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ABT-963, A Highly Potent and Selective Disubstituted Pyridazinone COX-2 Inhibitor

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Abbreviations:

EDTA, ethylenediamine-tetraacetic acid; PBS, phosphate buffered saline; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; RIA, radio-immune assay; PGHS prostaglandin H synthetase; PGE_2 prostaglandin E_2; TXB2, Tromboxane B_2; ELISA, enzyme linked immuno-assay; MRI, magnetic resonance imaging.

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Abstract

Nonsteriodal anti-inflammatory drugs (NSAIDS) are efficacious for the treatment of pain associated with inflammatory disease. Clinical experience with marketed selective COX-2 inhibitors (celecoxib, rofecoxib, and valdecoxib) has confirmed the utility of these agents in the treatment of inflammatory pain with an improved gastrointestinal safety profile relative to NSAID comparators. These COX-2 inhibitors belong to the same structural class. Each contains a core heterocyclic ring with two appropriately substituted phenyl rings appended to adjacent atoms. Here we report the identification of vicinally disubstituted pyridazinones as potent and selective COX-2 inhibitors. The lead compound in the series, ABT-963 (2-(3,4-Difluoro-phenyl)-4-(3-hydroxy-3-methyl-butoxy)-5-(4-methanesulfonyl-phenyl)-2H-pyridazin-3-one), has excellent selectivity (ratio of 276, COX-2/COX-1) in human whole blood, improved aqueous solubility as compared to celecoxib and rofecoxib, high oral anti-inflammatory potency *in vi*vo, and gastric safety in the animal studies. Following oral administration, ABT-963 reduced PGE_2 production in the rat carrageenan air pouch model (ED_{50} of 0.4 mg/kg) and reduced the edema in the carrageenan induced paw edema model with an ED_{30} of 1.9 mg/kg. ABT-963 dose-dependently reduced nociception in the carrageenan hyperalgesia model (ED_{50} of 3.1 mg/kg). After 14 days of dosing in the adjuvant arthritis model, ABT-963 had an ED_{50} of 1.0 mg/kg in reducing the swelling of the hind paws. MRI examination of the diseased paws in the adjuvant model showed that ABT-963 significantly reduced bone loss and soft tissue destruction. ABT-963 is a highly selective COX-2 inhibitor that may have utility in the treatment of the pain and inflammation associated with arthritis.

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Introduction

Prostaglandins play a significant role in the maintenance of homeostasis and in the body's response to the environment. Two isozymes of cyclooxygenase are responsible for the biosynthesis of these mediators commonly called cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). Xie et al., (1992) and Smith, (1992) have proposed that COX-2 generated prostaglandins are mediators of inflammation, cellular proliferation, and pain, while COX-1 generated prostaglandins are involved in homeostasis in the stomach, kidney and blood coagulation. Additional support for this proposal has come from *in vitro* and tissue expression studies as well as pharmacologic and clinical studies with recently discovered agents. The data from these studies have demonstrated that COX-2 expression is somewhat limited in that it is found in cells involved in the inflammatory process and in tissues that are undergoing accelerated proliferation such as those in cancer growth. COX-1 is more widely expressed and is primarily involved in homeostasis (Koki et al., 2002). Recent clinical and pharmacological studies demonstrate the benefit of selectively inhibiting cyclooxygenase-2 (COX-2) while leaving cyclooxygenase-1 (COX-1) active (Capone et al., 2003).

The search for selective COX-2 inhibitors has been a challenging one. The catalytic sites of the two enzymes are very similar (Garaveto et al., 2002) and thus it has been difficult to find selective compounds. Thus far, a number of published agents have the tri-cyclic general structure first described for DuP-697 (2-bromo-4-(4'-sulfonylmethyl)phenyl-5-(4'-fluoro)phenylthiophene) (Figure 1) (Gans et al., 1990). The currently marketed compounds share many of the structural characteristics of DuP-697. Therefore a new distinct chemical series may offer the opportunity for improved selectivity *in vitro*, pharmacologic superiority

and enhanced safety. Our laboratory has identified a novel, structurally distinct chemical series of COX-2 inhibitors that have high selectivity and potency both *in vitro* and *in vivo*. These compounds, containing a central pyridazinone ring, in general show improved potency and selectivity compared to previously published compounds. The lead compound in the series, ABT-963 (2-(3,4-Difluoro-phenyl)-4-(3-hydroxy-3-methyl-butoxy)-5-(4-methanesulfonyl-phenyl)-2H-pyridazin-3-one), has improved selectivity in human whole blood, enhanced aqueous solubility as compared to the currently marketed compounds, and high oral potency *in vi*vo and gastric safety in animal studies.

Materials and Methods

BCA protein assay reagents were purchased from Pierce Chemical, Rockford IL. HPLC columns were purchased from Regis Chemical Co., Morton Grove, IL. ELISA reagents were purchased from Cayman Chemical, Ann Arbor, MI or from PerSeptive Diagnostics, Cambridge, MA. RIA kits were from PerSeptive Diagnostics. A23187 was purchased from Sigma Chemical, St Louis, MO.

Human Whole Blood Eicosanoid Formation

Whole blood eicosanoid formation was measured essentially as described by Patrignani et al. (1994). Briefly, whole blood was collected in tubes either with or without heparin. For PGHS-1 activity, non-heparinized whole blood was used and one ml aliquots were placed in glass tubes and allowed to clot at 37^{o} C for one hour. In some assays, COX-1 activity was assessed by stimulating whole blood with 10 nM A23187. The clotted blood was then spun at 2000x g and the serum collected and frozen until TXB_2 was determined. For PGHS-2 activity one ml aliquots containing 10 IU of heparin were incubated with LPS for 24 hours. Plasma was separated by centrifugation at 2000x g for 10 minutes and the plasma was frozen until PGE_2 levels were determined by ELISA.

WISH Cell Assay

The WISH assay was conducted as described in Hulkower et al., (1997). Briefly, WISH cells were treated with IL-1β to induce and activate COX-2 for 18 hours. Compounds were added just prior to IL-1β. Culture supernatants were collected and assayed for PGE_2 levels.

Platelet Assay

Washed human platelets were obtained from donors as described by Baenziger and Majerus (Baenziger and Majerus 1974). Platelets were stimulated with 8.8 uM calcium ionophore A23187 for 10 minutes at 37^{o}C. Platelets were then pelleted and supernatents assayed for TXB_2.

Determination of Prostaglandins

TXB_2 and PGE_2 levels in plasma were determined after the samples were mixed with 4 volumes of ice cold MeOH, then centrifuged at 2000x g for 10 minutes. The immunoassays were conducted on the supernatants, as recommended by the manufacturer (Cayman Chemical, Ann Arbor, MI). The ELISAs were conducted in 96 well microtiter plates (Nunc Roskilde, Denmark) and optical density measured using a microplate reader (Vmax, Molecular Devices Corp., Menlo Park, CA).

For conditioned media samples (as from WISH assay) and in isolated enzyme incubations, the supernatant was analyzed directly without any further treatments, except appropriate dilutions in ELISA buffer.

