM (COX-2) arachidonic acid.

lectivity ($IC_{50} = 59 \pm 14$ nM for COX-1). At least a 1000 fold selectivity was observed for DFU in these assays with an IC_{50} value >50 μM in the CHO[COX-1] cell assay.

Mechanism of inhibition of purified recombinant human COX-2 by DFU

The kinetic mechanism of the inhibition of COX-2 by DFU was investigated with purified recombinant human enzyme. Preincubation of the enzyme with various concentrations of DFU for 15 min before arachidonic acid addition resulted in a dose-dependent inhibition of activity with an IC_{50} value similar to that of indomethacin (IC_{50} values of 0.31 and 0.92 μM, respectively). The level of inhibition of COX-2 by DFU was dependent upon the length of preincubation of enzyme and inhibitor before the addition of substrate (Figure 3). In the absence of a preincubation, no inhibition of the cyclo-oxygenase reaction rate was observed at the concentrations of DFU up to 100 μM. When COX-2 was preincubated with DFU the level of activity was reduced in a time-dependent manner, a higher inhibitor concentration resulting in a faster loss of activity as illustrated in Figure 3a for the curves obtained at 12 and 100 μM DFU. The inhibition of PGE_2 production by DFU in CHO[COX-2] cells was also time-dependent with IC_{50} values of >10 μM, 0.56 μM, 0.23 μM and 0.05 μM obtained with preincubation periods of 0, 1, 3 and 15 min, respectively (data not shown). The decrease in activity follows first order kinetics and was analysed according to a model (Scheme 1) where inhibition results from reversible binding of enzyme and inhibitor (characterized by K_i) followed by a first order inactivation process (characterized by k_2). The rate of reversal of this process (k_{-2}) is considered to be negligible on the timescale of the experiment. Average values of K_i and k_2 of 140 ± 68 μM and 0.11 ± 0.06 s^{-1} ($n=6$) were obtained by nonlinear regression analysis of the data (Figure 3b). As a comparison, the values for indomethacin were 62 ± 26 μM and 0.06 ± 0.01 s^{-1} ($n=2$). The inhibition by DFU is specific for the cyclo-oxygenase activity of COX-2 as no inhibition of H_2O_2-dependent peroxidase activity was observed at the highest concentration tested (100 μM).

The stoichiometry of the tightly bound enzyme-inhibitor complex (EI*, Scheme 1) was determined by a titration in which aliquots of purified COX-2 (3.4 μM) were treated with 0–8 μM DFU and the remaining cyclo-oxygenase activity measured after a 15 or 30 min preincubation period. A linear decrease in enzyme activity with increasing inhibitor concentration was obtained with the break point in the titration occurring at a concentration of 4.3 μM DFU (Figure 4). No increase in inhibition was evident at the longer preincubation time. This result is therefore consistent with the formation of an EI* complex having 1 : 1 stoichiometry. The results also show that for the EI* complex a residual activity of approximately 9% of the uninhibited enzyme was retained in the presence of an excess of DFU.

In order to determine if any inhibitor degradation or covalent modification of COX-2 occurs during the time-dependent inhibition by DFU, a study was performed to determine whether intact DFU could be recovered from the enzyme-inhibitor complex. Purified COX-2 was treated with one equivalent of DFU and incubated for 60 min. At this time the enzyme cyclo-oxygenase activity was approximately 4% of a vehicle-treated control. The enzyme-inhibitor complex was then denatured by treatment with acidified ethyl acetate/methanol and any released drug extracted from the protein. H.p.l.c. of the extract showed a single peak with the same retention time and u.v. spectrum as authentic DFU (data not shown). Comparison with an identically treated sample of DFU in the absence of enzyme indicated that the recovery of DFU was quantitative. These results are therefore consistent with the non-covalent nature of the enzyme-inhibitor complex.

The inhibition of COX-2 by a structurally related compound, DuP 697, was considered to be irreversible as no enzyme activity was recovered after a 5 h dialysis of the enzyme-inhibitor complex (Copeland *et al*., 1994). The reversibility of the complex between DFU and COX-2 was determined in two

