The Cyclooxygenase-2 Inhibitor GW406381X [2-(4-Ethoxyphenyl)-3-[4-(methylsulfonyl)phenyl]-pyrazolo[1,5-b]pyridazine] Is Effective in Animal Models of Neuropathic Pain and Central Sensitization


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ABSTRACT

The pathogenic form of the cyclooxygenase (COX) enzyme, COX-2, is also constitutively present in the spinal cord and has been implicated in chronic pain states in rat and man. A number of COX-2 inhibitors, including celecoxib and rofecoxib, are already used in man for the treatment of inflammatory pain. Preclinically, the dual-acting COX-2 inhibitor, GW406381X [2-(4-ethoxyphenyl)-3-[4-(methylsulfonyl)phenyl]-pyrazolo[1,5-b]pyridazine, where X denotes the free base], is as effective as rofecoxib and celecoxib in the rat established Freund’s Complete Adjuvant model with an ED50 of 1.5 mg/kg p.o. compared with 1.0 mg/kg p.o. for rofecoxib and 6.6 mg/kg p.o. for celecoxib. However, in contrast to celecoxib (5 mg/kg p.o. b.i.d.) and rofecoxib (5 mg/kg p.o. b.i.d.), which were without significant effect, GW406381X (5 mg/kg p.o. b.i.d.) fully reversed mechanical allodynia in the chronic constriction injury model and reversed thermal hyperalgesia in the mouse partial ligature model, both models of neuropathic pain. GW406381X, was also effective in a rat model of capsaicin-induced central sensitization, when given intrathecally (ED50 = 0.07 μg) and after chronic but not acute oral dosing. Celecoxib and rofecoxib had no effect in this model. Several hypotheses have been proposed to try to explain these differences in efficacy, including central nervous system penetration, enzyme kinetics, and potency. The novel finding of effectiveness of GW406381X in these models of neuropathic pain/central sensitization, in addition to activity in inflammatory pain models and together with its central efficacy, suggests dual activity of GW406381X compared with celecoxib and rofecoxib, which may translate into greater efficacy in a broader spectrum of pain states in the clinic.

The role of prostaglandins, formed through the action of the two isoforms of the cyclooxygenase (COX) enzyme, COX-1 and COX-2, in the pathophysiology of pain is well documented (Steinmeyer, 2000; Camu et al., 2003; Claria, 2003; Flower, 2003). COX-1 is believed to play a more homeostatic role, whereas the inducible form, COX-2, is up-regulated following persistent activation of nociceptive pathways (Hoffmann, 2000; Vanegas and Schaible, 2001; Svensson and Yaksh, 2002; Flower, 2003). A wider role for spinal COX-2 has been proposed based on the presence of constitutive COX-2 in the spinal cord and its up-regulation in animal models of inflammation (Ichitani et al., 1997; Willingale et al., 1997; Beiche et al., 1998; Vanegas and Schaible, 2001; Yaksh et al., 2001; Svensson and Yaksh, 2002; Seybold et al., 2003) and the fact that COX-2 inhibitors given intrathecally can inhibit both inflammation-induced thermal hyperalgesia and substance P-evoked prostaglandin release (Yaksh et al., 2001).

The introduction of selective inhibitors of COX-2 has led to treatments that are as effective as conventional nonsteroidal anti-inflammatory drugs in the treatment of both inflammatory and neuropathic pain states (Svensson and Yaksh, 2002; Flower, 2003). A key advantage of COX-2 inhibitors over traditional NSAIDs is their ability to selectively inhibit COX-2 in inflamed tissue while sparing COX-1 in non-inflammatory tissues, potentially reducing the risk of gastrointestinal complications commonly associated with NSAIDs.

ABBREVIATIONS: COX, cyclooxygenase; GW406381X, 2-(4-ethoxyphenyl)-3-[4-(methylsulfonyl)phenyl]-pyrazolo[1,5-b]pyridazine, where X denotes the free base; CNS, central nervous system; DMSO, dimethyl sulfoxide; PGE2, prostaglandin E2; EIA, enzyme immunoassay; LPS, lipopolysaccharide; TXB2, thromboxane B2; HPLC, high-pressure liquid chromatography; MS/MS, tandem mass spectrometry; CCI, chronic constriction injury; FCA, Freund’s Complete Adjuvant; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; ANOVA, analysis of variance; AA, arachidonic acid; HVA, homovanillic acid.
anti-inflammatory drugs in treating pain due to inflammation but without the GI toxicity associated with COX-1 activity (Cannon, 2000; Bertin et al., 2003). There are a number of selective COX-2 inhibitors on the market including celecoxib (Celebrex; G.D. Searle & Co., Skokie, IL) (Penning et al., 1997), rofecoxib (Vioxx; Merck, Whitehouse Station, NJ) (Prasit et al., 1999), and valdecoxib (Bextra; G.D. Searle & Co.) (Talley et al., 2000), and although all are effective at treating conditions such as osteoarthritis and rheumatoid arthritis (Clemett and Goa, 2000), the overall response rate and relief of pain is not maximal (Everts et al., 2000; Duncan and Capell, 2003), and they have little efficacy in neuropathic pain conditions.

Data are presented showing that the COX-2 inhibitor, GW406381X [2-(4-ethoxyphenyl)-3-[4-(methylsulfonyl)phenyl]-pyrazolo[1,5-b]pyridazine, where X denotes the free base] (Beswick et al., 2004) is more potent in vitro than already marketed compounds, with good pharmacokinetic characteristics, including superior penetration into the central nervous system (CNS). GW406381X is as effective as rofecoxib and celecoxib in animal models of inflammatory pain but, in addition, is highly effective in models of neuropathic pain after chronic oral dosing, and central sensitization when given intrathecally. The factors that may contribute to this differentiation among GW406381X, celecoxib, and rofecoxib are discussed, together with the possible implications.

