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Evaluation of COX-1/COX-2 selectivity and potency of a new class of COX-2 inhibitors

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ABSTRACT

A new class of selective cyclooxygenase-2 (COX-2) inhibitors has been identified by high throughput screening. Structurally distinct from previously described selective COX-2 inhibitors, these benzopyrans contain a carboxylic acid function and CF_3 functionality. The compound SC-75416 is a representative of this class. A range if *in vitro* and *in vivo* tests were employed to characterize its potency and selectivity. Using human recombinant enzymes, this compound displays a concentration that provides 50% inhibition (IC_{50}) of 0.25 μM for COX-2 and 49.6 μM for COX-1. A mutation of the side pocket residues in COX-2 to COX-1 had little effect on potency suggesting that these inhibitors bind in a unique manner in COX-2 distinct from COX-2 inhibiting diaryl heterocycles. Using rheumatoid arthritic synovial cells stimulated with interleukin-1β (IL-1β) and washed platelets the compound displayed IC_{50} of 3 nM and 400 nM respectively. Potency and selectivity was maintained but predictably right shifted in whole blood with IC_{50} of 1.4 μM for lipopolysaccharide (LPS) stimulated induction of COX-2 and >200 μM for inhibition of platelet thromboxane production. SC-75416 is 89% bioavailable and its *in vivo* half life is sufficient for once a day dosing. In the rat air pouch model of inflammation, the compound inhibited PGE_2 production with an effective dose that provides 50% inhibition (ED_{50}) of 0.4 mg/kg, while sparing gastric prostaglandin E2 (PGE_2) production with an ED_{50} of 26.5 mg/kg. In a model of acute inflammation and pain caused by carrageenan injection into the rat paw, the compound reduced edema and hyperalgesia with ED_{50}s of 2.7 and 4 mg/kg respectively. In a chronic model of arthritis the compound demonstrated an ED_{50} of 0.081 mg/kg and an ED_{80} of 0.38 mg/kg. In a model of neuropathic pain, SC-75416 had good efficacy. This compound's unique chemical structure and effect on COX enzyme binding and activity as well as its potency and selectivity may prove useful in treating pain and inflammation.

1. Introduction

Cyclooxygenase (COX) is a bi-functional enzyme that first catalysis the addition of two molecules of oxygen to arachidonic acid to form the hydroperoxide prostaglandin G_2 (PGG_2), then reduces the hydroperoxide to the alcohol, PGH_2, by a peroxidase activity (Smith and Song, 2002). Cyclooxygenase is found in two forms; COX-1, which is constitutively expressed in most cells and is responsible for the production of prostaglandins that maintain homeostasis; COX-2 by contrast is upregulated in inflammatory cells in response to an inflammatory stimulus (cytokines, lipopolysaccharide (LPS) etc) and responsible for the production of prostaglandins at the site of inflammation (Crofford, 1997; Masferrer and Seibert, 1994; Mitchell et al., 1993; Seibert and Masferrer, 1994). COX-2 is also expressed to a lesser extent constitutively in brain and kidney (Seibert et al., 1997).

The advantage of a COX-2 selective inhibitor soon became apparent and testing schemes were developed that relied on using partially purified enzymes expressed in insect cells (Gierse et al., 1995; Meade et al., 1993). *In vitro* recombinant enzyme assays provided a powerful means for assessing COX selectivity and potency and led to the discovery and clinical development of the first rationally designed COX-2 selective inhibitors, celecoxib (Gierse et al., 1999; Penning et al., 1997) and rofecoxib (Black et al., 1999). These assays also allowed for the rapid screening of millions of compounds against isolated human recombinant COX-1 and COX-2. As a result of this screening a class of compounds was identified with unique structural features. These compounds were benzopyrans which included a carboxylic acid. Following many rounds of optimization for *in vitro* and *in vivo* potency, SC-75416 emerged as the leading example of this class of COX-2 selective inhibitors (Fig. 1).