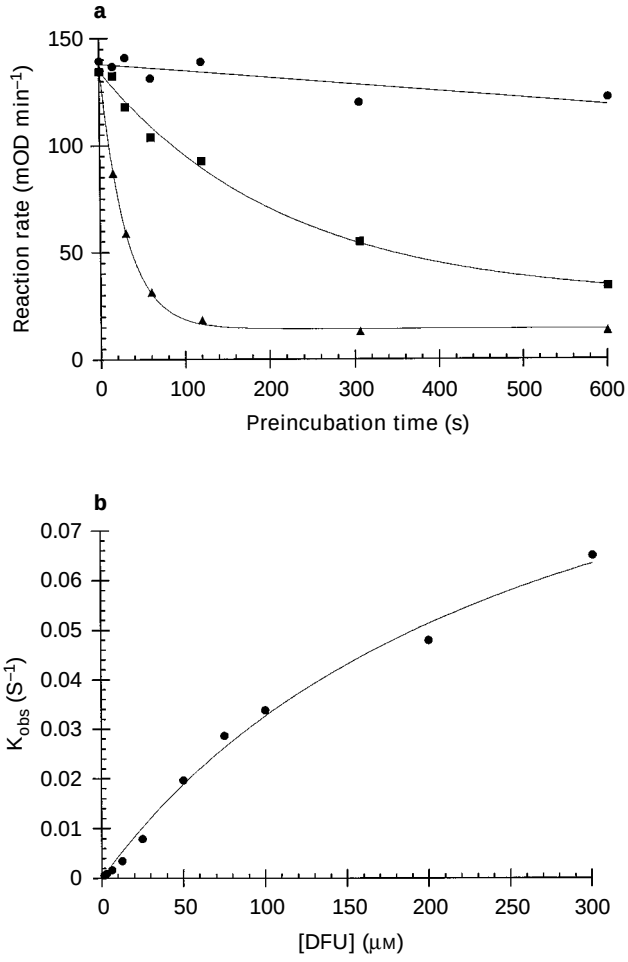

Figure 3 Time-dependent inhibition of purified recombinant human COX-2 by DFU. (a) The enzyme was preincubated for different periods of time without (●), or with 12.5 μM (■) or 100 μM (▲) DFU before initiation of the reaction with arachidonic acid. (b) Increase in the observed first order rate constant (k_{obs}) for the formation of the tight enzyme-DFU complex as function of inhibitor concentration.

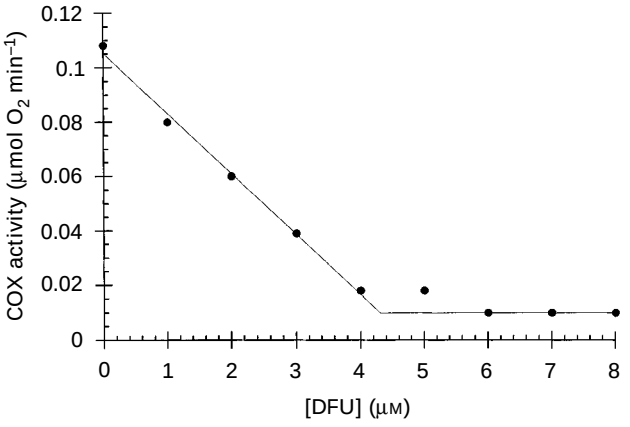

Figure 4 Determination of inhibitor stoichiometry of purified COX-2 with DFU. Aliquots of COX-2 were preincubated with the inhibitor for 15 min before measurement of the cyclo-oxygenase reaction. The concentration of COX-2 (subunit) was 3.4 μM.

separate experiments. Purified COX-2 was treated with 1 equivalent of ^{14}C-labelled DFU and after an extended preincubation, 10 equivalents of unlabelled DFU or vehicle was added. Aliquots were removed with time and any released [^{14}C]-DFU separated from enzyme-bound inhibitor by a micro concentrator device. Radioactivity measurements showed that 85% of the labelled inhibitor was initially bound to the enzyme and that the label was released in a first order fashion ($t_{1/2}=1.2\pm0.1$ h) following the addition of the 10 fold excess of unlabelled DFU (data not shown). Extrapolation of the data to $t=\infty$ shows that 85 ± 1% of the label was displaced, which is in fair agreement with the expected value of 91%. The second experiment to demonstrate inhibitor reversibility involved the extended dialysis of a 1 : 1 COX-2-DFU complex against a large excess of buffer. The time course for the recovery of activity followed a first order process with a $t_{1/2}$ of 3.1 ± 0.3 h. The slow reversal of inhibition of purified COX-2 by DFU was also observed with CHO[COX-2] cells treated with 150 nM DFU in the presence of cycloheximide to prevent further COX-2 expression. After treatment with the inhibitor, the production of PGE_2 by CHO[COX-2] cells was inhibited >95% compared to vehicle-treated control. However, following resuspension in fresh buffer, the cellular COX activity of DFU-treated CHO cells recovered slowly. COX-2 activity had recovered to 40% that of vehicle-treated controls at 5.5 h. The results indicate that the DFU-COX-2 inhibitor complex can dissociate slowly with a half-time of one to a few hours.

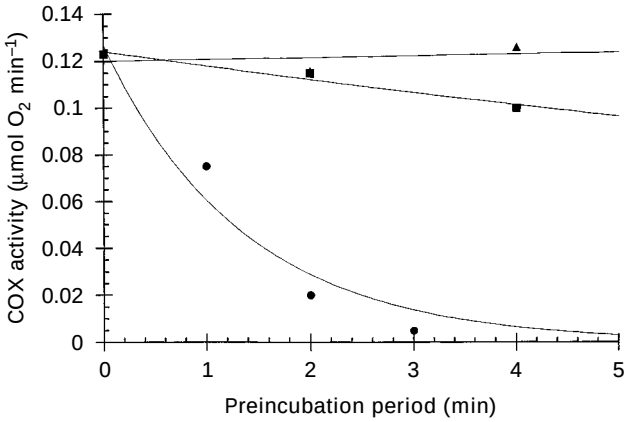

Figure 5 Effect of arachidonic acid on time-dependent inhibition of COX-2 by DFU. The enzyme was preincubated for different periods of time with 10 μM DFU and arachidonic acid at concentrations of 0 (●), 5 (■) and 30 μM (▲) under nonturnover conditions before dilution and assay of enzyme activity.

The effect of substrate arachidonic acid on the time-dependent inhibition of COX-2 by DFU was investigated. In the presence of 10 μM DFU, COX-2 was inhibited in a time-dependent manner with a $t_{1/2}$ of 1.0 min. The inclusion of arachidonic acid (5 and 30 μM) in the preincubation mixture reduced the rate of the onset of inhibition in a concentration-dependent manner (Figure 5), such that no inhibition was observed after 4 min in the presence of 30 μM arachidonic acid. The results are consistent with DFU binding COX-2 competitively with arachidonic acid.