Materials and Methods

In Vitro Assays

COS cells stably transfected with cDNA for human COX-1 and human COX-2 were incubated overnight in 24-well cell culture plates. The incubation medium was replaced with 250 μl of fresh, unsupplemented Dulbecco’s modified Eagle’s medium (DMEM) or DMEM was added to the wells in a volume of 1 μl. Plates were incubated for 1 h (37°C, 95% air/5% CO2) before addition of 10 μl of arachidonic acid (750 μM) to a final concentration of 30 μM. Plates were incubated for a further 15 min, and the incubation medium was removed from each well of the plates and stored at −20°C prior to determination of PGE2 levels using EIA (Biotrak enzyme immunoassay kit RPN222 Amersham Biosciences Inc., Piscataway, NJ).

Human Whole-Blood Assay

COX-2 Human Whole Blood (LPS-Stimulated PGE2 Production). Human whole blood was incubated with aspirin (10 μg/ml) for 30 min at 37°C to inactivate all constitutive cyclooxygenase enzyme before dispensing (1 ml) into Eppendorf tubes containing 10 μl of the test compound (100× required test concentration) or DMEM and 10 μl of LPS (Escherichia coli 026:B6; L-8274; 200 ng/ml; Sigma-Aldrich, St. Louis, MO) to give a final concentration of 2 μg/ml. Tubes were incubated at 37°C, 95% air and 5% CO2 for 24 h then centrifuged (150g; 2 min, Eppendorf bench-top centrifuge). Plasma was removed, and levels of PGE2 were determined by EIA (Brideau et al., 1996).

COX-1 Human Whole Blood [Coagulation-Induced Thromboxane B2 (TxB2) Production]. Spontaneous clotting of human whole blood resulted in the generation of TxB2 via the action of COX-1 (Patrignani et al., 1994). Human whole blood (1 ml) was dispensed into glass tubes containing 10 μl of test compound (100× required test concentration) or DMEM. The tubes were transferred to an incubator, and the blood was allowed to clot (37°C, 95% air, 5% CO2). Thirty minutes later, blood clots were released from the walls of the tubes and incubated for a further 30 min before centrifugation (2500 rpm, 10 min) and removal of serum. Levels of TxB2 were determined by EIA (Biotrak enzyme immunoassay kit RPN221; Amersham Biosciences Inc.).

Data Analysis. The inhibitory potency of the test compound was expressed as an IC50 value. This is defined as the concentration of the compound required to inhibit total prostaglandin release from the cells by 50% (cultured cell assay and whole-blood assay). The individual IC50 values obtained were then meaned across experiments to yield the mean ± S.E.M. value. The selectivity ratio of inhibition of COX-1 versus COX-2 was calculated by comparing respective IC50 values.

Clearance and CNS Penetration

Animals and Experimental Protocol. Male rats (Random Hooded, supplied by an approved Home Office supplier) were used in these studies. Prior to surgery, rats received antibiotic (Penbritin) and analgesic (Rimadyl). Each rat was cannulated via a femoral vein into the vena cava (for drug administration) and jugular vein (for blood sampling) while under isoflurane anesthesia. Cannulae were exteriorized at the back of the neck. The rats were placed in harnesses with tethers and allowed to recover for 3 days prior to dosing. Each rat was housed in a plastic solid bottom cage and had free access to food (Teklad 2014 rodent maintenance diet; Harlan, Indianapolis, IN) and water. Temperature was maintained at 21 ± 2°C and humidity at 55 ± 10%. At the end of the study, the rats were killed by injection of Euthatal through the jugular vein cannula.

Dosing. GW406381X was dissolved in 5% glucose containing 2% (v/v) DMSO and 10% (w/v) Encapsin HPB at concentrations of 0.1 mg/ml and infused over 14 h at 1 ml/kg/h to achieve target dose rate of 0.1 mg/kg/h. Celecoxib was dissolved in 5% glucose containing 2% (v/v) DMSO and 10% (w/v) Encapsin HPB at concentration of 0.05 mg/ml and infused over 12 h at 0.6 ml/kg/h to achieve a target dose rate of 0.03 mg/kg/h. Rofecoxib was dissolved in 5% glucose containing 10% (v/v) DMSO and 10% (w/v) Encapsin HPB at concentration of 0.04 mg/ml and infused over 12 h at 7.5 ml/kg/h to achieve a target dose rate of 0.3 mg/kg/h.

Samples. Blood samples (ca. 80 μl, collected into tubes containing potassium EDTA as anticoagulant) were taken over the last 2 h of the infusion to confirm that steady-state blood concentrations had been achieved. A 50-μl aliquot of each sample was transferred to a fresh micronic tube, diluted with an equal volume of purified water (total 100 μl), mixed, and stored at approximately −80°C prior to analysis by HPLC/MS/MS. At the end of the infusion period, rats were anesthetized with Euthatal, exsanguinated, killed by decapitation, and brains removed and homogenized with an equal volume of purified water. Triplicate 50-μl aliquots of the homogenates were transferred to fresh micronic tubes and stored at approximately −80°C prior to analysis by HPLC/MS/MS.

Sample and Data Analysis

Steady-State CNS Penetration Studies. Sample extraction was performed by means of protein precipitation using acetonitrile/10 mM ammonium acetate (250 μl, 80:20, v/v) also containing an appropriate internal standard (0.2 μg/ml). Following centrifugation, an aliquot of the supernatant was analyzed by reverse-phase HPLC/MS/MS using an API365 mass spectrometer (MDS Sciex, Concord, ON, Canada) fitted with a heat-assisted electrospray interface. An HTS PAL autosampler (Presearch, Hitchin, UK) and an appropriate internal standard (0.2 μg/ml) was added. At the end of the study, the rats were killed by injection of Euthatal through the jugular vein cannula. CNS penetration of GW406381X was confirmed by analysis of brain homogenates.