Determination of Drug Plasma Concentrations

ABT-963 was recovered from the plasma utilizing liquid-liquid extraction with an ethyl acetate-hexane mixture. In a 15 ml glass tube containing 0.1 ml internal standard and 6 ml of ethyl acetate:hexane (1:1) was added a 0.2-0.5 ml plasma aliquot (sample or spiked standard). The samples were vortexed vigorously for 60 seconds followed by centrifugation at 2800x g for 10 minutes (4° C). The upper organic layer was transferred to a 15 ml glass centrifuge tube

and evaporated to dryness with a gentle stream of dry nitrogen over low heat (~35°C). The samples were reconstituted in acetonitrile:water (1:1) by the addition of 0.1 ml acetonitrile and vortexed for 15 seconds followed by the addition of 0.1 ml water and again vortexed 15 seconds. Samples were transferred to autosampler vials with glass micro inserts for HPLC analysis. Spiked plasma standards were assayed interspersed evenly with the samples.

ABT-963 and an internal standard were separated from each other and any co-extracted contaminants by HPLC using a 5 cm x 3.0 mm Kromasil C_{18} column (Keystone) with an acetonitrile: 1% acetic acid mobile phase (65:35, v/v) at a flow rate of 0.7 ml/min with multiple reaction monitoring (MRM) detection of the 25 - 50 µl injection on a Sciex API III+ mass spectrometer. The assay method was linear (correlation coefficient > 0.99) over the concentration range 0.2-100 ng/ml with a mean percent standard deviation <15% for the analysis of triplicate standards at eight separate concentrations and an estimated limit of quantitation of ~0.3 ng/ml.

Solubility

Solubility was determined by shaking 1 mg of the experimental compounds in 1 ml 50mM NaH_2PO_4 (pH7.4) in a 2 ml glass vial for 72 hours at room temperature. The vial was then centrifuged at 6800x g for 20 minutes. A 50 µl aliquot of the supernatant (diluted with 450 µLs mobile phase) and a 10 µg/ml standard (in mobile phase) were then analyzed for drug by reverse phase HPLC. HPLC analytical conditions: mobile phase of 55:45(CH_3CN/0.1% TFA) at a 1.0 ml/min flow with a Luna C18 (100x4.6mm) and UV detection @ 210 nm.

Compound Dosing

Compounds were suspended/dissolved in 0.2% hydroxylpropylmethyl cellulose (Abbott Laboratories, Abbott Park, IL) and dosed in a volume of 5 ml/kg orally by the use of an oral gavage needle.

Carrageenan Induced Air Pouch Prostaglandin Biosynthesis Model

Air pouches were formed in the backs of male Sprague Dawley rats by injecting 20 ml of sterile air on day 0 after Segwick et al., (1983). Three days later the pouch was re-inflated with 10 ml of sterile air. On day 7, 1 ml of saline containing 0.2 % carrageenan was injected into the pouch to induce the inflammatory reaction, which was characterized by the release of prostaglandins. Compounds were dosed 30 minutes prior to carrageenan. Four hours after the carrageenan injection the animals were euthanized and the pouch was lavaged with PBS containing 1,000 units heparin/ml and the lavage fluid added to ice cold methanol to a final concentration of 30% methanol. The levels of immuno-reactive prostaglandins were determined by enzyme immuno-assay as described above. Percent inhibitions were calculated by comparing the response in animals that received vehicle to those that received compound.

Carrageenan Induced Paw Edema in Rats

Hind paw edema was induced in male rats as described by Winter et al., (1962). Male Sprague-Dawley rats weighing between 170 and 190 gms were dosed with experimental compound p.o. by gavage 1 hour prior to the sub-plantar injection of 0.1 ml of 1% sodium carrageenan (Sigma St Louis, MO) into the right hind paw. Right paw volumes (ml) were measured immediately following injection of carrageenan for baseline volume measurements using a Buxco plethysmograph (Buxco Electronics Inc., Troy, NY). After 3 hours, right paws

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were re-measured and paw edema calculated for each rat by subtracting the zero time reading from the 3-hour reading. Data are reported as percent inhibition and were analyzed when appropriate by ANOVA, $p<0.05$ was considered statistically significant.

Adjuvant Arthritis

Male Lewis rats (150-180 g) were weighed, and both hind paw volumes were measured using a Buxco plethysmograph (Buxco Electronics, Inc., Troy, NY). The acute arthritic response was initiated on day 0 by injecting rats subcutaneously in the right hind paw with a suspension of heat-killed, desiccated *Mycobacterium butyricum* (0.5 mg in 0.1 ml light mineral oil) as previously described by Stoerk et al. (1954) and Weichman (1989). On day 16, rats were again weighed and both hind paws re-measured. Animals demonstrating a typical secondary inflammation as evidenced by swelling of the left paw were randomized into groups of 8-10 animals. ABT-963, celecoxib, or vehicle was orally administered once daily for 13 consecutive days. On day 30, body weights and left hind paw volumes were measured, and the differences between day 0 and day 30 was calculated for both body weight and paw. Paw edema was evaluated for significance from control adjuvant edema using Dunnetts multiple comparison test where $p<0.05$ was considered statistically significant. MRI images were taken at initiation of dosing and after the last dose (Jacobson et al. 1999).

Carrageenan-induced thermal hyperalgesia

Carrageenan-induced hyperalgesia was induced by injecting 0.1 ml of a 1% solution of carrageenan in physiological saline into the plantar surface of the right hindpaw of the rat as previously described by Hargreaves et al., (1988). Paw withdrawal latencies of both injured and uninjured paws to thermal stimulation was determined 2 h later using a commercially

available paw thermal stimulator (UARDG, Department of Anesthesiology, University of California, San Diego, La Jolla, CA), modeled after the one described by Hargreaves et al., (1988). Paw withdrawal latencies were calculated as the mean of the two shortest latencies. Sensitivity to acute thermal stimulation in naïve rats was also assessed as a measure of acute thermal nociception.

Receptor Binding

The selectivity of ABT-963 as a COX-2 inhibitor was evaluated in a receptor binding selectivity screen of 75 receptors, enzymes and ion channels by the use of standard protocols (CEREP, Celle l'Evescault, France) as previously described (Jarvis et al., 2000).

CNS safety

CNS safety was evaluated by the use of standard behavior methods (Porsolt & Partners Pharmacology, Paris, France). ABT-963 was evaluated for potential CNS effects in the following tests: locomotor stimulation/depression, motor coordination (rotating treadmill, mouse, 3-100 mg/kg, po, reference compound: diazepam), hypnotic potentiation, pro convulsant and anticonvulsant activity, nociception (hot-plate, mouse, and tail flick, rat). Effects on rectal temperature were also measured (mouse, 3-100 mg/kg, po, reference compound: chlorpromazine). The gross physiological, behavioral and toxic effects ABT-963 were assessed in the mouse Irwin test (Irwin, 1969).

Cardiovascular Safety

Male beagle dogs (9 – 11 kg; n=6/group) were anesthetized with pentobarbital (35 mg/kg, intravenously) and immediately placed on a constant intravenous infusion (6.0 mg/kg/hour,

0.21 ml/min) to maintain a surgical plane of anesthesia, intubated with a cuffed endotracheal tube and ventilated with room air by means of a mechanical respiration pump (Harvard Apparatus, Model 613). Expiratory CO_2 was monitored with an end-tidal CO_2 monitor (Criticare Systems; Model POET TE) and maintained at 4-5%.