The overall dissociation constant (K_i*) for the purified COX-2-DFU complex (EI*, Scheme 1) can be calculated from the kinetic constants determined above for the equilibrium defined in scheme 1.

$$K_i^* = \frac{[E]\ [I]}{[EI^*]}$$

hence

$$K_i^* = K_i \frac{k_{-2}}{k_2}$$

Substituting in the values obtained (with the value of $k_2=0.0099\pm0.0004$ min^{-1} for the dissociation of [^{14}C]-DFU) gives an estimate of K_i* of 0.21 ± 0.13 μM.

Mechanism of inhibition of purified recombinant human COX-1 by DFU

Inhibition of purified human COX-1 by DFU is very weak. No inhibition of COX-1 could be detected at 100 μM DFU under the conditions of the TMPD assay in which the arachidonic acid concentration is 100 μM. However, an IC_{50} value of 63 ± 5 μM was obtained with the [^{14}C]-arachidonic acid h.p.l.c. assay at a lower substrate concentration (0.1 μM). In contrast to COX-2, the inhibition of purified COX-1 by DFU was not dependent on the time of preincubation between inhibitor and enzyme. The same degree of inhibition by 100 μM DFU (52 ± 14%) was observed with either the simultaneous addition of inhibitor and arachidonic acid (0.1 μM), or preincubation of the inhibitor with the enzyme for periods of up to 60 min before the addition of substrate. The increase in inhibition of COX-1 at lowered substrate concentration and time-in-

Table 1 Inhibition of PGE_2 production in CHO cells stably transfected with human COX-1 and COX-2

	IC_{50} values (nM) *COX-1*		*COX-2*		*COX-1/COX-2*
Flurbiprofen	1.8±0.4	(5)	4±1	(5)	0.4
Diclofenac	4±1	(5)	1.3±0.3	(3)	3
Ketoprofen	6.1±0.1	(2)	119*±52	(3)	0.05
Indomethacin	18±3	(7)	26±6	(13)	0.7
Sulindac sulphide	28±7	(10)	4±1	(10)	7
DuP 697	59±14	(8)	2.1±0.9	(5)	30
Naproxen	62±29	(3)	26*±13	(4)	2
Ibuprofen	470±60	(8)	670±140	(7)	0.7
Nimesulide	780±220	(3)	9*±3	(3)	90
Meloxicam	1,810±430	(4)	6±1	(6)	300
NS-398	1,900±600	(12)	6±1	(10)	300
Piroxicam	3,460±990	(4)	35*±7	(7)	100
6-MNA	2,290±530	(4)	~5,000*	(3)	~0.5
SC-57666	6,000±1,900	(2)	3.2±0.8	(2)	2,000
CGP 28238	8,100±3,700	(3)	8±5	(2)	1,000
SC-58125	12,000±8,000	(3)	10±3	(3)	1,000
L-745,337	~50,000	(5)	60*±14	(8)	~800
Etodolac	~50,000	(4)	41±18	(5)	~1,000
DFU	>50,000	(4)	41±14	(5)	>1,000

IC_{50} values are given±s.e.mean ($n>2$) or ±range ($n=2$). *Titrations did not reach complete inhibition.

dependent inhibition are consistent with reversible competitive behaviour by DFU.

Experiments were conducted to evaluate the reversibility of inhibition of COX-1 by DFU. Purified COX-1 was treated with a concentration of DFU (100 μM) that is approximately 1–2 times the IC_{50} value, before assaying for activity by dilution 30 fold into assay mixture containing substrate. The activities of the inhibitor-treated enzyme samples were the same as vehicle-treated controls. The lack of any inhibition after dilution of the inhibitor therefore demonstrates the rapidly reversible nature of the inhibition of COX-1 by DFU.

Evaluation of inhibitor selectivity with CHO transfected cells

The selectivity of DFU as a COX-2 inhibitor was compared to that of conventional NSAIDs and recently developed selective COX-2 inhibitors in CHO cells transfected with each human COX isozyme. IC_{50} values were determined in assays performed with a 15 min preincubation of the cells with inhibitors in serum-free medium. Arachidonic acid was used at a lower concentration (0.5 μM) in the CHO[COX-1] cells than in CHO-[COX-2] cells (10 μM) to increase the sensitivity of the COX-1 assay for competitive inhibitors. Data are summarized in Table 1. The potency of DFU as an inhibitor of cellular COX-2 activity was similar to that of naproxen, piroxicam and etodolac (IC_{50} = 25–40 nM), but lower than that of the less selective inhibitors diclofenac, flurbiprofen or sulindac sulphide (IC_{50} = 1–4 nM). Ibuprofen and 6-MNA, the active metabolite of nabumetone (Clarke *et al*., 1994), showed no selectivity of inhibition under the conditions used. NS-398, nimesulide, meloxicam and CGP 28238 (flosulide) were all potent (IC_{50} = 6–13 nM) and selective (90 to 1000 fold) inhibitors of cellular COX-2 activity, but were at least 6 to 60 times more potent inhibitors in the CHO[COX-1] assay than DFU. A 1000 fold or more selectivity was achieved with SC-57666, SC-58125, etodolac, L-745,337 and DFU, for which more sensitive assays are required to discriminate their relative effects on COX-1.

