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RO1138452 and RO3244794: characterization of structurally distinct, potent and selective IP (prostacyclin) receptor antagonists

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1 Prostacyclin (PGI_2) possesses various physiological functions, including modulation of nociception, inflammation and cardiovascular activity. Elucidation of these functions has been hampered by the absence of selective IP receptor antagonists.

2 Two structurally distinct series of IP receptor antagonists have been developed: 4,5-dihydro-1H-imidazol-2-yl)-[4-(4-isopropoxy-benzyl)-phenyl]-amine (RO1138452) and *R*-3-(4-fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxycarbonylamino]-propionic acid (RO3244794).

3 RO1138452 and RO3244794 display high affinity for IP receptors. In human platelets, the receptor affinities (pK_i) were 9.3 ± 0.1 and 7.7 ± 0.03, respectively; in a recombinant IP receptor system, pK_i values were 8.7 ± 0.06 and 6.9 ± 0.1, respectively.

4 Functional antagonism of RO1138452 and RO3244794 was studied by measuring inhibition of carbaprostacyclin-induced cAMP accumulation in CHO-K1 cells stably expressing the human IP receptor. The antagonist affinities (pK_i) of RO1138452 and RO3244794 were 9.0 ± 0.06 and 8.5 ± 0.11, respectively.

5 Selectivity profiles for RO1138452 and RO3244794 were determined *via* a panel of receptor binding and enzyme assays. RO1138452 displayed affinity at I_2 (8.3) and PAF (7.9) receptors, while RO3244794 was highly selective for the IP receptor: pK_i values for EP_1 (<5), EP_3 (5.38), EP_4 (5.74) and TP (5.09).

6 RO1138452 (1–10 mg kg^{-1}, i.v.) and RO3244794 (1–30 mg kg^{-1}, i.v.) significantly reduced acetic acid-induced abdominal constrictions. RO1138452 (3–100 mg kg^{-1}, p.o.) and RO3244794 (0.3–30 mg kg^{-1}, p.o.) significantly reduced carrageenan-induced mechanical hyperalgesia and edema formation. RO3244794 (1 and 10 mg kg^{-1}, p.o.) also significantly reduced chronic joint discomfort induced by monoiodoacetate.

7 These data suggest that RO1138452 and RO3244794 are potent and selective antagonists for both human and rat IP receptors and that they possess analgesic and anti-inflammatory potential.
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Abbreviations: CHO-K1, Chinese hamster ovary cell line; COX, cyclooxygenase; $cPGI_2$, carbaprostacyclin; I_2, imidazoline$_2$ receptor; IBMX, isobutylmethylxanthine; PAF, platelet activating factor; PBS, phosphate-buffered saline; PGI_2, prostacyclin; WGA-SPA, wheat germ agglutinin-coupled scintillation proximity assay

Introduction

Prostacyclin (PGI_2), a member of the eicosanoid family of lipid mediators, is a major product of arachidonic acid metabolism formed in the vascular endothelium and other tissues. It was first discovered in 1976 in the course of identifying unstable eicosanoids produced in blood vessels (Bunting *et al*., 1976). Like the majority of eicosanoid lipid mediators, PGI_2 is produced initially by metabolism of arachidonic acid to PGH_2 by cyclooxygenase (COX) enzymes. PGH_2 is then converted to PGI_2 by the action of PGI_2 synthase (Vane & Botting, 1995). In many respects, PGI_2 appears to have opposite physiological behaviour to the eicosanoid thromboxane A_2 (TXA_2), as the former is a potent vasodilator, antithrombotic, and antiplatelet agent, while the latter produces vasoconstriction, thrombosis and platelet aggregation (Coleman *et al*., 1994). In recognition of the potential beneficial cardiovascular effects of PGI_2, synthetic PGI_2 analogs have undergone clinical evaluation for a variety of indications (Poredos, 2000).