Due to the fact that varying kinetic mechanisms affect potency for COX-1 vs. COX-2, relying on just enzyme assays will not always predict eventual COX-2 potency and selectivity *in vivo* (Copeland et al., 1994). Potency and selectivity in human whole blood is used by many groups and has been accepted as a standard assessment of COX-2 potency and selectivity (Gierse et al., 2005; Patrignani et al., 1997). In order to more fully characterize COX-2 potency and selectivity of SC-75416, a series of cellular and *in vivo* assays were performed. Both serum free and whole blood assays have been used to assess COX inhibition. COX-2 has been

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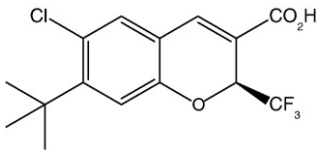

Fig. 1. Structure of SC-75416. S)-6-chloro-7-(1,1-dimethylethyl)-2-(trifluoromethyl)-2H-1-benzopyran-3-carboxylic acid.

shown to be induced in fibroblast cells collected from arthritis patients (Kishore et al., 2003) and these were used to assess COX-2 inhibition along with washed platelets as a source of COX-1 (Riendeau et al., 1997).

In addition, *in vivo* potency and selectivity can be assessed using a rat air pouch model of inflammation. Measurement of decreased PGE_2 in the inflamed pouch fluid as a marker for COX-2 inhibition can be accompanied by measurement of decreased PGE_2 levels in the gastric mucosa from the same animal as a marker of COX-1 inhibition (Masferrer et al., 1994).

In vivo efficacy was measured in several rat models of inflammation, arthritis, and pain. In carrageenan-stimulated models, inflammation and hyperalgesia can be blocked by pretreatment with non-steroidal anti-inflammatories (NSAIDs) or selective COX-2 inhibitors (Hargreaves et al., 1988; Seibert et al., 1994; Zhang et al., 1997). Only inhibition of COX-2 or the subsequent PGE_2 product reduces hyperalgesia and edema (Portanova et al., 1996; Smith et al., 1998). A chronic model of arthritis produced by injecting a rat paw subcutaneously with mycobacterium butyricum has proven to be useful in estimating human clinical doses (Gierse et al., 2005). Spinal nerve ligation was also used as a model of neuropathic pain and evaluated using Von Frey hairs to measure the tactile allodynia such pain produces. NSAIDs and COX-2 inhibitors have been historically poor in relieving this type of pain. Indeed, despite the numerous pharmacological agents available, a sub-population of pain patients remains underserved, due to untoward side effects or lack of efficacy for some individuals. A new chemical class of COX-2 inhibitors, such as SC-75416, may prove useful for this patient population. As with any new compound, clinical trials will be required to determine clinical benefit.

2. Materials and methods

2.1. Materials

Arachidonic acid (AA), supplied as the sodium salt (NuChek Prep, Elysian, MN); Prostaglandin E2 (PGE_2) and thromboxane B2 (TxB_2) enzyme linked Immunosorbant assay (ELISA) kits (Cayman Chemical Ann Arbor, MI); All cell culture reagents were obtained from Gibco (Grand Island, NY); carrageenan (FMC, Rockland, ME); Male Lewis and Sprague–Dawley rats (Harlan, IN); celecoxib, gabapentin and SC-75416 ((S)-6-chloro-7-(1,1-dimethylethyl)-2-(trifluoromethyl)-2H-1-benzopyran-3-carboxylic acid) were prepared by Pfizer Medicinal Chemistry Department; LPS, indomethacin and all standard buffer reagents were obtained from Sigma, Chemical Co. (St. Louis, MO).

2.2. Preparation of enzymes

Genes coding for human COX-1 and COX-2 were expressed in insect cells and the active enzymes isolated as described previously (Gierse et al., 1995). Briefly, insect cells were suspended in Tris buffer plus ethylenediaminettraacetic acid (EDTA) and diethyl dithio carbamate (DEDTC), and the COX enzymes were extracted by addition of 1% chaps detergent. Cell debris was removed by centrifugation at 48,000 ×g and the resulting supernatants were aliquoted and frozen for use in the PGE_2 ELISA assay.

2.3. Mutagenesis

The coding region of murine COX-2 (mCOX-2) was subcloned into the plasmid pALTER-1, and mutagenesis was performed using an Altered Sites II Kit (Promega, Madison, WI) to change valine 434 to isoleucine, arginine 513 to histidine, and valine 523 to isoleucine (IHI COX-2).

2.4. Human recombinant enzyme assay

Compounds were evaluated for potency and selectivity of inhibition *in vitro* using baculovirus-expressed recombinant human COX-1 and COX-2 enzymes as previously described (Gierse et al., 1995) and will be summarized here. Enzymes were pre-incubated with inhibitors for 10 min at 25 °C. The reaction was started by the addition of 10 μM arachidonic acid and allowed to proceed for 10 min. The reaction was terminated by dilution of the reaction in to buffer containing 25 μM indomethacin. The amount of PGE_2 formed was measured by ELISA.