Materials and Methods

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Chromatographic separation was performed with a Discovery Cyano, citation using 300 internal standard. Following vortex mixing and centrifugation, 350 A. Sayner, unpublished data) and 0.76 for rofecoxib (Halpin et al., using the blood/plasma ratios of 0.92 for GW406381X (R. Collins and D. studies, plasma concentrations were corrected to blood concentrations compare with the data obtained from the steady-state CNS penetration. Following centrifugation at 10°C, 2 ml of supernatant was standard was added and homogenized using an Ultraturrax homog-

\[ CL_b = \frac{Ro}{\text{BloodCss}} \] (1)

where CLb = blood clearance, Ro = rate of infusion, andCss = concentration at steady state, the study Css and infusion rate were used to calculate steady-state CLb. The brain concentration data obtained at the end of the study, corrected for residual blood content of 15 µg/g tissue (Brown et al., 1986), were assumed to represent steady-state brain concentrations. This was used with the bloodCss to calculate CNS penetration determined as the ratio:

Sample Analysis of Brains and Blood from the CCI Model. Plasma samples (100 µl) were extracted by means of protein precipitation using 300 µl of ice-cold acetonitrile containing 0.125 µg/ml of internal standard. Following vortex mixing and centrifugation, 350 µl of the resulting supernatant was evaporated to dryness under heated nitrogen at 40°C and reconstituted in 100 µl of 70:30 water/ acetonitrile for analysis by reverse-phase HPLC/MS/MS using an API365 mass spectrometer (MDS Sciex). Brain samples were weighed, and 5 ml of acetonitrile containing 0.125 µg/ml internal standard was added and homogenized using an Ultraturrax homogenizer. Following centrifugation at 10°C, 2 ml of supernatant was evaporated to dryness under heated nitrogen at 40°C and reconstituted in 200 µl of 70:30 water/acetonitrile for analysis by reversephase HPLC/MS/MS using an API365 mass spectrometer (MDS Sciex). For the GW406381X assay, GW614279X was selected as the internal standard. The nominal mass transitions were as follows (positive ion mode): GW406381X, m/z 394 to 287; and GW614279X, m/z 390 to 346. For the rofecoxib assay, GW551106X was selected as the internal standard. The nominal mass transitions were as follows (negative ion mode): rofecoxib, m/z 313 to 265; and GW551106X, m/z 434 to 249. Chromatographic separation was performed using a C18 SB-2, 2.1- × 50-mm, 5-µm column, using a timed gradient. For the GW406381X assay, the gradient was run using 0.05% trifluoroacetic acid in 0.05M ammonium acid in 95:5 water/acetonitrile and 0.05% trifluoroacetic acid in methanol. For the rofecoxib assay, the gradient was run using 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Flow was maintained at 800 µl/min for plasma analysis and 400 µl/min for brain analysis with run times of 2.4 min/sample. To compare with the data obtained from the steady-state CNS penetration studies, brain concentrations were corrected to blood concentrations using the blood/plasma ratios of 0.92 for GW406381X (R. Collins and D. A. Sayner, unpublished data) and 0.76 for rofecoxib (Halpin et al., 2000).

Behavioral Studies: Animals

Experiments were performed using adult, male Random Hooded rats (170–250 g) supplied by the rodent breeding unit at GlaxoSmithKline (Uxbridge, Middlesex, UK) or adult male C57BL6 mice (20–25 g) from Charles River (Margate, Kent, UK). Animals were housed in groups of five to seven on a 12-h light/dark cycle (lights on 7:00 AM) and fed on an RM1 chow pellet diet with free access to water. The number of animals and intensity of noxious stimuli were the minimum necessary to demonstrate consistent effects of drug treatments. All experiments were performed in accordance with Home Office guidelines on experimentation with animals, under supervision of a veterinarian, and complied with local GSK regulations regarding the care of experimental animals.

Experimental Protocol

Carrageenan Model. Carrageenan (2% w/v, 100 µl) was injected intraplantar into the left hind paw (n = 7 per group). GW406381X, celecoxib, rofecoxib (1–10 mg/kg p.o.), or vehicle was administered 30 min prior to the injection of carrageenan. Three hours after carra-
genan injection, the ability to bear weight on the inflamed left paw was measured using a dual-channel weight averager together with the development of paw edema, using a commercially available plethysmometer (Ugo Basile, Comerio, Italy).

Acute Inflammation-Induced Hyperalgesia. Freund’s Complete Adjuvant (FCA; 1 mg/ml Mycobacterium tuberculosis, 100 µl) was injected intraplantar into the left hind paw (n = 7 per group). GW406381, celecoxib (1–10 mg/kg p.o.), rofecoxib (0.3–3 mg/kg p.o.), or vehicle was administered 1 h prior to FCA, and the change in weight-bearing ability of the inflamed paw was measured 6 h postFCA together with the associated edema.

Established Inflammation-Induced Hyperalgesia. FCA (100 µl of 1 mg/ml M. tuberculosis (1 mg/ml)) was injected intraplantar into the left hind paw (n = 7 per group). GW406381X (1–10 mg/kg p.o.), rofecoxib (0.3–3 mg/kg p.o.), and celecoxib (3–15 mg/kg p.o.) were administered 24 h post-FCA. One hour later, the effect on weight bearing on the inflamed paw and associated edema was determined.

CCI Model. Under isoflurane anesthesia (3%), the common left sciatic nerve was exposed and tied with four loose ligatures of chromic gut (4.0), spaced 1 mm apart (Bennett and Xie, 1988) (n = 5–10 per group, depending on the time point). The wound was closed using suture clips. The surgical procedure was identical for the sham-operated animals, except the sciatic nerve was not ligated (n = 5–10 per group). Rats were allowed a period of at least 7 days to recover from surgery before behavioral testing began. The effect of compounds on CCI-induced decrease in paw withdrawal threshold (PWT) was measured using an algosimeter (Linton Instruments, Diss, Norfolk, UK). In the initial study, GW406381 was dosed chronically (5 mg/kg b.i.d. p.o. for 9 days) beginning on day 20 postsurgery, after stable reductions in PWT were established. In the second study, dosing with GW406381X or rofecoxib (5 mg/kg b.i.d. p.o. for 16 days) commenced 17 days postligation, when stable baselines had been established. Plasma samples were taken on days 22 and 28 (days 5 and 11 postdose) from different animals on each occasion (n = 5) and on days when behavioral testing did not occur. On the last day of dosing (day 16), terminal plasma and brains were taken from four CCI rats treated with GW406381X or rofecoxib for determination of drug levels. In a separate study, celecoxib (10 mg/kg b.i.d. p.o.) or rofecoxib (5 mg/kg b.i.d. p.o.) were dosed for 12 days (on days 17–28 postligation). To determine a dose-response relationship to GW406381, in a further study, CCI animals were prepared as de-
scribed above and chronically dosed with GW406381X (0.3–5.0 mg/kg p.o.) or vehicle for 5 days (on days 34–38 postligation). In all studies, mechanical hyperalgesia was assessed 2 h following the initial administration of drug and on subsequent days throughout the remainder of the study.