PGI_2 mediates its effects primarily through a membrane-associated G protein-coupled receptor termed the IP receptor (Smyth & Fitzgerald, 2002). A classification of prostanoid receptors (DP, EP, FP, IP and TP receptors) was developed initially based on a differential rank order of potency for PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and TXA_2, respectively, as well as the selective activity of synthetic agonists and antagonists (for a review, see Coleman *et al*., 1994; Ushikubi *et al*., 1995). In addition, EP receptors were further subdivided, through the use of selective agonists and antagonists, into EP_1, EP_2, EP_3

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and EP_4 receptor subtypes. Subsequently, prostanoid receptors corresponding to each of these subtypes were cloned from human and other tissues (see Ushikubi *et al*., 1995), providing molecular confirmation for the pharmacological subdivision of these receptors. However, in addition to a G protein-coupled cell surface receptor, several recent reports show that endogenously produced PGI_2 can also activate peroxisomal proliferator-activated receptors of the δ subtype (PPARδ) *in vivo* (reviewed by Lim & Dey, 2002).

In addition to a widely appreciated role in cardiovascular system regulation, PGI_2 is generated rapidly in response to mechanical injury or inflammation, and can sensitize or directly activate sensory nerves in painful or inflammatory conditions (reviewed by Bley *et al*., 1998). Thus, even though PGE_2 attained initially wide recognition as the primary pain-producing inflammatory prostanoid (Ferreira, 1979), the majority of recent studies directly comparing the effects of PGI_2 and PGE_2 have suggested that the former has more potent sensory neuron activating and sensitizing properties (e.g., Smith *et al*., 1998; Nakae *et al*., 2005a). Moreover, recent transgenic mouse data point to a primary role of IP receptors in experimental pain behaviours (Murata *et al*., 1997; reviewed by Kobayashi & Narumiya, 2002). Still, several EP receptor subtypes have been considered as viable targets for analgesia and at least one EP_1 antagonist has entered clinical evaluation for visceral pain (Sakar *et al*., 2003).

In contrast to DP, EP, FP and TP receptors, to date, there have been no selective antagonists described for IP receptors. Availability of an antagonist to the IP receptor would help to clarify many of the questions relating to the roles of PGI_2 and IP receptor physiology. For instance, the hypothesis that endogenous PGI_2 production prevents cardiovascular disease – suggested by some studies with transgenic mice (Murata *et al*., 1997; Zhang *et al*., 2003) – has remained speculative. In addition, IP receptor antagonists could potentially have therapeutic utility in the treatment of painful or inflammatory conditions. In light of these considerations, successful focused library screening and medicinal chemistry efforts yielded two distinct series of IP receptor antagonists. *In vitro* data on interactions with a wide variety of receptors and selected enzymes, as well as *in vivo* data from tests of inflammation and nociception, are presented in this paper for one compound from each series: RO1138452 and RO3244794. A brief report concerning the identification and synthesis of RO1138452 has appeared recently (Clark *et al*., 2004) and preliminary *in vivo* analgesic efficacy data for this compound have appeared in abstract form (Jett *et al*., 2005).

Methods

IP receptor binding

Native IP receptor in human platelets Human platelets were first centrifuged at low speed ($\sim 800 \times g$) for 5 min on a table top centrifuge. The supernatant was then centrifuged at 17,000 r.p.m. ($45{,}400 \times g$) for 30 min at 4°C. The pellet was resuspended in membrane buffer (20 mM Tris-HCl, 5 mM EDTA), homogenized using a polytron homogenizer (setting 5) and centrifuged again at $45{,}400 \times g$ for 30 min at 4°C. The pellet was then re-suspended in 20 mM Tris-HCl, 5 mM $MgCl_2$, homogenized, aliquoted and stored at −80°C until used. For competition displacement binding experiments, increasing concentrations of iloprost, RO1138452 and RO3244794 were used to compete with 10 nM ^{3}H-labelled iloprost binding. Briefly, 50 μg of platelet membrane was used for each reaction and nonspecific binding was determined in the presence of 10 μM unlabelled iloprost, while total binding was determined in the presence of 8.5 nM ^{3}H-labelled iloprost. The incubation time was 90 min at 25°C and the reaction was terminated by vacuum filtration using Whatman GF/B filtration plates. Scintillant was then added and the radioactivity counted using a TopCount microplate scintillation counter (Packard Instrument, CT, U.S.A.).