Polyethylene catheters were inserted into the right femoral vein and artery for infusion of test agents and collection of blood samples, respectively. A Swan-Ganz catheter (5.5 F) was advanced into the pulmonary artery via the right jugular vein for measurement of cardiac output via thermodilution utilizing a cardiac output computer (Abbott Laboratories, Oximetrix 3). Central venous pressure and pulmonary arterial pressure was measured through the distal port of the catheter. A dual tip micromanometer catheter (Millar, Model SPC-770, 7F) was advanced into the left ventricle of the heart via the right carotid artery for measurement of left ventricular and aortic blood pressure; indices of contractile function (dP/dt) were derived from the aortic pressure wave. A lead II ECG was recorded for measurement of the QT and PR interval. Body temperature was monitored and maintained at 36-38°C throughout the experiment. Blood samples were collected into standard blood tubes containing heparin at 15-minute intervals throughout the experimental protocol for determination of drug concentrations. A separate group of animals were administered vehicle alone (PEG-400; 0.02 ml/kg/min). Primary hemodynamic variables were recorded using commercial software and a signal processing workstation (BioReport, Modular Instruments Inc).

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Results

Early Pyridazinone Inhibitors

The discovery of selective COX-2 inhibitors was facilitated by the description of the crystal structure of human COX-1 and mouse COX-2 structures (Picot et al., 1994 and Luong et al., 1996) as well as early clues from known inhibitors. Examination of these data indicated the possibility of replacing some moieties of known inhibitors with other components. In particular, the replacement of the 5-membered central rings contained in DuP-697 and celecoxib (Figure 1) with a six-membered pyridazinone ring yielded potent inhibitors of COX-2. Early members of this series gave potent inhibition of recombinant COX-2 and good selectivity as assessed by cellular cyclooxygenase assays (Table 1 and S. Majest and R. Bell unpublished observations). Accordingly, a number of substituents were examined at C-2 (L Black, T Kolasa unpublished observations).

A-241611 (2,4-Bis-(4-fluoro-phenyl)-5-(4-methanesulfonyl-phenyl)-2H-pyridazin-3-one) represented an early lead from this series and quite selective for the inhibition of COX-2 as measured by cellular assays (Table 1). The selectivity, as assessed in these assays, was superior to celecoxib and similar to rofecoxib. In addition, the compound exhibited dose dependent activity *in vivo* in both inhibiting prostaglandin production and inflammation in the rat in acute models with oral doses of 10-30 mg/kg (Table 2).

Given the activity of A-241611 *in vivo* in acute models, we examined the compound in a more chronic established adjuvant arthritis model (Stoerk et al., 1954). At an oral dose of 1 mg/kg given over a two-week period, A-241611 produced a 73% inhibition of paw edema and was

comparable to celecoxib (Table 2). In addition, A-241611 reduced both paw tissue damage and bone destruction as accessed by MRI (data not show).

Although the initial anti-inflammatory activity of A-241611 was very encouraging in both acute and chronic models in the rat, further characterization of the compound revealed some deficiencies. One challenge was aqueous solubility. A-241611 and other compounds in the series required dosing in PEG 400 to achieve good oral bioavailability and efficacy. Another issue was the very slow elimination half-life for A-241611 of greater than 15 hours. Accordingly, chemical modifications were sought in A-241611 that might give more aqueous soluble compounds and improved clearance profiles.

Alkoxy containing pyridazinone inhibitors

A 4-butoxy substituted compound, A-282904 (4-[1-(3,4-Difluoro-phenyl)-5-isobutoxy-6-oxo-1,6-dihydro-pyridazin-4-yl]-benzenesulfonamide) (Figure 1) was found to have excellent anti-inflammatory and analgesic properties *in vitro* and *in vivo* (Tables 1, 2). In particular, this compound was more potent for COX-2 inhibition than A-241611. However, this compound still suffered from poor solubility. The addition of a tertiary alcohol to the terminal carbon of the alkoxy chain yielded ABT-963 (2-(3,4-Difluoro-phenyl)-4-(3-hydroxy-3-methyl-butoxy)-5-(4-methanesulfonyl-phenyl)-2H-pyridazin-3-one), a potent and selective COX-2 inhibitor that had significantly increased solubility as compared to the earlier compounds as well as celecoxib and rofecoxib (Figure 2). Further, ABT-963 demonstrated improved selectivity in whole blood assays (Table 1) and an improved pharmacokinetic profile as described below.

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Characterization of ABT-963

Selective Inhibition of Cyclooxygenase-2 *in vitro*

As described above, COX-2 was assessed using LPS challenged blood for 24 hours. As shown in Table 1, ABT-963 was very selective in these assays with an IC_{50} of 17 nM for COX-2 and 4.7 μM for COX-1. A selectivity ratio (COX-2/COX-1) of 276 was calculated from this data (Figure 3). ABT-963 was also found to be a potent inhibitor of PGE_2 formation with an IC_{50} of 130 nM in the IL-1 stimulated WISH COX-2 assay. Platelet eicosanoid production was also inhibited at micromolar concentrations (Table 1).

The potency of ABT-963 against isolated enzymes was also assessed in partially purified preparations of human COX-2 and COX-1 expressed in baculo virus. The enzymatic reaction was initiated with the substrate arachidonic acid and the production of PGE_2 assessed by enzyme immunoassay. Inhibition of COX-2 by several classes of selective inhibitors has been shown to be time-dependent while COX-1 inhibition is not (Copeland et al 1994). This was also seen with the early pyridazinone inhibitors such as A-282904. COX-2 inhibitory potencies have been shown to be assay condition dependent and have been the source of considerable confusion in the literature (Grossman et al 1995). In this system, ABT-963 yielded an IC_{50} of 2 μM with no inhibition of COX-1 at 300 μM. Thus the compound gave a selectivity ratio of > 150 in this assay. In comparison, celecoxib gave an IC_{50} 12 nM against COX-2 and 4 μM against COX-1 with a resulting selectivity ratio of 333. Rofecoxib gave an IC_{50} of 190 nM against COX-2 with no significant inhibition at 100 μM of COX-1. Although this type of assay has not been predictive of selectivity *in vivo*, it does indicate that ABT-963 is a direct inhibitor of human COX-2.

Additional Selectivity Studies

ABT-963 was examined in a battery of receptor binding, enzyme and ion channel assays at a concentration of 10 μM (CEREP). No significant activity was found in any of these assays.

Inhibition of prostaglandin formation in the rat

The primary *in vivo* assay used in the discovery of ABT-963 was the carageenan air pouch model. ABT-963 was a potent inhibitor of PGE_2 in this model yielding an ED_{50} of 0.4 mg/kg after oral dosing (Figure 4 a).

Rat carrageenan paw edema assay

The rat carrageenan paw edema assay was used by the project as a primary indication of anti-inflammatory activity. This model has been used classically to discover anti-inflammatory compounds (Winter et al., 1962). ABT-963 was active in the model and showed a dose dependent inhibition of the edema as shown in Figure 4b. Celecoxib and ABT-963 were nearly equipotent and rofecoxib was less potent (Table 2).