Mouse Partial Ligation Model. Animals were habituated to test chambers for 3 days prior to testing. On days –4 and –1, paw withdrawal latency to a thermal stimulus was measured for both paws using a localized heat source (Plantar Test Apparatus; Ugo Basile). On day 0, mice were anesthetized with isoflurane, the left sciatic nerve was exposed, and a tight ligature (1/00 Virgin Silk; Ethicon, Somerville, NJ) was tied around the dorsal third of the nerve and tied tightly before closing the wound with a suture (70 Mersilk; Ethicon). Mice were allowed to recover for 3 days before...
testing commenced. Paw withdrawal latencies (PWLs) were measured 3, 6, 7, 8, 10, 14, 17, and 21 days postoperatively. Following testing on day 6, mice were allocated into treatment groups such that mean PWLs were similar between groups. GW406381X (10 mg/kg p.o. b.i.d.) was dosed on days 7 to 10 postsurgery, and testing took place 60 min after the first dose.

Capsaicin-Induced Secondary Hyperalgesia

Acute Dosing. Rats were dosed orally with GW406381X (5 and 15 mg/kg), celecoxib, rofecoxib (10 mg/kg), or vehicle 30 min before being lightly anesthetized with isoflurane and injected intraplantar with capsaicin (10 μg in 10 μl) at the heel of the left hind paw (Gilchrist et al., 1996) (n = 5 per group).

Chronic Dosing. Rats (n = 5 per group), were dosed with GW406381X (5 mg/kg b.i.d. p.o.), rofecoxib (10 mg/kg b.i.d. p.o.), or vehicle for 5 days. Thirty minutes after the morning dose on day 5, the animals were lightly anesthetized with isoflurane and injected intraplantar with capsaicin (10 μg in 10 μl) into the heel of the left hind paw.

Intrathecal Dosing. Rats (n = 5 per group) were lightly anesthetized with isoflurane and received either GW406381X (0.0015–0.5 μg i.t.), celecoxib (20 μg i.t.), rofecoxib (5 and 15 μg i.t.), or vehicle immediately prior to being injected intraplantar with capsaicin (10 μg in 10 μl) into the heel of the left hind paw. In all experiments, the animals were left for a period of 30 min after capsaicin injection to allow the secondary hyperalgesia to develop before behavioral assessment. The presence of mechanical (tactile) allodynia was assessed by applying Von Frey hair monofilaments (range: 2.35–46.54 g) of increasing intensity. The presence of allodynia was assessed by applying Von Frey hair monofilaments (range: 2.35–46.54 g) of increasing intensity.

Data Analysis and Statistics

Carrageenan and FCA. Weight-bearing ability was recorded in grams and edema measured in milliliters for each foot separately and expressed as the difference (Δ) between the FCA-injected paw and the contralateral paw. The percentage reversal was calculated for each animal using the formula: (test – mean vehicle/base line) × 100, expressed as mean ± S.E.M., and an ED50 (with confidence limits) calculated using the statistical package SPSS (SPSS Inc., Chicago, IL). Drug-treated groups were compared with vehicle using Fisher’s test.

CCI. PWTs were measured in grams for the left and right paws separately and expressed as mean ± S.E.M. Drug-treated groups were compared with the vehicle-treated groups at each time point using an unpaired Student’s t test (where P < 0.05 was considered significant). Where appropriate, ED50 Values (50% of response observed in vehicle animals at a particular time point) were calculated as geometric means with 95% confidence intervals, of the form x1±y, where y±x form the 95% confidence interval. These were generated using the statistical package SPSS.

Mouse Partial Ligation. PWLs were measured in seconds, and the left PWL (ipsi) was expressed as a percentage of that for the right paw (contra) (%ipsi/contra). Only mice with an ipsi/contra ratio ≤ 80% on day 6 were included in the study. Data are expressed as mean ± S.E.M. and compared between groups using ANOVA followed by post hoc Duncan’s test.

Capsaicin-Induced Secondary Hyperalgesia. The frequency of paw withdrawal to a particular Von Frey hair was measured, and the mean force in grams required to elicit a 50% response (5 withdrawals to 10 stimulations; EG50) was calculated in Robosage Excel. A two-way ANOVA followed by the Benjamini-Hochberg approach for multiple comparisons was used to compare EG50. The percentage reversal was calculated from the mean EG50 data using the formula: (ipsi drug EG50 – ipsi vehicle EG50/contra vehicle EG50 – ipsi vehicle EG50) × 100. Percentage reversal was used to calculate an ED50 in Robosage Excel.

Compound and Other Substances: Preparation and Dosing. GW406381X (Beswick et al., 2004), celecoxib, and rofecoxib were dissolved in 1% DMSO, 66% polyethylene glycol 400, and 33% distilled water. For the capsaicin studies, GW406381X, celecoxib, and rofecoxib were dissolved in 1% DMSO, 49.5% polyethylene glycol 400, and 49.5% distilled water. Further dilutions were prepared in the appropriate vehicles. All compounds were freshly prepared before use, except chronic studies where the dissolved compound was stored in a refrigerator between doses.

The dose-response CCI study used GW406381X spray dried powder 49.0% (w/w) (wet bead milled) supplied by Pharmaceutical Development (GlaxoSmithKline). GW406381X [49% (w/w); 355.65 mg] was made to 1 g with water to make a 170 mg/g suspension. This suspension was diluted with Pharmacoat 603 (Hydroxypropylmethylcellulose, batch 009395, made up as a 4% solution in distilled water) to the required concentrations on each day of dosing. Capsaicin was dissolved in 10% ethanol, 10% Tween 80, and 80% saline for intraplantar injection. Carrageenan was prepared in distilled water to give a final concentration of 2%. Freund’s Complete Adjuvant (1 mg/ml M. tuberculosis) was supplied by Sigma-Aldrich.