2.5. Rheumatoid arthritic patient synovial fibroblast assay for COX-2

Synovial fibroblasts were cultured from the inflamed synovium of a female, rheumatoid arthritic patient undergoing total knee replacement and the established rheumatoid arthritic synovial fibroblasts (RASF) cell line was used at passages 7–16 for these studies. Cells were maintained in DMEM high glucose, 15% FBS (JRH Biosciences, Lenexa, KS), 2 mM L-glutamine and 100 U/ml penicillin/100 μg/ml streptomycin at 37 °C, 5% CO_2. Confluent, adherent cells were harvested from flasks via trypsinization and plated onto 96 well microtiter tissue culture plates at a concentration of 25,000 cells per well (100 μl/well) in RPMI and 1% FBS. The cells were incubated (37 °C, 5% CO_2) for at least 2 h to allow for cell attachment. Assay medium or test compound solution was added to the wells and the plates were incubated for 1 h. The cells were then stimulated with 1 ng/ml recombinant, human, IL-1β (R&D Systems, Minneapolis, MN) and incubated for an additional 24 h. The culture medium was then removed from the cells and analyzed for PGE_2 production using an ELISA.

2.6. Washed platelet assay for COX-1

Human blood (200 ml) was collected in sodium citrate vacutainers. The blood was centrifuged and the platelet rich plasma was pipetted off and placed with anti-coagulant solution (1.5 g citric acid, 2.5 g sodium citrate, 2 g glucose in 100 ml H_2O). Plasma was then mixed with 50% volume of Hanks balanced salt solution buffered with 15 mM HEPES, pH 7.4 (HBSS) and 30% anti-coagulant solution, collected by centrifugation and resuspended in HBSS. Inhibitor was added to platelets and incubated for 10 min. Platelets were activated by addition of 2 μM calcium ionophore and incubated for 10 min. The reaction was stopped by addition of methanol and centrifuged. TxB_2 produced was measured by ELISA.

2.7. Human whole blood assay

The assay of Patrignani et al. (1997) was used to assess COX inhibition in human whole blood. To evaluate COX-1 mediated thromboxane B_2 (TxB_2) production, venous blood from allowed to clot and amount of TxB_2 determined by ELISA. To evaluate compounds for COX-2 mediated PGE_2 production, venous blood from healthy human donors was collected in heparinized tubes. Blood was incubated with 100 μg/ml lipopolysaccharide and compound overnight. PGE2 produced was quantitated by ELISA.

2.8. Pharmacokinetics

The pharmacokinetics and bioavailability of SC-75416 were evaluated in Sprague–Dawley rats after a single 10 kg/kg oral and

intravenous (IV) dose administration. Blood samples were collected in heparinized tubes at various time points and compound was quantified by liquid chromatography/mass spectrometry. Pharmacokinetic calculations were performed using the non-compartmental approach.

2.9. Carrageenan-induced pouch inflammation

Male Lewis rats 170–210 g were used in the study. Air pouches were formed between the scapula of the shaved rat backs by subcutaneous injection of 20 ml sterile air, while the animals were lightly anesthetized with CO_2:O_2. One day later, rats were dosed orally with compound in 1 ml of vehicle (0.5% methylcellulose, 0.025% Tween-20). The rats in the saline and vehicle groups were dosed with 1 ml of vehicle alone. Thirty minutes after dosing, 2 ml of 1% carrageenan suspension in saline were injected into each rat's pouch. At 3 h post-injection, animals were euthanized and the pouch fluid, and samples of blood and stomach mucosa were collected for analysis.

Fluid from the inflamed pouch was collected after lavage with 5 ml of cold phosphate buffered saline and centrifuged. Prostaglandin levels from the supernatants were quantified by ELISA.

To determine whether orally administered compounds inhibited a physiological prostaglandin production in this model, the rat stomachs were surgically removed, opened and rinsed in saline. Several pieces of mucosa from each stomach were sampled, pooled, and weighed (20–40 mg). The tissue was homogenized in methanol and centrifuged and dried under nitrogen. Amount of PGE_2 was determined by ELISA.

2.10. Carrageenan-induced paw inflammation

Male Sprague–Dawley rats, weighing 180–200 g were used in this experiment. Rats were fasted with free access to water ~16 h prior to testing. Carrageenan was prepared as 1% suspension in saline, and a volume of 0.1 ml was injected into the footpad tissue of the right ipsilateral hind paw while the non-injected contralateral footpad of each animal served as a normal control. Indomethacin or SC-75416 was dosed orally in vehicle (0.5% methyl cellulose, 0.025% Tween-20), 30 min before the carrageenan injection.