Inflammatory thermal hyperalgesia

Another hallmark of prostaglandin production is hyperalgesia and pain. Cyclooxygenase inhibitors are effective in a number of animal models of pain and we used the carrageenan induced hyperalgesia model to determine ABT-963 anti-nociceptive activity (Hargreaves et al., 1988). As shown in Figure 5, ABT-963 was effective (ED_{50} = 3.1 mg/kg) in the model with activity similar to rofecoxib (ED_{50} = 4.6 mg/kg) and celecoxib (ED_{50} = 1.9 mg/kg) (Table 2).

Established adjuvant-induced arthritis in the rat

ABT-963 was examined in the established adjuvant assay in rats, a chronic model of inflammation and tissue destruction (Stoerk et al., 1954 and Weichman, 1989). ABT-963 inhibited the edematous response with potency similar to both rofecoxib and celecoxib (Figure 6a). MRI was used to analyze the effects of ABT-963 on bone and soft tissue. Representative images for each compound (3 mg/kg dose) and positive and negative controls are illustrated in Figure 6b. The compound gave very good inhibition of bone and tissue destruction as shown in Figure 6b and quantified in Figure 6c.

Pharmacokinetics in the rat,

The pharmacokinetic profile of ABT-963 was evaluated in rats (Table 3). The pharmacokinetic behavior of ABT-963 following a 3 mg/kg intravenous dose in rat was characterized by a plasma elimination half-life of 4.9 hours. Initial volume of distribution value (V_C) was modest (0.5l/Kg). Plasma clearance value was very low (0.14 L/hr•kg). ABT-963 was rapidly absorbed from a solution formulation (PEG-400) at low doses but more slowly at higher doses, with peak plasma concentrations observed from 1-6 hours after dosing. Peak plasma concentrations averaged 1.74 µg/ml following a 3 mg/kg oral dose in the rat. The plasma elimination half-life was similar to that noted following intravenous dosing, averaging 5.2 hours in the rat. Bioavailability of ABT-963 from the aqueous solution formulation was 93% in the rat.

Gastric Safety Profile

The gastric safety of ABT-963, celecoxib, and rofecoxib were evaluated in a rat model of gastric prostaglandin production for their ability to reduce the levels of prostaglandins. For the

prostaglandin measurements the compounds were given to fasted animals at a dose of 10 mg/kg and the animals euthanized 4 hours later. ABT-963, rofecoxib, and celecoxib did not significantly change prostaglandin production in the stomach relative to vehicle controls while indomethacin gave 66% inhibition.

A second model in which the compounds were examined for gastric safety utilized the dog. In these experiments the compounds were given orally for four days and then on the fifth day endoscopy was performed to access the integrity of the duodenum and the stomach. Standard views were used which included both the proximal and distal duodenum, the pyloric antrum, greater curvature and the cardia. Significant damage to the stomach and the duodenum was seen in the indomethacin (10 mg/kg) treated animals. In the ABT-963 treated animals (20 mg/kg, plasma level at endosocpy 21.8 μM) three animals gave normal endoscopy. In the fourth animal a few small punctate blemishes were seen. Celecoxib (20 mg/kg, plasma level at endoscopy 0.49 μM) showed these type of lesions in 2 of 4 dogs, while rofecoxib (20 mg/kg, plasma level at endoscopy <0.05 μM) gave numerous small lesions in all four dogs. These small lesions were not ulcers and thus are not the same as seen for indomethacin where three of the four dogs had frank ulceration (see Figure 7). It is unclear what the significance of the small lesions was. However in this study the GI safety of ABT-963 was equivalent or superior to both marketed selective COX-2 inhibitors especially in light of the plasma concentrations that were determined from blood samples taken at the time of endoscopy.

CNS Safety Pharmacology

Doses of 3 to 100 mg/kg, po, of ABT-963 caused no significant effects on locomotor activity, motor coordination, hypnotic potentiation, pentylenetetrazol seizures, nociception or rectal temperature. Taken together, these results suggest that ABT-963 had no clear CNS effects in rats and mice in the dose-range tested.

Cardiovascular safety

ABT-963 was administered as a series of three, 30-minute intravenous infusions (0.2, 2 and 20 mg/kg). At the end of each infusion period, respective plasma concentrations of ABT-963 achieved were 0.31 ± 0.12, 2.36 ± 0.54 and 31.24 ± 2.11 μg/mL. Sixty minutes following termination of infusion plasma concentration declined to 15.02±0.65 μg/mL.

Efficacious levels in the animal models of inflammation were in the range of 0.25 to 1 μg/mL. In the anesthetized dog model and at the highest plasma concentrations tested (31.2 ± 2.1 μg/mL), ABT-963 produced modest effects on mean arterial pressure (MAP) but had no physiologically relevant effects (compared to vehicle controls) on any other parameter measured including: heart rate, cardiac output, pulmonary arterial pressure, indices of cardiac contractile function, systemic and pulmonary vascular resistance, the QT interval corrected for heart rate (Bazett's; QTcB) and the PR interval. During the high dose infusion, ABT-963 produced a 25% reduction in MAP during the last 10 minutes of the infusion period. During the subsequent 60-minute post-treatment period MAP tended to remain at these levels but was not significantly different from vehicle controls. Thus, plasma concentrations of ABT-963 as

high as 31 μg/mL or 30 to 124-fold over the efficacious concentration produced little to no effect on cardiovascular and hemodynamic function in the anesthetized beagle dog.

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Discussion

Prostaglandins modulate the pain and edema observed in a variety of inflammatory disorders. These compounds also play a role in protection of the gastric lining, hemodynamic functions such as platelet aggregation, as well as having a role in normal renal function. The first enzyme in the prostaglandin biosynthetic pathway is prostaglandin H synthase and this enzyme catalyzes two activities, cyclooxygenase and hydroperoxidase (Smith 1992). Usually both activities are termed cyclooxygenase. There are two isozymes of cyclooxygenase, termed cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Herschman 1996). A wealth of work indicates that COX-1 is constitutively expressed in most tissues while COX-2 is not normally expressed but is induced by cytokines, hormones, and growth factors (Herschman, 1996, Smith, 1992).

Until recently, most clinically used inhibitors of prostaglandin formation inhibit both isozymes of cyclooxygenase. The result of inhibiting both isozymes of the enzyme is clinically significant inhibition of pain and inflammation accompanied by a significant incidence of gastrointestinal distress and renal complications. The gastrointestinal distress seen in the clinic can be modeled in rats or dogs by giving high doses of conventional NSAIDS. Experimental compounds with greater selectivity for COX-2 versus COX-1 have markedly attenuated gastrointestinal damage in rodents. The recent approval of COX-2 selective agents for use in human disease has been accompanied by initial safety results indicating a clear advantage for selective agents versus mixed inhibitors, Capone et al., (2003). This and other evidence indicates that of the two known isozymes, COX-1 is responsible for gastric protection and hemodynamic balance, while COX-2 produces prostaglandins in inflammation and cytokine/growth factor induced processes.