Recombinant IP receptor in Chinese hamster ovary (CHO)-K1 cells Human IP receptor-transfected cells (Genbank Accession Number: NM000960) were grown in Ham's F-12 medium with 10% foetal bovine serum, G418 (250 μg ml^{-1}), geneticin (150 μg ml^{-1}) and streptomycin/penicillin (30 μg ml^{-1} and 20 U ml^{-1}) at 37°C in 7% CO_2. Confluent cells were washed once by suspending the cells in phosphate-buffered saline (PBS) and collecting them by centrifugation at $1000 \times g$ for 10 min at 4°C and harvested by incubating the cells in PBS containing 2 mM EDTA. Cells were then washed twice with PBS and left on ice for 15 min and homogenized in buffer (50 mM Tris-HCl, 5 mM EDTA, pH 7.5 at 4°C) using a Polytron homogenizer at setting of 6 for 10 s. The homogenate was centrifuged at $39{,}000 \times g$ for 30 min at 4°C and the pellet was resuspended in the assay buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4 at 25°C) at $\sim$4 mg protein ml^{-1}.

For displacement of ^{3}H-labelled iloprost with RO1138452, membranes were precoupled to wheat germ agglutinin-coupled scintillation proximity assay beads (WGA-SPA beads by suspending the beads in assay buffer at 500 mg/25 ml. Equal volumes of suspended beads and resuspended membranes were mixed and placed on an orbital shaker (VWR, DS-500) maintained at 300 r.p.m. for 2 h. The receptor precoupled beads were then centrifuged at $300–500 \times g$ for 7 min and the pellet was washed once with assay buffer. The final pellet was brought up to the original volume with assay buffer. For displacement of ^{3}H-labelled iloprost by cold iloprost and RO3244794, regular filtration methodology was used as described for the native human platelets. Increasing concentrations of iloprost, RO1138452 and RO3244794 were used to displace 7.5 nM (for WGA-SPA assay) or 12 nM (for filtration assay) ^{3}H-labelled iloprost.

All competition binding data were analyzed by iterative curve fitting to a four parameter logistic equation. Hill coefficients and IC_{50} values were obtained directly. pK_i (−log of the molar K_i) of competing ligands were calculated from IC_{50} values using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). For reference, the K_d for ^{3}H-labelled iloprost was between 2.9 and 3.5 nM, based on radioligand binding isotherm studies in CHO-K1 cells, and was between 10 and 11 nM in human platelet membranes. For all binding experiments, pK_i values are presented as the mean ± s.e.m.

Functional assay (cAMP) of IP receptor antagonism

IP receptor-transfected CHO-K1 cells were cultured in Ham's F-12 nutrient media supplemented with 10% foetal bovine serum and G418 (300 μg ml^{-1}), harvested at 90% confluence, washed twice with PBS and detached with VERSENE for

5 min at 37°C. Cells were then re-suspended in 40 ml of stimulation buffer (Hank's buffered salt solution with 5 mM HEPES, 0.1% bovine serum albumin) and centrifuged at $800 \times g$ for 5 min. After centrifugation, the pellet was suspended in stimulation buffer (with 0.5 M isobutylmethyl xanthine, IBMX). Cells were diluted to the appropriate number of cells ml^{-1} for a plating density of 100,000 cells $well^{-1}$. cAMP detection was carried out using the AlphaScreen™ assay platform in a 96-well format. For inhibition experiments, 5 μl of either RO1138452 or RO3244794 (in stimulation buffer) was dispensed to a 96-well plate in triplicate. Cell suspensions (10 μl) were added with anti-cAMP acceptor beads in stimulation buffer to each plate and incubated for 15 min at room temperature (in the dark or covered with a black plate). Then 5 μl of agonist or vehicle was added to each well; for the wells containing antagonist, 10 nM of carbaprostacyclin ($cPGI_2$), a stable PGI_2 analogue, was used. Plates were incubated for 30 min at room temperature (in the dark or covered with a black plate) before the addition of 10 μl donor beads with biotin-cAMP in lyses buffer (5 mM HEPES, 0.3% Tween-20, 0.1% bovine serum albumin). Plates were incubated for 1 h with gentle shaking (in the dark or covered with a black plate). Plates were read on an AlphaScreen™ Fusion analyzer (Perkin Elmer, Boston, MA, U.S.A.).