Edema was expressed as the increase in paw volume caused by the inflammatory response. Individual volumes of each hind paw were determined by water displacement plethysmometer and the volume of the contralateral paw was subtracted from the volume of the inflamed ipsilateral paw for each animal.

Hyperalgesia was determined by timing the withdrawal response to a thermal stimulus applied to the hind paws. Rats, individually confined in plexiglass chambers, temperature controlled at 30 °C, were exposed to focal heat from a high intensity projector bulb positioned sequentially beneath each hind paw. The difference in the withdrawal latency between inflamed ipsilateral and contralateral (control) paws served as a measurement of pain.

2.11. Adjuvant induced arthritis

Male Lewis rats 150–200 g were used in the study. Each rat received a subcutaneous injection of 1 mg of Freund's Complete Adjuvant (heat-killed Mycobacterium butyricum, Difco Laboratories, Detroit MI), suspended in light mineral oil (White Light, MF), into the plantar surface of the right hind paw. This injection causes an inflammation of the extremity joints, initially in the ipsilateral paw and spreading systemically to the contralateral hind paw and forepaws as time ensues. Fourteen days after injection, the volume of the paw/joint contralateral to the injected paw from each rat is inflamed and was measured using a plethysmometer. After 14 days post adjuvant injection, the volumes of contralateral paws in rats are larger than normal rat paws. Only injected rats in which the left (contralateral) hind paw/joint volume was 0.37 ml greater than the mean left paw/joint volume of normal matched control rats were selected for use. These rats were randomly assigned to treatment groups of 5 rats each. On day 20 after adjuvant injection, rats were dosed orally with 1 ml vehicle (0.5% methylcellulose/0.025% Tween 20) or 1 ml vehicle in which doses of SC-75416 (0.08 or 0.8 mg/kg) were suspended.

2.12. Model of peripheral nerve injury: spinal nerve ligation

Adult male Sprague–Dawley rats weighing 250–350 g were used. The left L5 and L6 spinal nerves were tightly ligated using 8-0 silk thread three months before the experiments. Naproxen (30 mg/kg), acetaminophen (100 mg/kg), SC-75416 (1, 3 and 10 mg/kg) and gabapentin (30 mg/kg) were given by oral gavage, suspended in vehicle (0.5% methylcellulose, 0.025% Tween-20, Sigma, St. Louis) and morphine (1 mg/kg, Henry Schein, Indianapolis) was dissolved in saline and injected subcutaneously. Tactile allodynia was determined by measuring paw withdrawal thresholds after probing of the plantar surface of the hindpaw ipsilateral to the surgery with a series of calibrated fine filaments (Von Frey hairs, Semmes-Weinstein Aesthesiometer, Stolting Co., Wood Dale, IL). Rats were placed in clear plastic cages on an elevated mesh floor and acclimated for 30 min before testing. Von Frey hairs were applied over the midplantar skin of the left hindpaw 2 consecutive times in rapid succession. All behavioral observations were performed 2 h following compound administration except morphine after 15 min and were performed with the observer blinded as to the identity of the group.

2.13. Statistical analysis

For plate based assays, inhibitory concentration at 50% inhibition (IC_{50}) were determined from a 4-parameter log fit of the data from a dose curve run in duplicate, with experimental controls of 0 and 100% of maximal activity fixed. Statistical analysis for behavioral observations was carried out on paw withdrawal thresholds measurements using ANOVA followed by Dennett's test.

2.14. Animal care and use

\All studies were carried out under the supervision and approval of the Institutional Animal Care and Use Committee. All studies were carried out in a dedicated facility where the animals are housed at a controlled temperature of 23 °C with a 12:12-h light dark cycle and the rats were given clean bedding and filtered tap water ad libitum.

3. Results

3.1. Human recombinant enzymes

Potency and selectivity of SC-75416 was demonstrated by inhibition of human recombinant enzymes, with an IC_{50} of vs. COX-1 and COX-2 of 49.6 μM and 0.25 μM respectively for a COX-1/COX-2 selectivity ratio of 198. This potency and selectivity ratio is similar to that previously observed with celecoxib, 15 μM COX-1 and 0.05 μM COX-2 (Gierse et al., 2005). Assessment of the interactions of SC-75416 with the side pocket mutant of COX-2 resulted in a slight decrease in potency 0.25 μM for COX-2 vs. 1.9 μM for IHI COX-2 (Fig. 2). This is in contrast to the diarylheterocycle compound celecoxib, were the side pocket mutation changes the mutant COX-2 IC_{50} to an IC_{50} that equals COX-1, 16.9 vs. 15 μM. These data suggests that there is little interaction with SC-75416 with the “side pocket” of COX-2 and that the mechanism of selectivity is unique to this class of compounds and different than that of diaryl heterocycles.