The discovery of agents that selectively inhibit COX-2 and not COX-1 has proven challenging. This difficulty is derived from three sources. First, the active sites of the two proteins are very similar. There are few amino acid changes in the surface amino acids that form the active site pocket, Garavito et al., (2002). One important difference may be the substitution of Val 509 for Ile. This change may open a pocket that allows for some flexibility of binding. Other differences in the second layer of amino acids allows for some increase in size of the right hand side of the active site (Garavito et al 2002). The active site also is very hydrophobic which allows very little hydrophilicity to be incorporated in the inhibitors (Garavito et. al., 2002 and unpublished observations). The second source of difficulty in providing selective agents is providing agents that do not inhibit prostaglandin formation by inhibiting the release of arachidonic acid. Arachidonic acid is as a substrate for both COX-1 and COX-2 and therefore agents that inhibit arachidonic acid are non-selective.

The third difficulty is accessing the compound's selectivity. Several methods had been published utilizing different cells and stimuli with standard inhibitors (Grossman et al., 1995). Based upon this, our approaches to assess *in vitro* selectivity for COX-2 versus COX-1 evolved over the course of discovering ABT-963. Initially, the early series selectivity values were determined in cell assays using the WISH cell line, which upon IL-1 stimulation, COX-2 is dramatically induced and large amounts of PGE_2 are formed (Hulkower et. al., 1997). Cox-1 activity was determined in platelets, which only express COX-1 (Pairet et al., 1996). The later alkoxy and hydroxy alkoxy series were discovered using two whole blood assays performed essentially as described by Patrignani et al (1994). In our hands, the clotting COX-1 assay and the ionophore whole blood assay for COX-1 gave similar results for compounds that inhibit COX-1. A number of studies examining COX-2 expression in LPS stimulated

whole blood and product formation profiles indicated that a 24-hour assay was required to selectively measure COX-2 inhibition (S. Majest and R. Bell, unpublished) in this assay.

In vitro, ABT-963 selectively inhibits recombinant human COX-2 enzyme as well as COX-2 driven cellular production of prostaglandins while leaving platelet COX-1 thromboxane production intact at concentrations where COX-2 is completely inhibited. In whole blood assays ABT-963 is particularly potent and selective. Compared to other COX-2 inhibitors such as rofecoxib and celecoxib, ABT-963 was 11 fold more potent against COX-2 than celecoxib, while rofecoxib was 30 fold weaker against COX-2. ABT-963, celecoxib, and rofecoxib were all equally active at inhibiting COX-1. Thus the selectivity ratios in blood were 157 for ABT-963, 6 for rofecoxib and 14 for celecoxib. Etoricoxib has appeared in the literature (Riendeau et al 2001). This compound has potencies that are in the same range as ABT-963, however it appears to be more potent in the rat assays. From the published selectivity data ABT-963 appears to be comparable to this agent.

The present *in vivo* data show that ABT-963 is an effective anti-inflammatory agent. The carrageenan air pouch model had been previously used to assess *in vivo* inhibition of both leukotrienes and prostaglandins (deBrito 1989). More recently Seibert et al., (1994) showed that the lining of the carageenan challenged air pouch contains a significant level of induced COX-2 enzyme and implied that the majority of the PGE_2 formed was from COX-2. This model gave good reproducibility and selective agents gave nearly complete inhibition of PGE_2. ABT-963 inhibited prostaglandin formation in the air pouch model with oral doses of less than 1 mg/kg (ED_{50} = 0.3 mg/kg). This potency was similar to that seen with both rofecoxib and celecoxib. The compound also inhibited prostaglandin driven inflammation and pain in acute

rat models. In the carrageenan paw edema model, ABT-963 was effective at reducing the edema caused by the release of prostaglandins with an ED_{50} of 1.9 mg/kg. The compound was also effective in the carrageenan induced hyperalgesia model with an ED_{50} of 3.1 mg/kg. These data confirm the results with earlier selective agents that COX-2 derived prostaglandins are involved in the inflammatory reaction in these models (Penning et. al. 1997, Chan et. al. 1999).

ABT-963 was also examined in an established adjuvant arthritis model in the rat. The established model exhibits pronounced soft tissue and synovial inflammation between day 16 and 30, and is accompanied by a marked progression of periosteal reactions, pannus formation, internal bone inflammation, fibrosis in the joints and end-stage ankylosis (Weichman, 1989). Classical paw edema measurements were made as well as examinations of paw architecture using MRI techniques described by us (Jacobson et al, 1999). Daily oral doses of ABT-963 from day 14-28 gave significant inhibition of paw swelling as measured by plethysmography and MRI. In addition, MRI analysis showed that COX-2 inhibition arrested the progression of the disease at the time of initial dosing. Similar results were also seen for indomethacin suggesting that, in the rat, prostaglandins play a significant role in the progression of bone loss seen in this model (Weichman, 1989).

ABT-963 was examined in a dog model of cardiovascular safety and the data from these experiments suggest that ABT-963 may not have cardiovascular effects in man. Only a minor affect was seen at the highest plasma level, which was 30-124 fold over the efficacious levels in the models of inflammation.

Most importantly ABT-963 dosed daily in dogs for 4 days at doses 20-fold above effective anti-inflammatory doses gave no gastrointestinal damage as assessed by endoscopy. These data, although preliminary, indicate that ABT-963 has the potential to have increased gastric safety. The higher level of exposure of the animals to the drug coupled with the increased selectivity further suggest that ABT-963 will have improved gastric safety in man. In summary, ABT-963 has a preclincal anti-inflammatory and safety profile that suggests that this compound may be safe and effective in man. Continued clinical evaluation of ABT-963 will determine the human safety and efficacy profile of this compound.

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Figure Legends

Figure 1. Structures of the inhibitors used in this study

DuP-697 (2-bromo-4-(4'-sulfonylmethyl)phenyl-5-(4'-fluoro)phenylthiophene), A-241611 (2,4-Bis-(4-fluoro-phenyl)-5-(4-methanesulfonyl-phenyl)-2H-pyridazin-3-one), A-282904 (4-[1-(3,4-Difluoro-phenyl)-5-isobutoxy-6-oxo-1,6-dihydro-pyridazin-4-yl]-benzenesulfonamide), ABT-963, (2-(3,4-Difluoro-phenyl)-4-(3-hydroxy-3-methyl-butoxy)-5-(4-methanesulfonyl-phenyl)-2H-pyridazin-3-one).

Figure 2. Aqueous solubility of various COX-2 inhibitors

Solubility was determined at equilibrium. Levels were measured by HPLC.

Figure 3. Inhibition of COX-2 and COX-1 Driven Eicosanoid Production in Human Whole Blood by ABT-963

COX-1 (squares) and COX-2 (diamonds) eicosanoid production was assessed in 5-6 human donors. IC_{50}'s were 17 nM (95% confidence limits 3-86 nM) for COX-2 and 4.7 μM (0.4-10.9) for COX-1.

Figure 4. Anti-inflammatory activity of ABT-963

Panel A: Carrageen air pouch

Air pouches were formed in the rats at day 0 with 10 mL of sterile air. On day 3 the air pouches were re-inflated. On day 7 the animals were dosed with ABT-963 30 minutes prior to the injection of 1.0 mL of 1% carrageenan. Four hours later the animals were euthanized and the pouches lavaged. Prostaglandin levels in the lavage fluid were determined by ELISA. The control levels of PGE_2 this experiment were 23 ng/animal +/-3.9. ED_{50} for ABT-963 was 0.4 mg/kg (confidence limits of 0.1-0.1.2)

Panel B Carrageenan Paw Edema

ABT-963 was given to the animals 30 minutes prior to the injection of 0.1 mL of 1% carrageenan. Two hours later the paw volumes were determined. Control paw volume for this experiment was 1.29+/-0.11 mL. ED_{30} was 1.9 mg/kg (confidence limits of 0.6-3.2).