Receptor/enzyme profiling

Selectivity was determined by the ability of RO1138452 or RO3244794 (10 μM) to displace specific binding of standard radioligands at 51 receptors (Cerep, Celle L'Evescault, France). When significant displacement of radioligand was observed (>70% for RO1138452 and >30% for RO3244794), complete concentration-dependent displacement curves (in triplicate) were constructed to generate IC_{50} values. Displacement binding at the EP_3 receptor was performed at Roche Palo Alto. Enzyme inhibition assays were also conducted by Cerep according to standardized protocols. RO1138452 was evaluated at 10 μM in triplicate for inhibition of COX isoforms: COX-1 (ram seminal vesicle), COX-2 (sheep placenta and human umbilical vein). Arachidonic acid was used as a substrate and PGE_2 accumulation was detected by Flash Plate™. For RO3244794 (10 μM), human COX-2 activity was determined using arachidonic acid as a substrate (with PMA as COX-2 inducer) and PGE_2 accumulation was determined by radioimmunoassay.

Tests of nociception hyperalgesia and inflammation

All animal studies were carried out in accordance with the principles and policies of the Institutional Animal Care and Use Committee at Roche Palo Alto. Rats were housed at 21 ± 2°C on a 12 : 12 h light–dark cycle beginning at 0700 for at least 7 days prior to and during experimentation. Water and rat chow were provided *ad libitum*.

Abdominal constrictions test Male Sprague–Dawley rats (~120 g, Charles River, Hollister, CA, U.S.A.) were intravenously (i.v.) administered vehicle (1 ml kg^{-1}), indomethacin (5–10 mg kg^{-1}) or test compound ($n = 6$–8). After 60 min, acetic acid (1%, 2 ml kg^{-1}) in deionized water was injected into the peritoneum as described previously (Jett *et al*., 1999). The number of abdominal constrictions followed by dorsiflexion and extension occurring during a 15-min period beginning 15 min after acetic acid administration was counted. The data are expressed as mean (±s.e.m.) number of abdominal constrictions per 15 min period.

Carrageenan-induced paw hyperalgesia test Male Sprague–Dawley rats (~120 g, $n = 10$, Charles River, Hollister, CA, U.S.A.) were anesthetized with halothane (5%) and administered 100 μl of vehicle or carrageenan (1% in saline) subcutaneously (s.c.) on the plantar surface of the left hind paw, as described previously (Jett *et al*., 1999). Vehicle (1 ml kg^{-1}) or test compound were administered orally (p.o.) 2 h after carrageenan administration and 1 h before evaluation of hind paw mechanical hyperalgesia, measured as the change in paw withdrawal threshold (g) at which a rat removes its hind paw, vocalizes or struggles, using the Ugo Basile Analgesy meter (Stoelting, Wood Dale, IL, U.S.A.). Data are expressed as mean (±s.e.m.) paw withdrawal threshold (g).

Carrageenan-induced edema formation test Male Sprague–Dawley rats (~130–140 g, $n = 10$, Charles River, Hollister, CA, U.S.A.) were assigned to treatment groups so that each group was weight balanced and administered vehicle, indomethacin (positive control) or test compound. Immediately thereafter, the rats were anesthetized with halothane (5%) and administered 50 μl of vehicle or carrageenan (0.5% in saline) s.c. on plantar surface of the left hind paw, as described previously (Jett *et al*., 1999). After 3 h, the volume (Plethysmometer, Stoelting, Wood Dale, IL, U.S.A.) or weight of the treated and untreated hind paws was recorded and the difference between the paws was calculated. Both methods used to measure edema formation produced comparable results. Data are expressed as mean (±s.e.m.) difference in volume (ml) or weight (g), as a reflection of edema formation.

Monoiodoacetate-induced osteoarthritis test To induce symptoms of osteoarthritis, male Wistar rats (170–180 g, $n = 10$, Charles River, Hollister, CA, U.S.A.) were anesthetized with isoflurane (2% in O_2), administered a single intraarticular injection of sodium monoiodoacetate (mIOA; 1 mg in 50 μl) and returned to the animal colony (Bove *et al*., 2003). After 14 days, the rats were evaluated for joint discomfort, using an Incapacitance Tester (Linton Instrumentation, Norfolk, U.K.), which measures the weight distribution between the right (injected) and left (control) hind paws, with the difference in weight distribution being an index of joint discomfort. The rats were tested before and after administration of vehicle, rofecoxib (positive control) or test compound. Data are expressed as mean (±s.e.m.) difference in weight distribution (g) between the osteoarthritic and control hind paws.