Enzyme Kinetics

COX-2 Transfection. Human COX-2 cDNA was cloned into a BamHI site of the baculovirus expression vector pBlueBacIII (Invitrogen, Carlsbad, CA). The resulting plasmid (COX-2 pBlueBacIII) was used to transfect Sf9 Spodoptera cells in a similar fashion to that described by Cromlish et al. (1994). The Sf9 cells were infected at a multiplicity of infection of three to five and harvested 72 h postinfection. The washed cell pellets were immediately frozen at −80°C.

COX-2 Fluorogenic Assay. The preparation of a microsomal cell extract broadly followed the method of Percival et al. (1994). The fluorogenic COX-2 assay is based on the paper of Mekv et al. (1985). Due to the slow, tight-binding nature of the diarylheterycyles, the dissociation of inhibitor from the pseudooirreversible end complex was analyzed by monitoring the reaction in peroxidase activity over the initial pseudolinear phase of the progress plot because cyclooxygenase self-inactivates with time as it turns over arachidonic acid (AA) (Wu et al., 2003). The putative dissociation rate provides a relative method of ranking the affinities of the tested diarylheterycyles for COX-2. The assay utilizes homovanillic acid (HVA) as a reducing cosubstrate to donate electrons to the reaction resulting in the dimerization of HVA and production of a fluorescent signal. The assay was performed on a LS-50B spectrophurometer (PerkinElmer Life and Analytical Sciences, Boston, MA) with excitation at 315 nm and emission at 425 nm. The assay buffer consisted of 50 mM HEPES, 5 mM EDTA, 1 mM HVA, and 1 μM hematin, pH 8.0. COX-2 enzyme was thawed on ice prior to a 1:200 in the assay buffer dilution and 10-min equilibration. Compounds were dissolved in DMSO and added to the enzyme/buffer mix to final concentration of 200 nM, 0.1% (v/v) DMSO. Following a 40-min preincubation of enzyme and inhibitor at room temperature, the reaction was initiated by the addition of 40 mM AA in ethanol to give a final AA concentration of 40 μM. Putative dissociation rates were extracted by fitting the integrated rate equation (Morrison and Walsh, 1988) to the initial 300 s of pseudolinear data from the progress plots using Grafit 5.0 (Erithacus Software, Horley, Surrey, UK).

Results

In Vitro Assays

GW 406381X, celecoxib, and rofecoxib produced concentration-related inhibition of PGE2 release from human COX-2 transfected COS cells but had little or no effect on PGE2 release from human COX-1 transfected COS cells. In human whole-blood assays, GW 406381X, celecoxib, and rofecoxib inhibited LPS-induced PGE2 release from human whole blood (COX-2 assay) in a concentration-dependent manner.
but had little effect in the COX-1 assay. GW406381X was more potent and selective than either celecoxib or rofecoxib in both these in vitro assays (Table 1).

**Clearance and CNS Penetration**

Blood clearance and CNS penetration were determined following a constant-rate intravenous infusion to steady state. GW406381X is a low-clearance compound similar to rofecoxib, whereas celecoxib has extremely low clearance (Table 1). CNS penetration, as determined from steady-state brain and blood levels, was highest for GW406381X and lowest for celecoxib, with rofecoxib giving a value that was intermediate between GW406381X and celecoxib (Table 1).

**Behavioral Studies**

**Carrageenan-Induced Inflammatory Hyperalgesia.**

Three hours after intraplantar carrageenan (100 μl of 2% w/v), there was a decrease in weight-bearing ability on the injected paw and an increase in paw volume (e.g., for vehicle-treated rats, weight-bearing percent reversal = 21.8 ± 2.7%; paw volume, left carrageenan-injected paw = 2.6 ± 0.09 versus 1.2 ± 0.04 ml for uninflamed right paw). GW406381X (1–10 mg/kg p.o.) produced a dose-related reversal of the decrease in weight bearing on the carrageenan-injected paw, which was maximal at 10 mg/kg (88 ± 8%) with an ED₅₀ of 3.9 (2.7–5.9) mg/kg p.o. (Table 2). There was also a significant reversal of the associated paw edema (Table 2). Neither rofecoxib nor celecoxib had any significant effect on the associated paw edema (Table 2).

GW406381X (5 mg/kg p.o. b.i.d.) had no effect on the first day of dosing, 2 h after the first dose but by day 3 of dosing had fully reversed ipsilateral PWT to levels that were similar to the contralateral paw (day 22 postdose, ipsi = 109 ± 7.2 g; contra = 107.5 ± 6.7 g) and were significantly different from vehicle-treated CCI rats (day 22 postdose, ipsi = 86.0 ± 4.5 g; contra = 106.0 ± 6.5 g). This effect was maintained until the end of the dosing period on day 28 postsurgery (GW406381X ipi = 115.0 ± 9.8 g, contra = 111.0 ± 3.9 g; vehicle ipi = 79.0 ± 5.9 g, contra = 103.5 ± 6.0 g).

In another study, both GW406381X and rofecoxib were dosed and blood samples taken during the dosing period. Terminal plasma and brain samples were taken at the end of the dosing period. Again, following CCI, there was a significant decrease in the mean PWT of the ipsilateral hind paw (ipsi) compared with the contralateral (contra) paw, and there was no significant difference between the vehicle- and GW406381X-treated groups before dosing commenced on day 20 postsurgery (predose PWT, CCI vehicle group ipsi = 84.5 ± 3.6 g, contra = 95.5 ± 5.1 g; CCI GW406381X group ipsi = 79.5 ± 5.6 g, contra = 97.0 ± 5.8 g). GW406381X (5 mg/kg p.o. b.i.d.) had no effect on the first day of dosing, 2 h after the first dose but by day 3 of dosing had fully reversed ipsilateral PWT to levels that were similar to the contralateral paw (day 22 postdose, ipsi = 109 ± 7.2 g; contra = 107.5 ± 6.7 g) and were significantly different from vehicle-treated CCI rats (day 22 postdose, ipsi = 86.0 ± 4.5 g; contra = 106.0 ± 6.5 g). This effect was maintained until the end of the dosing period on day 28 postsurgery (GW406381X ipi = 115.0 ± 9.8 g, contra = 111.0 ± 3.9 g; vehicle ipi = 79.0 ± 5.9 g, contra = 103.5 ± 6.0 g).