Figure 5. Inhibition of carrageenan induced hyperalgesia by ABT-963

Rats were dosed with ABT-963 30 minutes prior to the injection in the right paw with 0.1 mL of 0.5% carrageenan. Two hours later the hyperalgesia was determined using a focused light. The above graph shows results from two separate experiments.

Figure 6. Dose responses of ABT-963 and celecoxib in Established Adjuvant Arthritis

Rats were injected on day 0 with 0.1 mL of Fruends complete adjuvant. On day 16, rats were again weighed and both hind paws re-measured. Animals demonstrating a typical secondary inflammation as evidenced by swelling of the left paw were randomized into groups of 8-10 animals. The animals were then dosed with ABT-963, rolecoxib, or celecoxib daily for 14 days. At the end of the treatment period paw volumes were again determined and MRI imaging was conducted. Panel A shows the effects on edema. Panel B shows typical MRI images of the paws. Panel C shows the graded scores of the MRI images. The MRI images were scored blind using a standard scoring form. Ten images per treatment group were scored. Data is average +/- sem. Control paw swelling 2.25 +/- 0.11 mL ED_{50} for ABT-963 was 0.9mg/kg (confidence limits 0.7-1.3), ED_{50} for rofecoxib 1 mg/kg (confidence limits 0.7-1.3), and ED_{50} for celecoxib 0.6 mg/kg (confidence limits 0.2-0.9).

Figure 7. Endoscopy of dogs treated with either indomethacin or rofecoxib

Dogs were treated with compound for four days, and then they were fasted for 24 hours prior to the endoscopy.

Table 1- Inhibition of COX-1 and 2 in Cells and Blood

	Intact Cells		Human Blood	
Inhibitor	WISH COX-2 IC_{50} (μM)	Platelet COX-1 IC_{50} (μM)	LPS COX-2 IC_{50} (μM)	Ionophore COX-1 IC_{50} (μM)
A-241611	0.185* (0.080-0.41)	29.0 (22-39)	ND	ND
A-282904	0.011 (0.003-0.052)	0.467 (0.393-0.551)	2.8 (2.2-3.9)	64.9 (27-220)
ABT-963	0.130 (0.080-0.190)	12.6 (0.4-25)	0.017 (0.008-0.086)	4.7 (0.4-10.9)
celecoxib	0.015 (0.007-0.034)	0.16 (0.11-0.22)	0.470 (0.200-3.00)	6.4 (0.9-38)
rofecoxib	0.068 (0.043-0.110)	6.0 (1-24)	1.70 (0.35-6.00)	10.2 (0.3-42)

*Values represent means (95% confidence intervals) from 3-6 observations.
ND Not Determined

Table 2- Inhibition Rat Models of Inflammation and Pain

Inhibitor	Air Pouch[a] ED_{50} mg/kg	Carageenan Paw Edema ED_{30} (mg/kg)	Adjuvant Edema ED_{50} (mg/kg)	Infammatory Thermal Hyperalgesia ED_{50} (mg/kg)
A-241611	ND	<30	73% (1 mg/kg)	ND
A-282904	0.5* (0.1-1.2)	3.8 (1.2-5.4)	1.1 (0.56-3.2)	8.4 (4.2-11.2)
ABT-963	0.4 (0.1-0.9)	1.9 (0.6-3.2)	0.9 (0.7-1.3)	3.1 (1-4.5)
celecoxib	0.2 (0.1-0.3)	1.7 (1.0-2.1)	0.6 (0.2-0.9)	1.9 (0.4-3)
rofecoxib	1.0 (0.6-1.5)	4.5 (3.9-5.1)	1.0 (0.7-1.3)	14.6 (10.2-16.6)

*Values represent means (95% confidence limits)

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Table 3: Pharmacokinetics of ABT-963 after a Single IV or Oral (solution) Dose in Rat

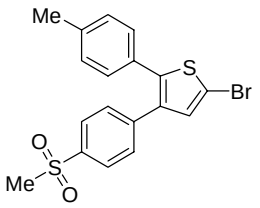
Intravenous Dose

Species	Dose	$t_{1/2}$ (hr)	V_c (L/kg)	$V\beta$ (L/kg)	AUC_{0-8} (µg•hr/ml)	CL_p (l/hr•kg)	n
Rat	3	4.9	0.5	1.0	21.2	0.14	3

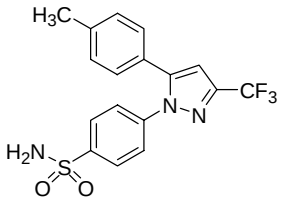
Oral Dose

Species	Dose mg/kg	$t_{1/2}$ (hr)	C_{max} (µg/ml)	T_{max} (hr)	AUC_{0-8} (µg•hr/ml)	F (%)	n
Rat	3	5.2	1.7	3.5	19.7	92.9	6
	30	6.0	11.1	4.7	211.8	99.4	3
	100	5.6	25.8	10.5	538.0	76.1	2

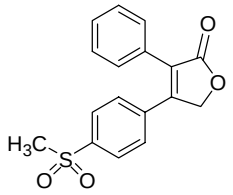
Figure 1



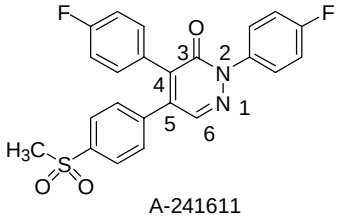
DuP-697



celecoxib



rofecoxib



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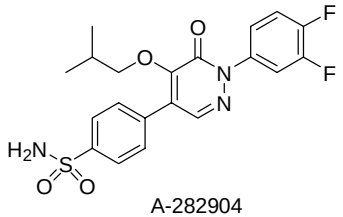

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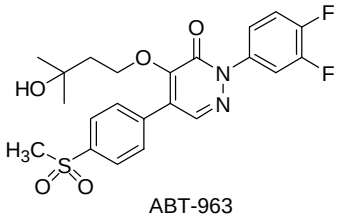