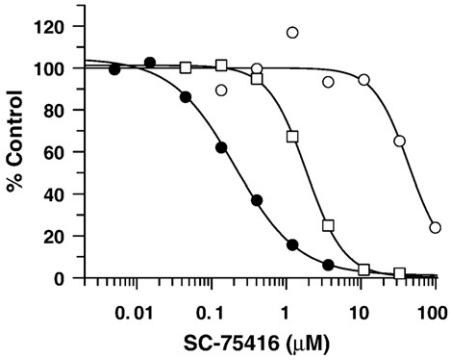

Fig. 2. Potency and selectivity of PGE_2 production from human recombinant enzymes. Either COX-1 (open circles), COX-2 (closed circles) or the mutant IHI COX-2 (open squares) were incubated with SC-75416 for 10 min, and the amount of PGE_2 produced following a 10 min incubation with 10 μM AA was measured by ELISA.

3.2. Cell and human whole blood assays

RASF cells produce large amounts of PGE_2 when stimulated with 1 ng/ml IL-1β for 24 h. The average positive control ranged from 15 to 38 ng/ml, with the average baseline control (-IL-1β) is below the limit of detection. Likewise, platelets activated with 2 μM calcium ionophore produced approximately 100–150 ng/ml of TxB_2. SC-75416 had an IC_{50} of 0.003 μM for inhibition of PGE_2 production in RASFs (a marker of COX-2) and 0.4 μM for inhibition of thromboxane in washed platelets (a marker of COX-1) (Fig. 3). The selectivity demonstrated by SC-75416 is similar to that obtained for celecoxib with IC_{50} of <0.001 and 0.4 μM respectively. This selectivity was maintained in the human whole blood assay with an IC_{50} of 1.4 μM for LPS induced production of PGE_2 and >200 μM for inhibition of platelet thromboxane as compared to the previously reported values for celecoxib of 0.3 μM and 8.3 μM respectively (Gierse et al., 2005) (Fig. 4). The whole blood potencies were right shifted as expected due to high protein binding of these compounds (<99%).

3.3. Pharmacokinetics

The mean maximum observed concentration (Cmax) in the rat after oral administration of 10 mg/kg SC-75416 was 9 μg/ml. The time to achieve maximum observed concentration (Tmax) ranged from 1 to 4 h, indicating varied rate of drug absorption among the rats in this study. The half life (T1/2) of SC-75416 in the rat following oral dosing was 17.4 h (Fig. 5), with a volume of distribution of 736 ml/kg and bioavailability of 89.4%.

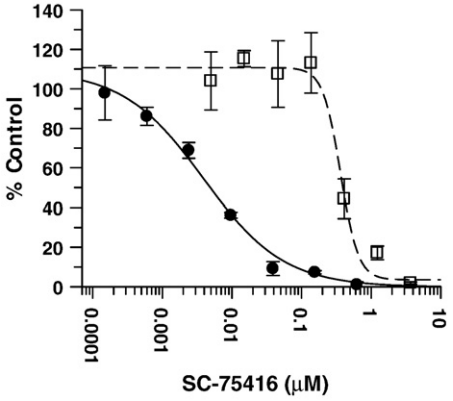

Fig. 3. Potency and selectivity of SC-75416 in isolated serum free cells. COX-2 inhibition was assessed by RASFs (closed circles) incubated with IL-1β plus compound for 24 h. PGE_2 produced was measured by ELISA. COX-1 inhibition was assessed by washed platelets (open squares). Platelets were activated by calcium ionophore in the presence of compounds. TxB_2 was measured by ELISA.