Inhibition of TXB_2 synthesis by human platelets

The production of TXB_2 by Ca^{2+} ionophore-stimulated human platelets was used to evaluate further the potency of inhibitors at blocking COX-1 mediated prostanoid production. In this assay, indomethacin and diclofenac were potent inhibitors (IC_{50} = 2–4 nM) and NS-398, piroxicam and meloxicam were about 3 to 4 fold more potent inhibitors of TXB_2 production (IC_{50} = 500–800 nM) than of PGE_2 production in CHO[COX-1] cells. A higher sensitivity to inhibition of TXB_2 production was also observed for etodolac, SC-57666, SC-58125 and CGP 28238 with IC_{50} values of 0.4 ± 0.1 μM, 2.1 ± 0.2 μM, 4.8 ± 1.4 μM and 6.0 ± 1.7 μM, respectively. In contrast, preincubation of the platelets for 15 min with DFU caused less than 50% inhibition of TXB_2 release over the range of 0.05–100 μM (33 ± 6% inhibition, n = 8, at the highest dose of 100 μM). The effect of DFU was also investigated with a more sensitive assay in which platelets were stimulated by a sub-optimal concentration of arachidonic acid (1 μM) instead of 2 μM calcium ionophore. Under these conditions, inhibition of the arachidonic acid-stimulated production of TXB_2 by DFU was detected with an IC_{50} value of 12 ± 5 μM (n = 3).

Table 2 Inhibition of COX-1 from U937 cell microsomes

	IC_{50} (nM)	
Diclofenac	7 ± 3	(3)
Indomethacin	20 ± 1	(23)
Naproxen	29 ± 7	(3)
Nimesulide	117 ± 37	(3)
Meloxicam	143 ± 55	(2)
Piroxicam	163 ± 17	(3)
NS-398	295 ± 124	(4)
SC-57666	480 ± 130	(3)
SC-58125	762 ± 68	(4)
CGP 28238	1,010 ± 70	(3)
Etodolac	1,200 ± 260	(4)
6-MNA	1,300 ± 600	(4)
L-745,337	2,780 ± 280	(3)
DFU	12,600 ± 2,400	(11)

IC_{50} values are given ± s.e.mean (n > 2) or ± range (n = 2). Numbers in parentheses show n value.

Inhibition of microsomal COX-1 from U937 cells at low arachidonic acid concentration

In order to characterize further the effects of inhibitors on COX-1, a sensitive assay was developed based on the production of PGE_2 by U937 cell microsomes following incubation with a low, sub-saturating concentration of arachidonic acid (0.1 μM). Potent non-selective inhibitors such as diclofenac, indomethacin and naproxen showed IC_{50} values in the low nM range in this assay (Table 2). Meloxicam, nimesulide and piroxicam inhibited with IC_{50} values ranging from 100–200 nM. Among the compounds that showed the highest selectivity in the cell-based assays, DFU (IC_{50} = 13 ± 2 μM) was about 10 to 40 fold less potent at inhibiting COX-1 than NS-398, SC-58125, SC-57666, CGP28238 or etodolac and 5 fold less potent that L-745,337 (Table 2).

COX-2 human whole blood assay (LPS-induced PGE_2 production)

The production of PGE_2 by LPS-challenged whole blood has been used to evaluate the efficacy of COX-2 inhibitors *in vitro* (Brideau *et al*., 1996). The control, unstimulated human blood at time zero, contained only small amounts of PGE_2 (0.28 ± 0.04 ng ml^{-1}, n = 12). In contrast, a substantial increase in PGE_2 was observed following incubation with LPS for 24 h, resulting in a net increase of 35.8 ± 5.5 ng ml^{-1} (n = 12). The LPS-induced production of PGE_2 was inhibited by DFU or indomethacin in a concentration-dependent manner with 100% inhibition observed with both compounds (Figure 6a). The potency of DFU was similar to that of indomethacin with IC_{50} values of 0.28 ± 0.04 μM (n = 12) and 0.68 ± 0.17 μM (n = 14), respectively.

COX-1 human whole blood assay (coagulation-induced TXB_2 production)

Spontaneous clotting of human blood resulted in the generation of large amounts of TXB_2 in the serum (72.9 ± 8.9 ng ml^{-1}). Indomethacin inhibited the production of TXB_2 in a concentration-dependent manner, with an IC_{50} value of 0.19 ± 0.02 μM (n = 38) (Figure 6b). In contrast, DFU inhibited the production of TXB_2 by only 38% even at the maximal concentration tested (97 μM). Thus, indomethacin is approximately equipotent at inhibiting both COX-1 and COX-2 whereas DFU is greater than 300 fold more selective for COX-2.

Carrageenan-induced rat paw oedema

In the control group, the paw volume increased by 1.04 ± 0.01 ml (n = 10) 3 h after injection of carrageenan. DFU (1 h pretreatment) inhibited the carrageenan-induced oedema response dose-dependently (ED_{50} = 1.1 mg kg^{-1}, Figure 7a). DFU was slightly more potent than indomethacin (ID_{50} = 2.0 mg kg^{-1}) in this assay.