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Figure 2

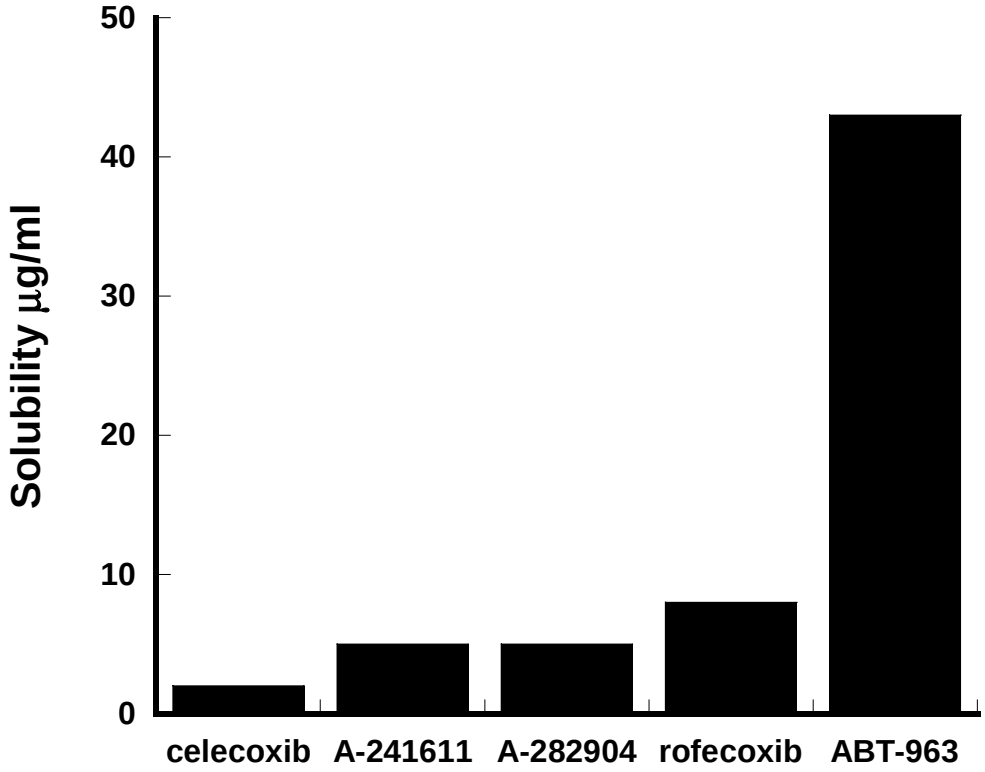


Figure 3

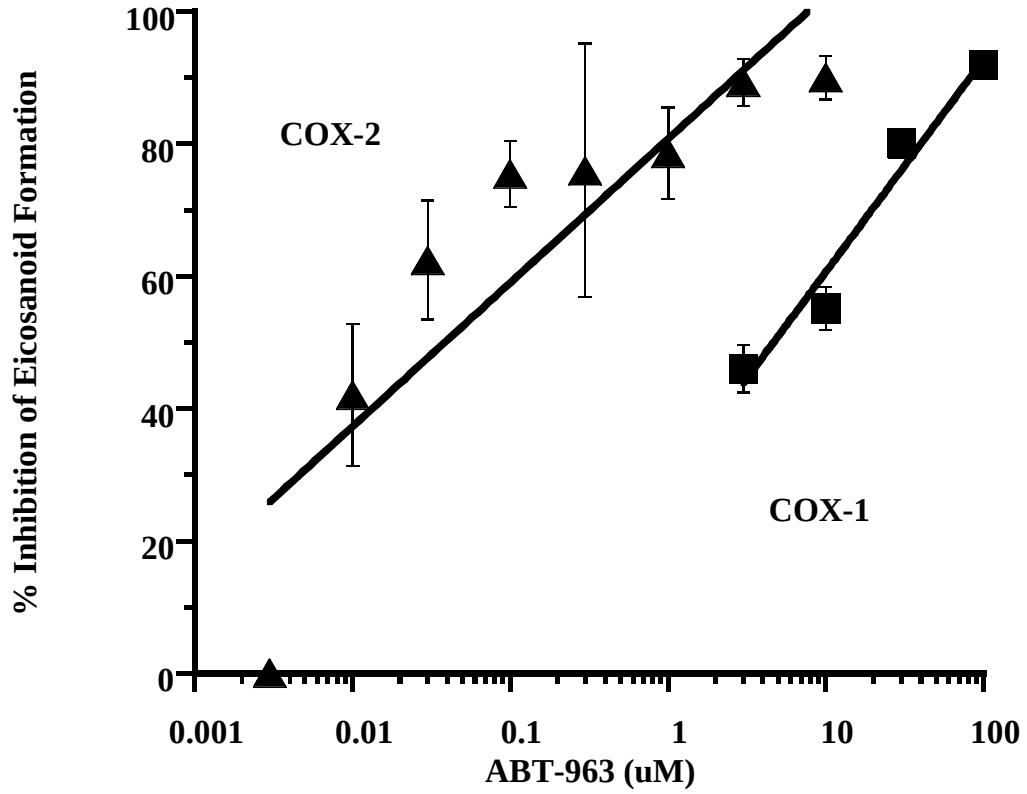
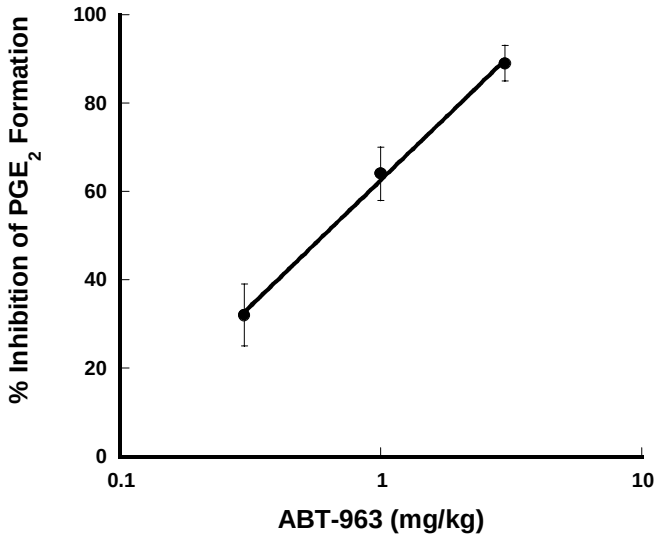