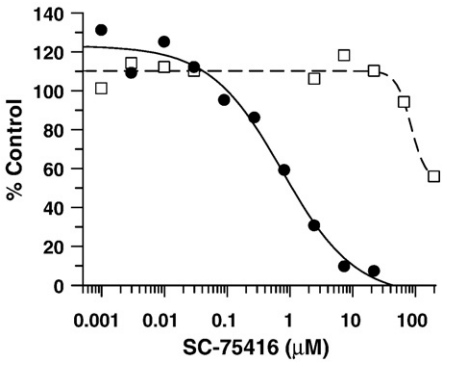

Fig. 4. Potency and selectivity of SC-75416 in human whole blood. COX-2 inhibition was assessed by incubating blood with LPS overnight with compound (closed circles). PGE_2 produced was measured by ELISA. COX-1 inhibition was assessed by allowing platelets to clot in the presence of compounds (open squares).

3.4. Pouch inflammation

Oral administration of SC-75416 inhibited prostaglandin production in the inflamed air pouch in Lewis rats (Fig. 6) in a dose-dependent manner with an ED_{50} of 0.43 mg/kg. A higher dose of SC-75416 was required to limit the production of COX-1-derived stomach mucosal PGE_2, with an ED_{50} of 26.5 mg/kg.

3.5. Carrageenan paw inflammation

Carrageenan injection induced acute inflammation (swollenness and increased sensitization to the hyperalgesic stimulus) in the rat hind paw. In dose response assays, a range of doses of SC-75416 was administered 30 min before the carrageenan stimulation. The results from this assay indicate a dose-dependent inhibition of edema and hyperalgesia (Fig. 7A). The ED_{50}s from this assay are 2.7 mg/kg for reducing paw edema and 4.0 mg/kg for the reversal of hyperalgesia. From 3 to 5 h after the injection, the paw withdrawal latency in response to the thermal stimulus on the inflamed paw was significantly decreased (~1 to 3 s) in comparison to the contralateral paw (~11 to 14 s). This difference served as a measurement of pain. The analgesic effect after oral administration of SC-75416 (10 mg/kg) was noticed at the first observation time point. SC-75416 rapidly reduced hyperalgesia by 41% at 30 min after dosing (Fig. 7B). The maximum inhibition occurred 1 to 2 h after dosing. The results indicate that SC-75416 posses a rapid onset of analgesic effect.

3.6. Adjuvant induced arthritis

An increase in contralateral paw volume was evident from 11 to 14 days after adjuvant injection in the ipsilateral hind paw in this model of inflammation, because the inflammation becomes systemic

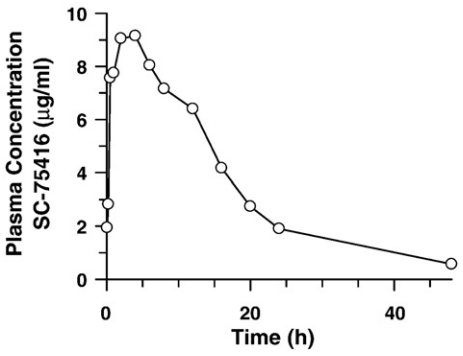

Fig. 5. Plasma concentrations of SC-75416 vs. time after oral administration of 10 mg/kg SC-75416 in Sprague–Dawley rats. Plotted is the mean of $N=3$ rats.

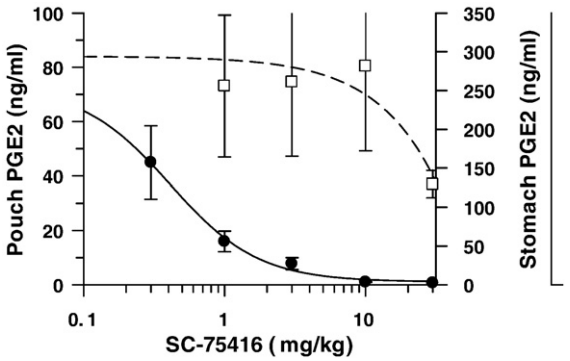

Fig. 6. Potency and selectivity of SC-75416 in rat air pouch model of inflammation. Vehicle or compound was dosed orally 30 min prior to carrageenan injection. Potency against COX-2 activity was measured as the inhibition of PGE_2 formed in an inflamed pouch 3 h post dosing (black circles). Selectivity for COX-2 over COX-1 was assessed by comparison of IC_{50} to the IC_{50} dose required to inhibit PGE_2 produced in the stomach (open squares). Plotted is mean measurements ±S.E.M. of N=4–6 rats/group.

at this time. Compounds were administered starting at day 15 post adjuvant injection. After 11 days of dosing, indomethacin (2 mg/kg/day) reduced paw edema by 76% compared to the vehicle group. SC-75416 reduced paw edema in a dose-dependent manner (Fig. 8). The ED_{50} is 0.081 mg/kg and the ED_{80} is 0.38 mg/kg.