Carrageenan-induced rat paw hyperalgesia

Intraplantar injection of carrageenan induces marked paw

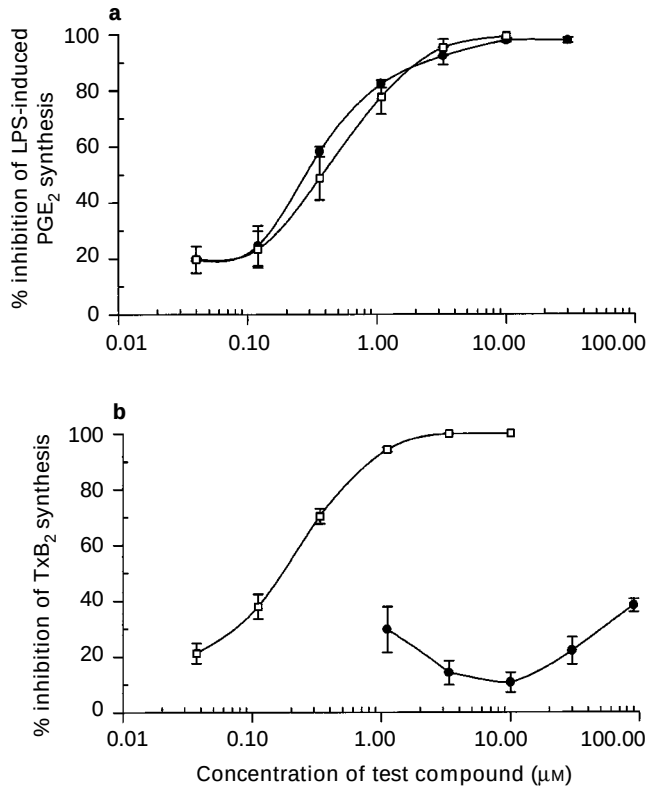

Figure 6 Effects of DFU (●) or indomethacin (□) in human (a) COX-2 and (b) COX-1 whole blood assays. $n=12$ donors each for DFU COX-1 and COX-2 assays. $n=38$ for indomethacin COX-1 assay and $n=14$ for indomethacin COX-2 assay. Vertical lines show s.e.mean.

oedema and hyperalgesia to mechanical compression. Oral administration of DFU 1 h before the hyperalgesia measurement dose-dependently reversed carrageenan-induced hyperalgesia with an ED_{50} of 0.95 mg kg^{-1}, DFU being slightly more potent than indomethacin ($ED_{50}=1.5$ mg kg^{-1}, Figure 7c). The hyperalgesic responses could be completely reversed by DFU at 10 mg kg^{-1}.

Endotoxin-induced pyretic response in rats

In the saline-injected rats, the rectal temperature was maintained at a steady level throughout the course of the experiment, being at 36.32 ± 0.08, 36.46 ± 0.11, 36.38 ± 0.06 and 36.36 ± 0.07°C ($n=10$) at 0, 5, 6 and 7 h, respectively. In contrast, the corresponding values at these time points in the LPS-injected group were 36.50 ± 0.15, 38.80 ± 0.08, 38.92 ± 0.07 and 38.96 ± 0.08°C, respectively. This represented a net increase of 2.58 ± 0.07°C in the LPS-treated vehicle control group 7 h after LPS injection compared to the saline-injected group. Oral administration of DFU at the plateau of temperature elevation (5 h) reversed the LPS-induction pyrexia in a dose-dependent manner (ED_{50} of 0.76 mg kg^{-1}, Figure 7b). The potency of DFU was comparable to that of indomethacin ($ED_{50}=1.07$ mg kg^{-1}).

^{51}Cr faecal excretion in rats

Acute dosing of indomethacin (10 mg kg^{-1}), meloxicam (3 mg kg^{-1}) and etodolac (10–30 mg kg^{-1}), caused a significant increase in faecal ^{51}Cr excretion in a 48 h period following injection of ^{51}Cr-labelled red blood cells (Figure 8). In chronic dosing studies, administration of diclofenac at 3 mg kg^{-1}, b.i.d., for 5 days resulted in a significant increase in faecal ^{51}Cr excretion. In chronic dosing studies with indomethacin at 3 mg kg^{-1}, one of five animals died of gastrointestinal side effects after 4 days of dosing. The remaining 4 animals showed overt clinical symptoms (loss of appetite, loss of body weight, constipation, jaundice) and ^{51}Cr excretion experiments could not be performed. In contrast, DFU at 100 mg kg^{-1}, b.i.d., for 5 days had no effect on faecal ^{51}Cr excretion in either male or female rats. The plasma levels of DFU were 5.8 μM and 6.7 μM, respectively, in male and female rats 1 h after the morning dose at day 4.

^{51}Cr faecal excretion in squirrel monkeys

In the control group, oral administration of 1% methocel (4–5 days b.i.d.) resulted in excretion of faecal ^{51}Cr of 0.58 ± 0.11% over 24 h ($n=10$). DFU at 100 mg kg^{-1} for 5 days resulted in 0.97 ± 0.47% ($n=3$) excretion, a value not significantly different from the methocel control. Plasma concentrations of drug were 10.3 μM 1 h after the last dose. In contrast, the non-selective NSAID diclofenac at 1 mg kg^{-1}, b.i.d., for 4 days, resulted in a significant 24 h ^{51}Cr excretion of 1.91 ± 0.70% ($n=6$; $P<0.05$ compared with methocel controls) (Figure 9), with a plasma concentration of 0.4 μM at 1 h (no drug detectable at 8 h). Similarly, naproxen at 5 mg kg^{-1} for 5 days b.i.d. caused a significant excretion of 2.21 ± 0.20% ($n=4$) of the administered radioactivity, with a plasma concentration of 193 μM at 1 h after the last dose.