Figure 4

Panel A



Panel B

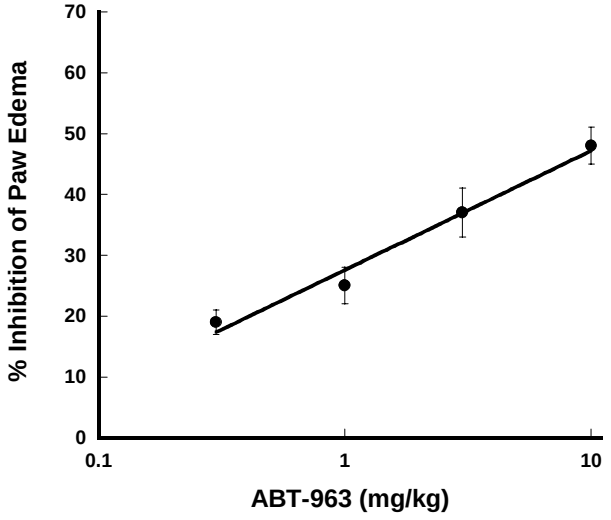


Figure 5

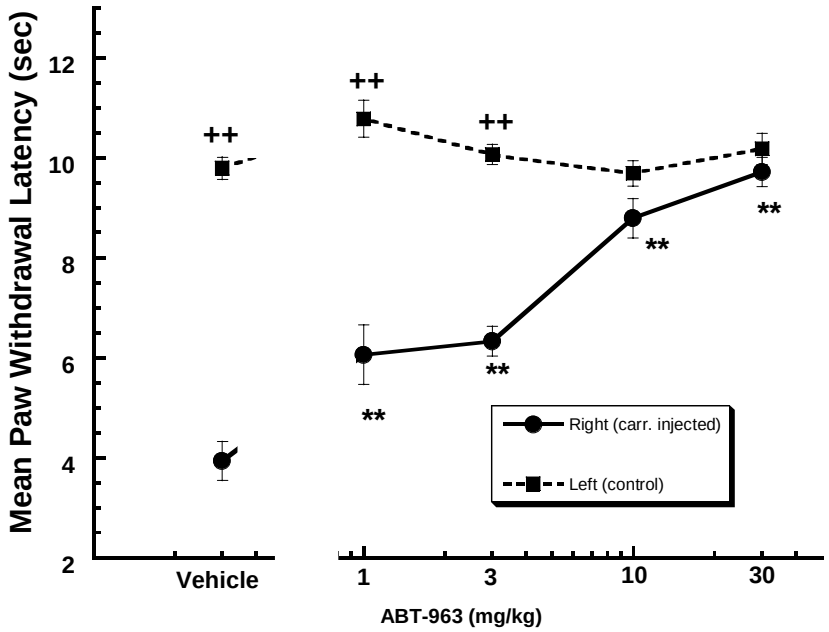
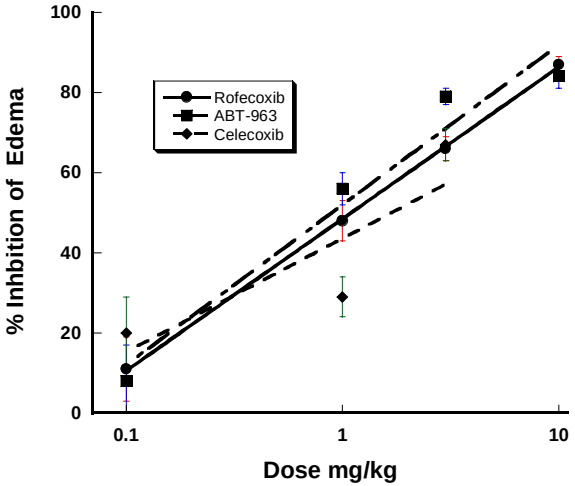
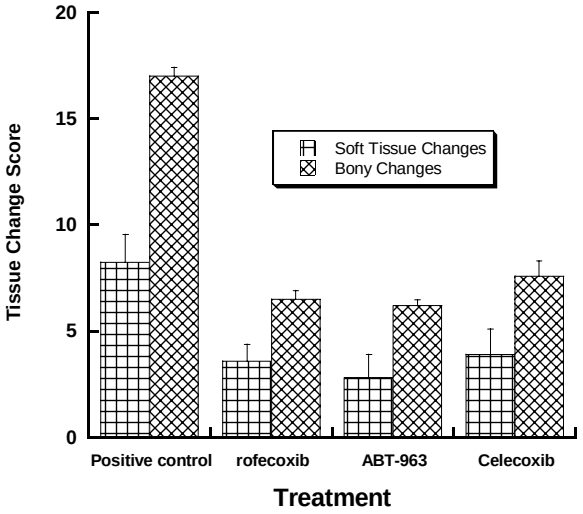


Figure 6

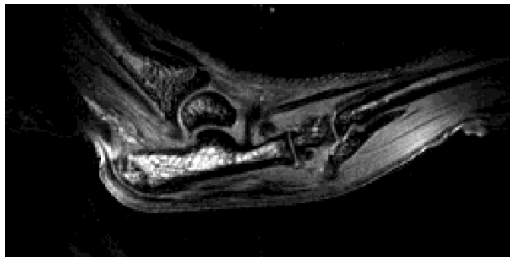


Panel A Effect of the compounds on edema in adjuvant arthritis



Panel C Scores from MRI analysis of changes in both bone and soft tissue.

Panel B MRI Images



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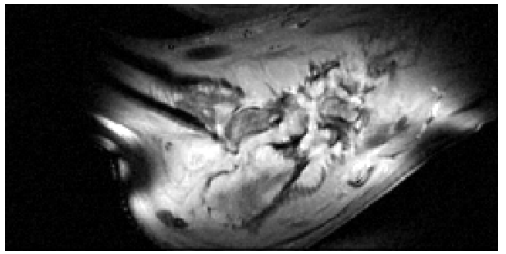

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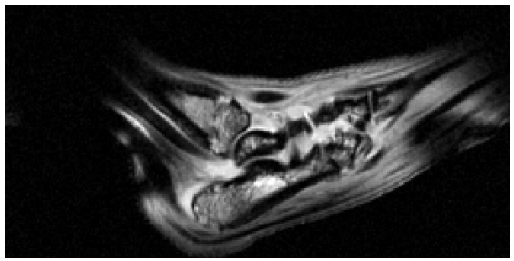

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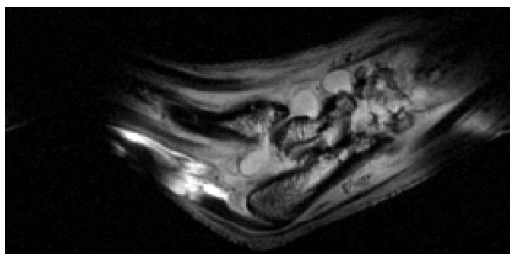
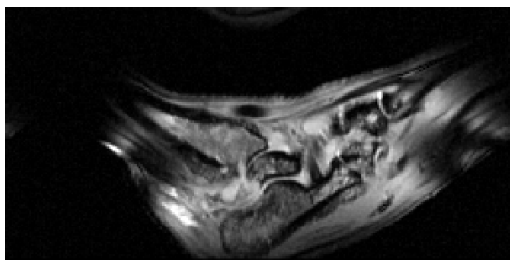


Celecoxib



rofecoxib

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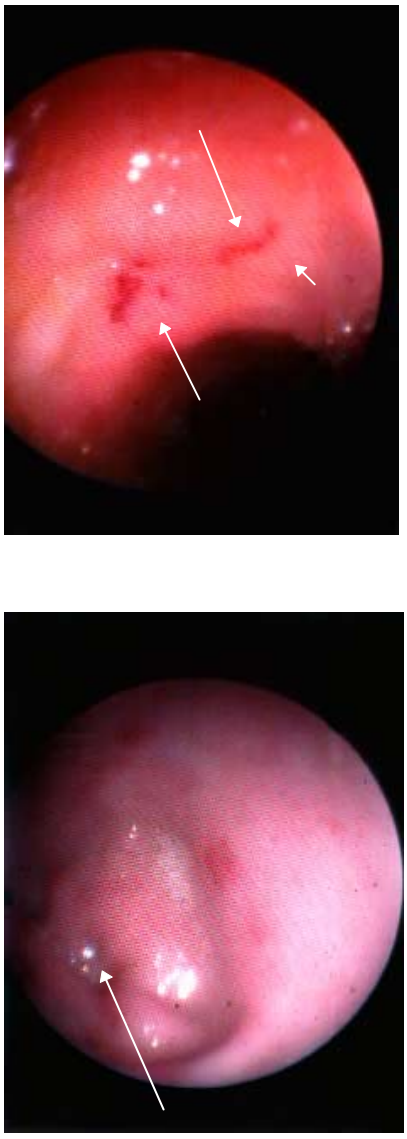

Figure 7