**Established Inflammatory Hyperalgesia.** GW406381X dosed 24 h post-FCA produced a dose-related reversal of the decrease in weight bearing on the injected paw when measured 6 h postdose. A maximal reversal was seen at 10 mg/kg (91 ± 9%) with an ED₅₀ of 1.5 (1.1–2.9) mg/kg p.o. (Table 2). Celecoxib and rofecoxib also produced a dose-related reversal of the decrease in weight bearing on the FCA-injected paw when measured at the same time postdose. For celecoxib, the ED₅₀ was 6.6 (1.2–10.9) (maximum reversal = 70 ± 15% at 15 mg/kg), and for rofecoxib, the ED₅₀ was 1.0 (0.6–1.4) mg/kg p.o. (maximum reversal 102 ± 14; Table 2). None of the compounds reduced the associated increase in paw volume in this model.

**CCI Model.** Following CCI, there was a significant decrease in the mean mechanical PWT of the left ipsilateral hind paw (ipsi) compared with the contralateral (contra) paw, and there was no significant difference between the vehicle- and GW406381X-treated groups before dosing commenced on day 20 postsurgery (predose PWT, CCI vehicle group ipsi = 84.5 ± 3.6 g, contra = 95.5 ± 5.1 g; CCI GW406381X group ipsi = 79.5 ± 5.6 g, contra = 97.0 ± 5.8 g). GW406381X (5 mg/kg p.o. b.i.d.) had no effect on the first day of dosing, 2 h after the first dose but by day 3 of dosing had fully reversed ipsilateral PWT to levels that were similar to the contralateral paw (day 22 postdose, ipsi = 109 ± 7.2 g; contra = 107.5 ± 6.7 g) and were significantly different from vehicle-treated CCI rats (day 22 postdose, ipsi = 86.0 ± 4.5 g; contra = 106.0 ± 6.5 g). This effect was maintained until the end of the dosing period on day 28 postsurgery (GW406381X ipi = 115.0 ± 9.8 g, contra = 111.0 ± 3.9 g; vehicle ipi = 79.0 ± 5.9 g, contra = 103.5 ± 6.0 g).

In another study, both GW406381X and rofecoxib were dosed and blood samples taken during the dosing period. Terminal plasma and brain samples were taken at the end of the dosing period. Again, following CCI, there was a significant decrease in the mean PWT of the ipsilateral hind paw, compared with the sham-operated group (e.g., for the vehicle group, CCI = 73.5 ± 7.0 g; sham = 111.0 ± 6.0 g; P < 0.01). GW406381X (5 mg/kg p.o. b.i.d.) had no effect on the first day of dosing, 2 h after the first dose, but by 5 days postdose, the ipsilateral PWT was not different from sham vehicle levels (CCI PWT 406381X = 111.5 ± 16.5 g; CCI PWT vehicle = 83.5 ± 5.6 g; sham vehicle = 111.9 ± 9.0 g) (Fig. 1a). This reversal was maintained for a further 5 days and was not significantly different from vehicle by the end of the dosing period (day 16 postdose). In contrast to GW406381X, rofecoxib (5 mg/kg p.o. b.i.d.) had no effect on the CCI-induced

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GW406381X</th>
<th>Celecoxib</th>
<th>Rofecoxib</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCOX-1 (nM)</td>
<td>&gt;84,000</td>
<td>1689</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>hCOX-2 (nM)</td>
<td>3</td>
<td>68</td>
<td>32</td>
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<td>Selectivity</td>
<td>28,067</td>
<td>24,8</td>
<td>&gt;3125</td>
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<tr>
<td>HWB COX-1 (nM)</td>
<td>31,557</td>
<td>10,194</td>
<td>15,500</td>
</tr>
<tr>
<td>HWB COX-2 (nM)</td>
<td>42</td>
<td>336</td>
<td>260</td>
</tr>
<tr>
<td>Selectivity</td>
<td>749</td>
<td>30.3</td>
<td>59.6</td>
</tr>
<tr>
<td>CLb (mL/min/kg)</td>
<td>9.01 ± 1.34</td>
<td>0.76 ± 0.05</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Blood Css (μM)</td>
<td>0.46 ± 0.06</td>
<td>1.68 ± 0.10</td>
<td>2.47 ± 0.31</td>
</tr>
<tr>
<td>Brain Css (μM)</td>
<td>0.71 ± 0.24</td>
<td>0.23 ± 0.01</td>
<td>1.97 ± 0.43</td>
</tr>
<tr>
<td>Brain/blood ratio</td>
<td>1.53 ± 0.401</td>
<td>0.14 ± 0.01:1</td>
<td>0.80 ± 0.16:1</td>
</tr>
</tbody>
</table>

HWB, human whole-blood assay; CLb, blood clearance; Css, concentration at steady state.
decrease in PWT at any time point over the same dosing period (Fig. 1a).

Analysis of serial blood samples taken on days 5 and 11 and terminally on day 16 postdose (days 21, 27, and 32 postsurgery) showed similar micromolar concentrations of GW406381X and rofecoxib at each time point, suggesting that steady-state conditions had been reached for both compounds. Mean blood concentrations over the dosing period for GW406381X and rofecoxib were $1.8 \pm 0.08$ and $2.4 \pm 0.1$ μM, respectively, and represented values 591 times the IC$_{50}$ determined in vitro for GW406381X (3 nM) and 76 times the IC$_{50}$ for rofecoxib (32 nM). However in the brain, the concentration of GW406381X was approximately 5× that of rofecoxib ($2.3 \pm 0.2$ and $0.5 \pm 0.03$ μM, respectively. The brain/blood ratio calculated for both GW406381X (1.3) and rofecoxib (0.2) was less than that obtained from steady-state studies following intravenous administration (Table 1).