3.7. Spinal nerve ligation model of neuropathic pain

In a neuropathic pain model, SC-75416 dose-dependently reversed tactile allodynia induced by spinal nerve ligation. At the lowest concentration tested (1 mg/kg) there was significant reversal (P<0.05) vs. baseline with an approximate 50% reversal at 10 mg/kg (Fig. 9). In this model naproxen and acetaminophen had no effect on tactile allodynia at 30 mg/kg and 100 mg/kg, respectively following oral administration. Gabapentin and morphine dose-dependently reversed tactile allodynia with ED_{50} of 30 mg/kg and 0.5 mg/kg, respectively.

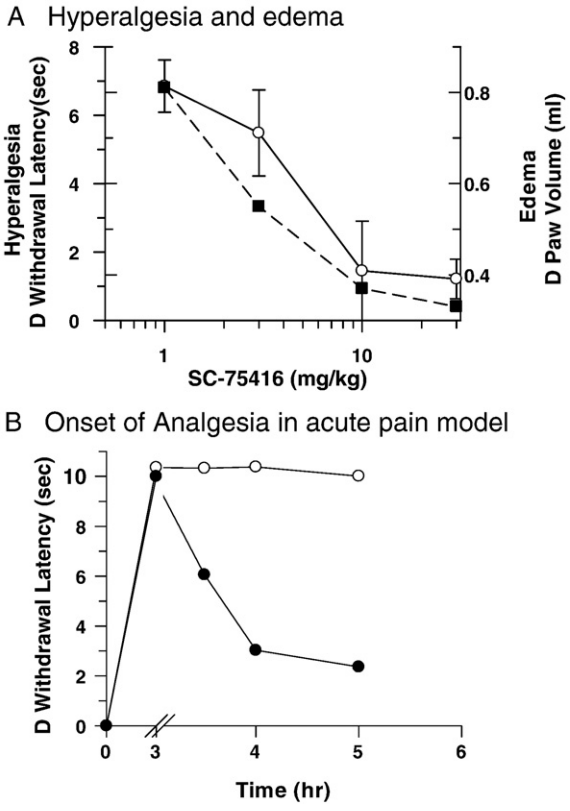

Fig. 7. Potency of SC-75416 in an acute model of inflammation. Vehicle or compound was dosed orally 30 min prior to carrageenan injection into the ipsilateral paw. Paw edema (closed squares) and hyperalgesia (open circles) were measures 3 h after carrageenan injection. Plotted is the mean ±S.E.M. of N=5 rats/group. B) Onset of analgesia. Either vehicle (open circles) or SC-75416 (closed circles) was administered 3 h after carrageenan injection. Hyperalgesia was measured at 30 min, 1 and 2 h after dosing. Plotted in the mean ±S.E.M. of N=5 rats/group.

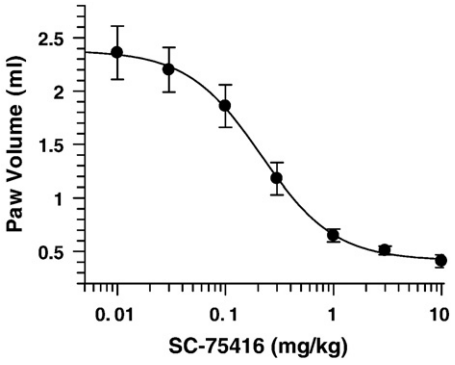

Fig. 8. Potency of SC-754156 in chronic model of arthritis. Vehicle or compound was dosed orally BID from day 15 to 26 post adjuvant injection. Plotted is the mean of contralateral paw volume ±S.E.M. of 7–8 rats at day 26, in this model of systemic inflammation.

4. Discussion

Enzyme assays, employed to screen a class of benzopyran compounds, identified SC-75416 as a COX-2 selective inhibitor and a potential clinical candidate. Although many labs have demonstrated that enzyme assays can be misleading in efforts to predict eventual *in vivo* potency and selectivity, the human recombinant enzyme assay has proven to be useful as a screening tool to identify COX-2 selective inhibitors. The addition of a compound-plus-enzyme preincubation step takes advantage of the unique kinetic mechanism of COX-2 inhibitors, displaying time dependent inhibition of COX-2 and not COX-1. In addition we have found that a 10-minute-endpoint assay as opposed to an initial-rate enzyme assay negates the complex time dependency that is displayed by weakly binding time dependent inhibitors and accurately reflects what can be expected in *in vivo* systems. We have found that by assessing potency and selectivity in an assay that measures initial rates of oxygen consumption, this class of compounds did not appear to be selective (data not presented here). X-ray crystal structure of these compounds in the active site of the COX-2 molecule, suggests that these compounds bind to COX-2 in a manner similar to traditional NSAIDs. Without an additional ring structure that inserts into the side pocket of COX-2, these compounds did not demonstrate a three step model of inhibition (Walker et al., 2001). There is no