Pharmacokinetic analysis

Male Sprague–Dawley rats ($n = 3$) were administered RO1138452 or RO3244794 (5 mg kg^{-1}, i.v.). At various times after dose administration, the rats were anesthetized by halothane (5%), blood was collected by orbital bleed into a heparinized syringe and a plasma fraction was obtained by centrifugation of the blood at $2600 \times g$ for 5 min in a clinical centrifuge. The level of test compound in each sample was determined by high-performance liquid chromatography with detection by mass spectrometry. Data are expressed as mean

(±s.d. mean). Values for plasma half-lives, volume of distribution, etc., were calculated using WinNonlin (Pharsight Corp., Mountain View, CA, U.S.A.).

Compound synthesis and materials

The HCl salts of RO1138452 ((4,5-dihydro-1H-imidazol-2-yl)-[4-(4-isopropoxy-benzyl)-phenyl]-amine), RO3244794 (*R*-3-(4-fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxy-carbonylamino]-propionic acid) and the sodium salt of indomethacin (Sigma-Aldrich Corp. (St Louis, MO, U.S.A.)) were synthesized by the Roche Palo Alto Department of Medicinal Chemistry using methods described in previous publications or patents from our laboratory (Cournoyer *et al*., 2001; Clark *et al*., 2004). RO1138452 and RO3244794 are structurally depicted in Figures 1a and b, respectively. U-44069 (9,11-dideoxy-9α-epoxymethano-prosta-5Z, 13E-dien-1-oic acid) was used as a standard in the TP receptor radioligand binding assay (Cerep, Cerep (Celle L'Evescault, France). RO1138452 (2–10 ml kg^{-1}, $<$10 mg ml^{-1}) was readily dissolved in saline or water. RO3244794 (1 ml kg^{-1}, $<$30 mg ml^{-1}) was dissolved in one of several vehicles: (a) 100 mM Trizma® base; (b) 10% DMSO, 50% propylene glycol in deionized water and (c) 5.6% sodium benzoate, 0.5% benzoic acid, 85% propylene glycol or suspended in 0.5% carboxymethylcellulose, 0.9% sodium chloride, 0.4% polysorbate, 0.9% benzyl alcohol in deionized water.

Human platelets were obtained from BRT laboratories (BRT Laboratories Inc., Baltimore, MD, U.S.A.). ^{3}H-labelled iloprost was obtained from Amersham Biosciences (Piscataway, NJ, U.S.A.); all other prostanoids were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.), unless specified otherwise. The reagents used for cell culture – Ham's F-12 media, foetal bovine serum, G418 sulfate (geneticin), gentamycin, streptomycin/penicillin, PBS, Versene, HEPES, EDTA – were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Unlabelled Iloprost and WGA-SPA beads were obtained from Amersham Biosciences (Buckinghamshire, U.K.). Whatman GF/B filtration plates were from Whatman Inc., Florham Park, NJ, U.S.A. and the scintillant (Readysafe) was from Beckman Coulter Inc. (Fullerton, CA, U.S.A.). Bovine serum albumin (BSA), IBMX, Tris-HCL monosodium iodoacetate, acetic acid, λ-carrageenan and $MgCl_2$ was obtained from Sigma-Aldrich Corp. (St Louis, MO, U.S.A.). The Alpha-Screen™ kit (Perkin Elmer, MA, U.S.A.), DS-500 Orbital Shaker (VWR, West Chester, PA, U.S.A.) and 96-well plates (Corning, NY, U.S.A.) were also used.