In a separate study, celecoxib (10 mg/kg b.i.d. p.o. for 5 days) also had no effect on PWTs at any time during the dosing period, and rofecoxib (5 mg/kg b.i.d. p.o. for 5 days) once again was without effect (Fig. 1b). When the dose-response relationship for GW406381X (0.3, 1, and 3 mg/kg p.o. b.i.d.) was investigated, a dose-related attenuation of the CCI-induced decrease in PWT was seen. On the 5th day of dosing, when other studies have shown GW406381X to be maximally effective, an ED$_{50}$ of $0.85 (0.45–1.6)$ mg/kg p.o. was calculated.

### Mouse Partial Ligation Model.

Partial ligation of the sciatic nerve produced a decrease in PWL from a presurgery baseline of $102.8 \pm 3.8\%$ to $42.6 \pm 4.3\%$ and $42.6 \pm 4.7\%$ [expressed as (ipsilateral PWL/contralateral PWT) $\times 100$] in the animals allocated to the GW406381X group and vehicle groups, respectively. GW406381X (10 mg/kg p.o. b.i.d. for 4 days) had no significant effect on the first day of dosing, due to a high vehicle response, but had produced a complete reversal of thermal hyperalgesia back to baseline levels after 3 days of dosing (GW406381X $111.6 \pm 10.0\%$; vehicle $41.6 \pm 7.5\%$, $n = 15–17$ per group), which was maintained to the end of the dosing period (Fig. 2). In the same study, rofecoxib (10 mg/kg p.o. b.i.d. for 4 days) was not well tolerated, and this arm of the study was terminated.

### Capsaicin-Induced Secondary Hyperalgesia.

Capsaicin injection into the plantar surface of the heel decreased
the EG$_{50}$ to Von Frey hair application (e.g., 42.2 g on the contralateral paw to 5.6 g on the ipsilateral paw in vehicle-treated rats in oral acute administration study). Single, oral doses of GW406381X (5 or 15 mg/kg) (Fig. 3a), and in a separate experiment, celecoxib (10 mg/kg) (Fig. 3b), had no effect on this decrease compared with vehicle. However, following chronic dosing, GW406381X (5 mg/kg b.i.d. p.o. for 5 days) significantly reversed the EG$_{50}$ from 5.7 g in vehicle-treated rats to 16.0 g (Fig. 3c). In contrast, in the same study, rofecoxib (10 mg/kg b.i.d. p.o. for 5 days) had no significant effect (Fig. 3c).

When given intrathecally, acute doses of GW406381X (0.015–0.5 µg) significantly attenuated the capsaicin-induced decrease in EG$_{50}$ from 6.9 to 27.9 g at 0.5 mg/kg (Fig. 4a). An ED$_{50}$ of 0.07 µg i.t. was calculated. Neither celecoxib (20 µg) (Fig. 4b), nor rofecoxib (5 and 15 µg) given intrathecally, had any effect on capsaicin-induced hyperalgesia (Fig. 4b). In the rofecoxib experiment, GW406381X (0.05–0.5 µg) produced a similar profile to that seen previously.

**Enzyme Kinetics.** The DMSO control showed the expected self-inactivation of COX-2 with time in the presence of AA (Fig. 5). Rofecoxib was displaced rapidly with a putative dissociation rate of 0.698 min$^{-1}$ with all of the COX-2 being inactivated to the DMSO control level. Celecoxib was displaced less rapidly with a putative dissociation rate of 0.066 min$^{-1}$, also reaching the same level as the DMSO control but over a longer period of time. In contrast, GW406381 was displaced at a significantly slower rate (0.0041 min$^{-1}$). This is 16- and 170-fold slower than celecoxib and rofecoxib, respectively (Fig. 5).

**Fig. 3.** a, GW406381X (5 and 15 mg/kg p.o., 60 min pretest) had no effect on the frequency of paw withdrawal to a range of Von Frey hair applications. b, celecoxib (10 mg/kg p.o. 60 min pretest and 20 µg i.t. 30 min pretest) had no effect on the capsaicin-induced secondary allodynia. c, GW406381X (5 mg/kg p.o. b.i.d. for 5 days) significantly attenuated capsaicin-induced secondary allodynia, whereas rofecoxib (5 mg/kg p.o. b.i.d.) was ineffective. Data are expressed as mean ± S.E.M. for the left paw (capsaicin injected) with the contralateral paw of the vehicle-injected group acting as a control and the frequency of paw withdrawal to 10 presentations of each Von Frey hair recorded. *P < 0.05, ANOVA followed by the Benjamini-Hochberg approach for multiple comparisons (n = 5 rats per group).

**Fig. 4.** a, GW406381X (0.015–0.5 µg i.t.) given 30 min prior to testing dose-dependently reversed capsaicin-induced secondary allodynia. b, rofecoxib (5 and 15 µg i.t.) given 30 min pretest had no effect on capsaicin-induced secondary allodynia, whereas GW406381X showed dose-related reversal of this parameter that was similar to that seen in (A). Data are expressed as mean ± S.E.M. for the left paw (capsaicin injected) with the contralateral paw of the vehicle-injected group acting as a control and the frequency of paw withdrawal to 10 presentations of each Von Frey hair recorded. *P < 0.05, ANOVA followed by the Benjamini-Hochberg approach for multiple comparisons.
Discussion

In models of inflammatory pain such as the carrageenan and FCA models, the COX-2 inhibitor GW406381X was as effective as gold standard compounds such as rofecoxib and celecoxib, which are used to treat inflammation-induced pain in man (Cannon, 2000; Clemett and Goa, 2000). However, in marked contrast to rofecoxib and celecoxib, orally administered GW406381X was also effective in three models of neuropathic pain (central sensitization, rat CCI, mouse partial ligation, and capsaicin-induced allodynia). This is the first time that this action has been demonstrated for a COX-2 inhibitor, and we suggest that GW406381X has dual activity insofar as it possesses activity in both inflammatory and neuropathic pain models.