Discussion

The anti-inflammatory agent DuP 697 (Gans *et al*., 1990) has been the prototype for a series of diaryl tricyclic selective COX-2 inhibitors containing thiophene (Bertenshaw *et al*., 1995; Leblanc *et al*., 1995), pyrroles (Wilkerson *et al*., 1995), benzene (Li *et al*., 1996), pyrazole (e.g. SC-58125) (Seibert *et al*., 1994), cyclopentene (e.g. SC-57666) (Reitz *et al*., 1994) or various heterocycles (Gauthier *et al*., 1996) as the central ring. DFU is a structurally related analogue containing a 5,5-dimethyl furanone ring, which has been optimized for potency and selectivity of inhibition of COX-2 by use of a variety of enzyme, cell-based and *in vivo* assays.

The results of this study are consistent with the inhibition of purified COX-2 by DFU occurring via a two-step mechanism characterized by a relatively high K_i value for rapid reversible binding (140 μM) followed by a slow ($t_{1/2}=6$ s) isomerisation to a tightly bound complex (EI*) with an overall dissociation constant for inhibition of approximately 0.21 μM. In contrast, inhibition of purified human COX-1 by DFU is much weaker, rapidly reversible and detectable only at low arachidonic acid concentrations. If competitive inhibition is assumed, the IC_{50} value (60 μM) measured for COX-1 at low substrate concentration should approximate the K_i value for inhibitor binding. Considering the similarity of the K_i values for COX-1 and COX-2, the selectivity of DFU for COX-2 relies on the slow formation of the tight EI* complex. This mechanism results in a highly selective COX-2 inhibition that translates in the various enzyme and cell assays.

The difference between the mechanism of inhibition of COX-1 and COX-2 observed for DFU has been noted previously for other selective COX-2 inhibitors (Copeland *et al*., 1994; Ouellet & Percival, 1995) and makes it difficult to determine accurately the selectivity ratio of the various COX inhibitors. In the present assay procedures for inhibition of COX-2, compounds were preincubated with enzymes or cells before the addition of arachidonic acid, conditions which favour inhibition by time-dependent inhibitors (Harris Callan *et al*., 1996). Because arachidonic acid delayed time-dependent inhibition of COX-2 by DFU, the effectiveness of DFU should depend on both arachidonic acid concentration and its time of supply during COX-2 induction. This is a complex situation for COX-2 since cellular endogenous arachidonic acid appears to be channelled to enzyme following cellular stimulation by