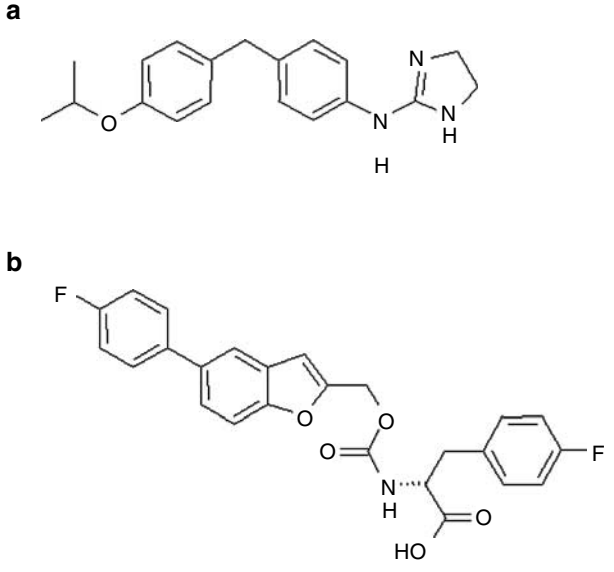

Figure 1 Structures of (a) RO1138452 ((4,5-dihydro-1H-imidazol-2-yl)-[4-(4-isopropoxy-benzyl)-phenyl]-amine), and (b) RO3244794 (*R*-3-(4-fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxy-carbonylamino]-propionic acid).

Data analysis

In vitro data was analyzed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, U.S.A.). For *in vivo* data from the abdominal constriction and carrageenan-induced paw edema tests, all groups were analyzed using a nonparametric method (the Kruskal–Wallis test) followed by *post hoc* comparisons of each dose group *versus* vehicle using the Wicoxon rank-sum exact test. For data from the carrageenan-induced paw hyperalgesia test and the mIOA osteoarthritis test, all groups were compared using a one-way analysis of variance (ANOVA) followed by *post hoc* comparisons of each dose group *versus* vehicle using Dunnett's test.

Results

Affinity estimates of RO1138452 and RO3244794 for human IP receptors

We estimated the binding affinity (pK_i) of RO1138452 and RO3244794 in two independent systems using competition displacement assays from 4 to 5 independent experiments: human platelet membranes (Figure 2a) and CHO-K1 membranes expressing the recombinant human IP receptor (Figure 2b). For the native and the recombinant human IP receptor systems, the affinity estimates of iloprost were 8.3±0.2 and 8.4±0.1, respectively. RO1138452 was more potent than RO3244794 in displacing ^{3}H-labelled iloprost in both systems studied. In human platelets, affinities of RO1138452 and RO3244794 were 9.3±0.1 and 7.7±0.03, respectively. In the recombinant IP receptor system, the affinities of RO1138452 and RO3244794 were estimated to be 8.7±0.1 and 6.9±0.1, respectively. In both the platelet and the recombinant systems, the Hill coefficients did not differ significantly from unity.

Functional antagonism of cPGI$_2$-induced cAMP accumulation

In order to determine whether RO1138452 and RO3244794 behave as functional antagonists of the IP receptor, we tested whether these two compounds could block cAMP accumulation in CHO-K1 cells overexpressing the human IP receptor in 4–5 independent experiments (Figure 3). $cPGI_2$ was used as the agonist to stimulate the IP receptor in these cells and showed a potency (pEC_{50}) of 10±0.08. For cAMP inhibition experiments, 10 nM $cPGI_2$ was used to drive the cAMP signalling pathway after incubating cells with various concentrations of RO1138452 and RO3244794. Consistent with the binding affinities, RO1138452 was a more potent antagonist of the