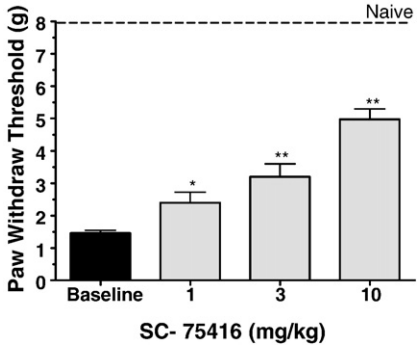

Fig. 9. SC-75416 dose-dependently reversed tactile allodynia 2 h following oral administration. Baselines were taken on the day of the experiment before dosing. Withdrawal threshold is expressed as grams (g). For naïve rats (no surgery) withdrawal threshold is 8 g. Data represent mean ±S.E.M. of 5 rats. $^{**}P$<0.01, $^{*}P$<0.05 vs. baseline.

insertion of a sulfonamide or methyl sulfone into the side pocket of COX-2, which has been shown to constitute the third step in inhibition of COX-2 by diaryl heterocycles. Key differences in the active site between COX-1 and COX-2 beyond the side pocket must be responsible for the selectivity of SC-75416. Indeed, it has been demonstrated by many that COX-2 selective inhibitors can be made based on traditional NSAID templates.

Selectivity of SC-75416 was demonstrated in a range of enzyme, cell, and *in vivo* assays. Platelets and RASF were found to be useful cellular assay systems to demonstrate the generation of TxB_2 and PGE_2 by COX-1 and COX-2 enzymes, respectively. Moreover, these assay systems can be utilized to reproducibly and accurately quantitate the potency and selectivity of inhibitors of COX-1 and COX-2. SC-75416 displayed selectivity in each of these systems, with the expected right shift in potency in the whole blood assay. The benzopyran class of inhibitors has demonstrated a high degree of protein binding and the whole blood assay has not been as predictive of efficacious blood levels, which tend to be quite a bit lower. This is a phenomenon that at this point we do not quite understand.

The advantage of COX-2 selective inhibitors in the digestive tract is that, at therapeutic doses, PGE-2 levels, which protect mucosa from ulceration, are mainly unaffected. In the air pouch model of inflammation, SC-75416 spared gastric PGE_2 production at doses that completely blocked PGE_2 production in the inflamed pouch.

SC-75416 displayed good analgesic properties. The compound potently inhibited both paw edema and hyperalgesia in an acute model of inflammation, rat carrageenan paw. In a model of neuropathic pain, spinal nerve ligation, SC-75416 significantly reduced tactile allodynia, whereas most conventional NSAIDs and other COX-2 selective inhibitors did not. This result is surprising because although other known inhibitors of COX-2 can markedly inhibit nerve injury induced upregulation of PGE2, most have only a modest effect on pain behavior (Schafers et al., 2004). Inhibition of neuropathic pain responses may rely on brain penetration since the volume of distribution of this class is larger than that of diaryls (data not shown). Another example of this may be the compound GW406381X, an inhibitor of COX-2 that has good central penetration and was the first compound to be effective in this model when chronically dosed (oral), or dosed intrathecally (Bingham et al., 2005). Pharmacokinetic evaluation of the compound indicates a large volume of distribution. The role of COX-2, however, in neuropathic pain is not completely understood. Another chronic model of pain, however, rat adjuvant arthritis has proven to be predictive of human clinical dosing for inflammatory pain, and SC-75416 is the most potent compound we have tested to date.

Clinical experience has also demonstrated enhanced potency with SC-75416 (Kowalski et al., 2007). In a clinical trial of post surgical dental pain, SC-75416 demonstrated superior efficacy to that achieved with ibuprofen. This compound's unique chemical structure and effect on COX enzyme binding and activity as well as its potency and selectivity may prove useful in the pharmacological arsenal against pain and inflammation, and may provide the relief needed for those patients underserved by the anti-pain medications available today. Further clinical trials may provide the necessary evidence to show the potential value of this compound for human patients.

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