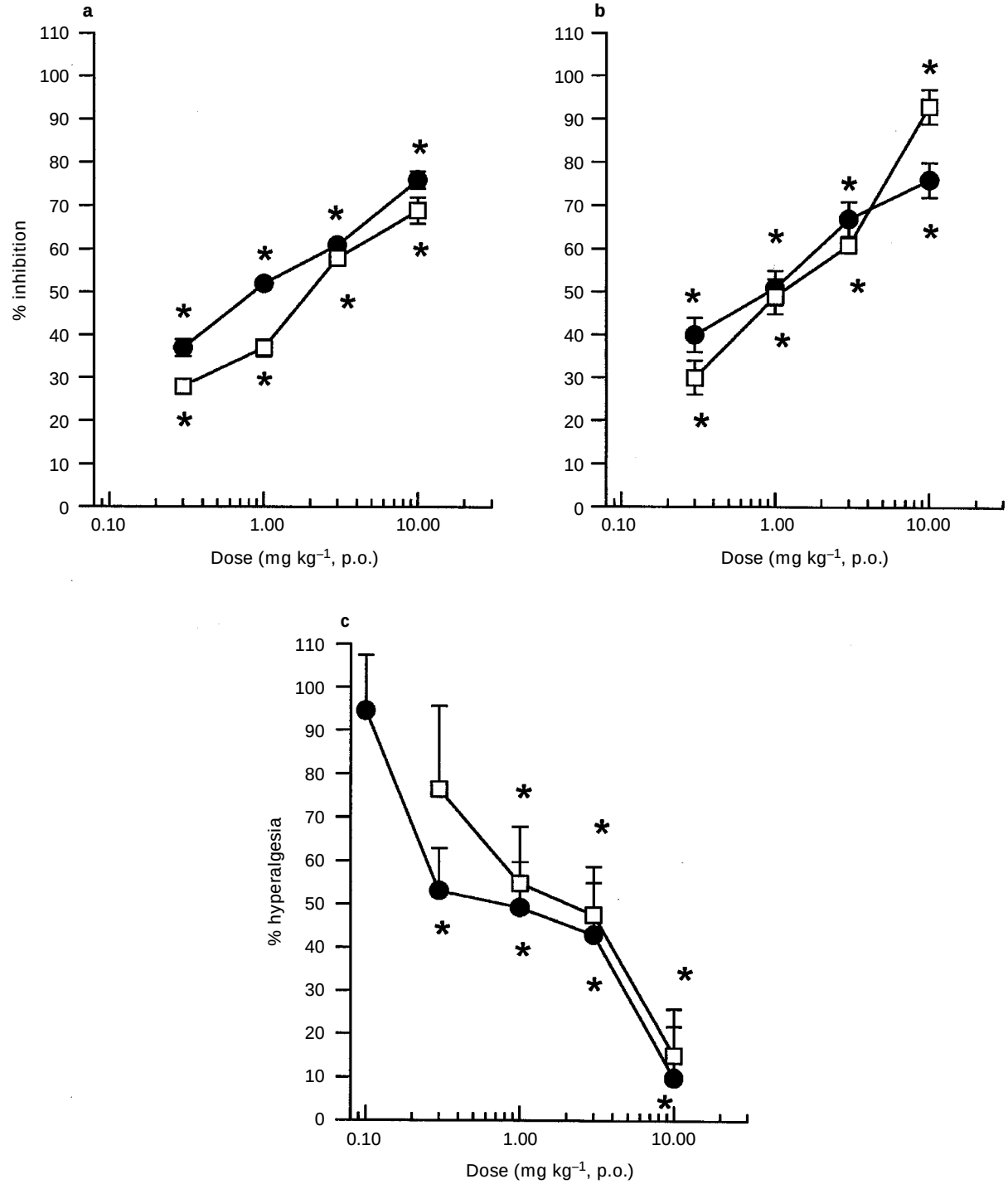

Figure 7 Dose-dependence of the inhibitory effects of DFU (●) and indomethacin (□) in (a) carrageenan-induced rat paw oedema assay, DFU ($n=15$), indomethacin ($n=5$); (b) LPS-induced pyresis in rats, DFU ($n=10$), indomethacin ($n=5$) and (c) carrageenan-

mitogens or LPS (Herschman, 1996). The fact that DFU can inhibit 100% of LPS-induced PGE_2 synthesis in whole blood assays (where plasma concentrations of arachidonic acid have been shown to increase to 15 μM, Brideau *et al.*, 1996) and shows efficacy in various *in vivo* models of inflammation indicates that the kinetic characteristics of COX-2 inhibition by this compound allow effective inhibition of the activity of the inducible enzyme.

DFU did not cause inhibition of prostanoid synthesis ($IC_{50} > 50$ μM) in several COX-1 mediated assays including the production of PGE_2 by arachidonic acid-stimulated CHO-[COX-1] transfected cells or U937 cells, the release of TXB_2 by Ca^{2+} ionophore-challenged human platelets and the production of TXB_2 following blood coagulation. However, inhibition of TXB_2 by DFU could be observed with platelets challenged with 1 μM arachidonic acid or in assays with purified COX-1 or U937 cell microsomes at a low arachidonic acid concentration (IC_{50}s = 10 – 60 μM). Assays at low arachidonic acid concentration permitted the detection of COX-1 inhibition with all selective COX-2 inhibitors tested and such assays were found to be useful in the comparison of their relative potencies as COX-1 inhibitors. SC-57666, CGP 28238, etodolac and SC-58125 were found to be about 10 fold more potent COX-1 inhibitors than DFU in such assays.

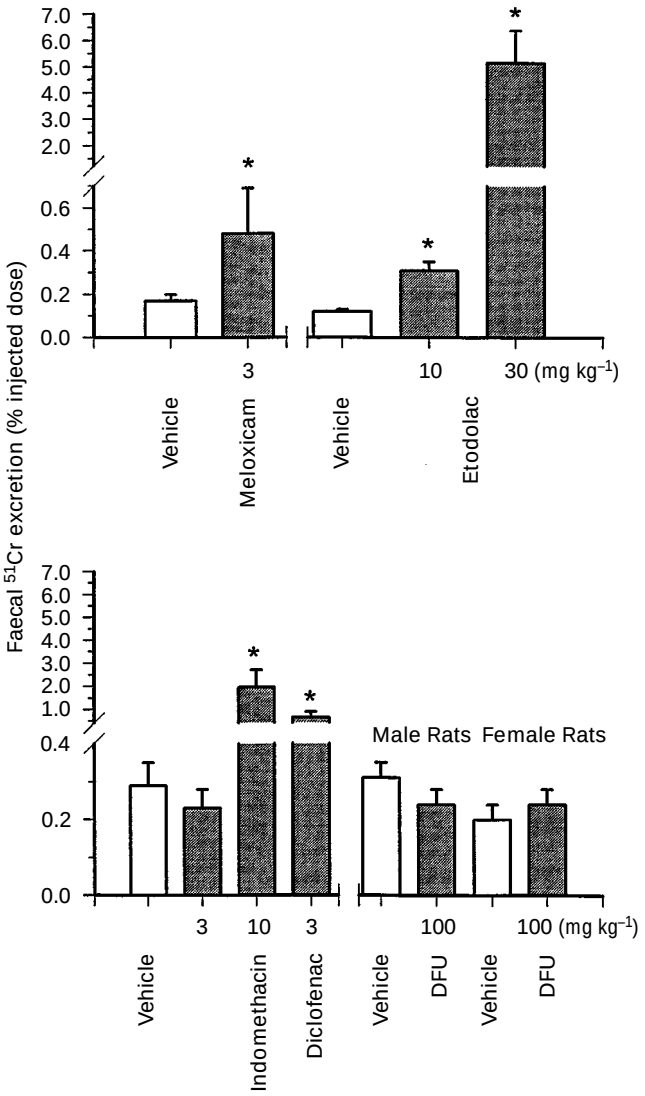

Figure 8 Effects of DFU, diclofenac, indomethacin, meloxicam or etodolac on faecal ^{51}Cr excretion in rats. Indomethacin was administered at a single dose of 3 ($n=8$) or 10 mg kg^{-1}, ($n=12$); diclofenac at 3 mg kg^{-1}, b.i.d. for 5 days ($n=4$), vehicle control ($n=11$); DFU at 100 mg kg^{-1}, b.i.d. for 5 days ($n=6$, both male and female rats), vehicle control ($n=6-8$); meloxicam at 3 mg kg^{-1} ($n=6$), vehicle control ($n=6$) and etodolac at 10 mg kg^{-1} ($n=4$) or 30 mg kg^{-1} ($n=4$), vehicle control ($n=8$). *$P<0.05$ vs matching vehicle control.