The CCI model has a large inflammatory component, which may be directly at the site of injury (Lindenlaub and Sommer, 2000) or indirect via neurogenic inflammation distal to the site of injury (Daemen et al., 1998). COX-2 is upregulated in injured nerve, and locally administered ketorolac (mixed COX-1/COX-2 inhibitor) reduces allodynia in neuropathic pain models (Ma and Eisenach, 2003); therefore, it could be argued that this is the site of action of GW406381X in this model. However, if this were the case, there is nothing to preclude rofecoxib also acting at this peripheral site. Indeed, in the CCI model, when blood samples were taken from rats treated for 16 days with either GW406381X or rofecoxib both at 5 mg/kg b.i.d., mean blood levels of the two compounds were similar (1.8 and 2.4 μM, respectively). Blood levels of GW406381X and rofecoxib in the CCI model were 591 and 76 times the IC₅₀ in vitro, respectively, based on potency and efficacy in the FCA model.

It could be argued that activity would have been seen with rofecoxib had blood levels been a similar multiple of the IC₅₀ to that of GW406381. However, this might be difficult to achieve because rofecoxib has dose-limited exposure and non-linear kinetics (Halpin et al., 2000). In addition, although the potency of GW406381X in vitro in COS cells expressing COX-2 is an order of magnitude greater than that of rofecoxib (IC₅₀ for GW406381X = 3 versus 32 nM for rofecoxib) and approximately 23 times that of celecoxib (IC₅₀ = 68 nM), this does not translate into same difference in potency in vivo, at least in the established FCA model, where the ED₅₀ values for GW406381X, celecoxib, and rofecoxib all fall within a similar range (1.5, 6.6, and 1.0 mg/kg, respectively).

In inflammatory and neuropathic pain, central sensitization models, there is thought to be sensitization of nociceptive pathways at both peripheral and central sites (Woolf and Salter, 2000; Ji and Woolf, 2001), and up-regulation of COX-2 is implicated in both these mechanisms (Ichitani et al., 1997; Beiche et al., 1998; Ma and Eisenach, 2002). Indeed, the COX-2 inhibitor 1-[(4-methylsulfonyl)phenyl]-3-tri-fluoromethyl-5-(4-fluorophenyl) pyrazole (SC58125) inhibits carrageenan-induced thermal hyperalgesia when given intrathecally prior to carrageenan (Yaksh et al., 2001), suggesting the generation of a central COX-2 component, which can be important in inflammation-induced pain, if the COX-2 inhibitor is given access. Furthermore, if access to a central site of action is limited, then potency at the target may then become a consideration.

Under steady-state conditions, celecoxib is poorly CNS penetrant, whereas rofecoxib has good CNS penetration, and GW406381X is more CNS penetrant still. In the CCI chronic dosing study, brain penetration of GW406381X and rofecoxib was less than that when dosed to true steady state but still greater for GW406381X compared with rofecoxib. In the capsaicin and CCI models, chronic dosing was required to show efficacy when GW406381X was administered orally, whereas in the capsainic model, intrathecal dosing was effective acutely. These data support a central role for COX-2 in these models and suggest that access to this central compartment may be a factor in the efficacy of GW406381X.

Brain levels for GW406381X and rofecoxib were determined in the CCI model at the end of the dosing period and showed that the concentration of GW406381X (2.3 μM) was similar to that in the blood and 772 times the IC₅₀ in vitro. In contrast, brain levels of rofecoxib were much lower (0.5 μM) and only 14 times the IC₅₀ in vitro.

Although these data might suggest that following systemic chronic dosing, concentrations of rofecoxib are not sufficient in the CNS to inhibit CCI-induced decreases in PWT, the fact that rofecoxib was also unable to inhibit capsaicin-induced allodynia when given intrathecally suggests that it is not simply access to a central site of action that is important. Indeed, the mixed COX-1/COX-2 inhibitor, ketorolac, and the COX-2 inhibitor N-[2-(cyclohexyloxyl)-4-nitrophenyl]-methane sulfonamide (NS-398) can produce antiallodynia in neuropathic pain models when delivered intrathecally (Ma et al., 2002). Another possibility is that the kinetics of cyclooxygenase inhibition may be a factor in determining this differentiation. The data for putative dissociation rates of GW406381X, celecoxib, and rofecoxib from COX-2 show that there are considerable differences in the ability of AA to displace various diarylheptanes from COX-2, with GW406381X demonstrating by far the slowest rate of dissociation from the COX-2 enzyme, by over two orders of magnitude in comparison with rofecoxib. The putative dissociation rate for celecoxib is in reasonable agreement with published data, being 4- to 5-fold higher than the figure of 0.014 min⁻¹ quoted by...
Bennett GJ and Xie YK (1988) A peripheral mononeuropathy in rat that produces pain.

Hood et al. (2003), which used unlabeled compound instead of the natural substrate to compete off-labeled compound.

These data showing that there is differentiation at the level of the enzyme are the strongest pieces of evidence for a possible mechanism underlying the differentiation of GW406381X in vivo but still cannot be the sole explanation. These data generated suggest that for the neuropathic pain and central sensitization models, a central site of action is important that is possibly independent of peripheral input. Therefore, to be effective in these models, COX-2 inhibitors must be able to access the CNS at sufficient concentrations to inhibit COX-2. Once there, a compound such as GW406381, with its high affinity for the COX-2 enzyme and its ability to block the active site for long periods of time, thereby preventing AA turn over, may reduce the amount of prostaglandins produced and, hence, reduce central sensitization.

These data suggest that, at the time points studied in these experiments in the carrageenan and established FCA models, the central COX-2 contribution to hyperalgesic mechanisms is more dependent on peripheral drive since celecoxib and rofecoxib were fully effective at reversing decreases in weight bearing ability due to inflammation. Presumably, these effects at the level of the enzyme are also operating peripherally, but plasma concentrations of celecoxib and rofecoxib are such that they are still able to effectively inhibit the production of prostaglandins.

In conclusion, these properties of GW406381X (greater CNS penetration and slower dissociation rate from the enzyme) together with its potency may explain its differentiation, when given orally, in models of neuropathic pain and central sensitization, the first time that such an activity has been described for a COX-2 inhibitor. This novel finding and associated data suggest that GW406381X has dual activity in vivo but still cannot be the sole explanation.

References