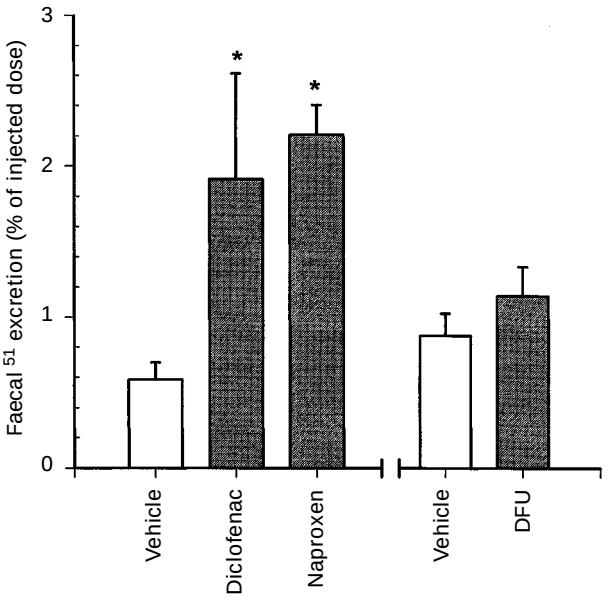

Figure 9 Effects of DFU, diclofenac or naproxen on faecal ^{51}Cr excretion in squirrel monkeys. Diclofenac, 1 mg kg^{-1}, b.i.d., 4 days ($n=6$); naproxen, 5 mg kg^{-1}, b.i.d., 5 days ($n=4$); DFU, 100 mg kg^{-1}, b.i.d., 5 days ($n=6$); vehicle control ($n=6-10$). *$P<0.05$ vs matching vehicle control.

DFU is active with high potency in different *in vivo* models of acute inflammation, pyresis and hyperalgesia with an approximate ED_{50} value of 1 mg kg^{-1}. At this dose, the plasma concentration of DFU is below the detection limit of the h.p.l.c. system (<0.2 μM) and is below that required to inhibit platelet COX-1 ($IC_{50}>97$ μM in human COX-1 whole blood assay). Thus, it is quite clear, at least in animal models, that inhibition of COX-1 is not required to achieve anti-inflammatory, anti-pyretic and analgesic effects, consistent with the hypothesis that COX-2 plays a dominant role in acute inflammation. In addition to acute inflammation and pain, DFU is also active in a model of chronic arthritic inflammation (Visco *et al*., 1996), supporting a role of COX-2 in chronic inflammatory conditions such as osteoarthritis and rheumatoid arthritis.

The major difference between COX-2 inhibitors and non-selective NSAIDs is that COX-2 inhibitors have a good separation between their functional efficacy and ulcerogenic potentials. COX-1 is the major COX isozyme present in the gastrointestinal tract of many species (Kargman *et al*., 1996a) and ulcerogenic sparing selective COX-2 inhibitors do not inhibit stomach PGE_2 production in contrast to non-selective NSAIDs (Futaki *et al*., 1993a; Masferrer *et al*., 1994; Chan *et al*., 1995). Nimesulide (Magni, 1993; Tavares *et al*., 1995), etodolac (Glaser *et al*., 1995; Laine *et al*., 1995), meloxicam (Engelhardt *et al*., 1996) and CGP 28238 (Klein *et al*., 1994) have been shown to inhibit preferentially COX-2 and to show an improved gastrointestinal tolerability as compared to non-selective NSAIDs. It should be noted that a higher degree of selectivity for COX-2 was observed in the CHO cell assays (90 to 1000 fold) than in whole blood assays where selectivity ratios of 6 to 43 fold have been obtained for these compounds (Glaser *et al*., 1995; Young *et al*., 1996; Brideau *et al*., 1996; Noble & Balfour, 1996), as compared to >350 fold for DFU. In the U937 microsomal COX-1 assay, nimesulide and meloxicam were about 100 times more potent inhibitors than DFU, while etodolac and CGP 28238 were about 10 times more potent. ED_{50} values for gastric ulceration in rats of 2.5 mg kg^{-1} day^{-1} and of 30 mg kg^{-1} have been determined for meloxicam (Engelhardt *et al*., 1995) and etodolac (Melarange *et al*., 1995), respectively. Thresholds of 30 mg kg^{-1} and of 100 mg kg^{-1} day^{-1} have been determined for a faecal blood loss by CGP 28238 (Wiesenberg-Bottcher *et al*., 1989) and nimesulide (Carr *et al*., 1986). When administered orally at 200 mg kg^{-1} day^{-1} for 5 days, DFU had no detectable ulcerogenic effect in rats in a sensitive faecal ^{51}Cr excretion assay which measures the integrity of the gastrointestinal tract. This would give DFU a margin of safety of greater than 200 fold between efficacy ($ED_{50}\cong 1$ mg kg^{-1}) and ulcerogenicity (threshold dose >200 mg kg^{-1}), in marked contrast to indomethacin or diclofenac which have a narrow margin of safety in these animal studies. The ulcerogenic sparing effect of DFU was observed not only in rats, but also in squirrel monkeys. The present findings with DFU are in accord with the notion that the inhibition of COX-1 is a major contributing factor in NSAID-induced gastropathy. However, COX-1-deficient mice have been shown recently to have no gastric pathology and to be less sensitive to indomethacin-induced ulceration than wild type mice (Langenbach *et al*., 1995), suggesting that either compensatory mechanisms for cytoprotection are present in the transgenic

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