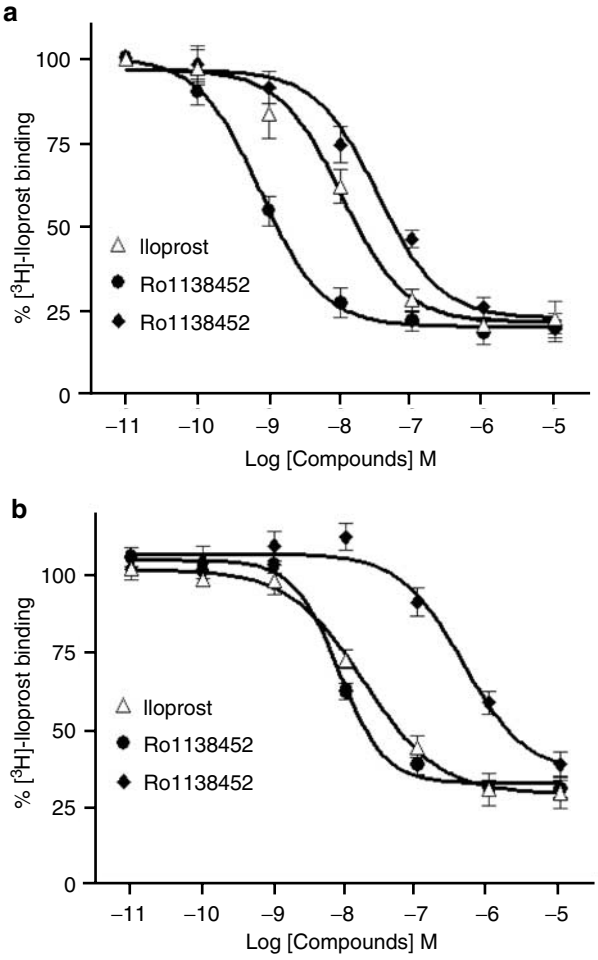

Figure 2 Binding affinity (pK_i) estimates of RO1138452 and RO3244794 at the human IP receptor. Iloprost, RO1138452 and RO3244794 displaced ^{3}H-labelled iloprost (7.5–12 nM) binding in a concentration-dependent manner in native human platelets (a) and in CHO-K1 cells expressing the IP receptor (b). In both the platelet and the recombinant systems, the Hill coefficients did not differ significantly from unity. Data are presented as total binding and represents mean of 4–5 independent experiments with bars indicating s.e.m.

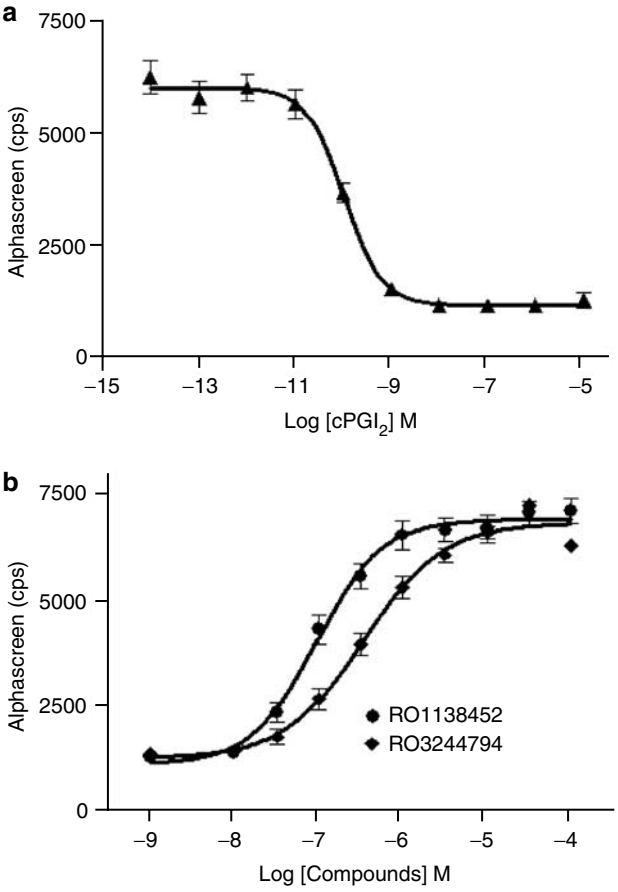

Figure 3 Functional antagonism of RO1138452 and RO3244794 in CHO-K1 cells expressing the human IP receptor. In this assay, cAMP level is inversely proportional to the raw counts (cps) shown on the *Y*-axis. $cPGI_2$ increased cAMP accumulation in a concentration-dependent manner (a). RO1138452 and RO3244794 attenuated 10 nM $cPGI_2$-induced cAMP accumulations (b). Points represent mean of 4–5 independent experiments with bars indicating s.e.m.

human IP receptor than RO3244794. The pIC_{50} values of RO1138452 and RO3244794 in attenuating cAMP accumulation were 7.0 ± 0.07 and 6.5 ± 0.06, respectively. Using the Cheng–Prusoff correction factor (Cheng & Prusoff, 1973), functional estimates of affinities for RO1138452 and RO3244794 were 9.0 ± 0.06 and 8.5 ± 0.11, respectively. The reversibility of both compounds remains to be characterized. Neither RO1138452 nor RO3304794 showed any agonist activity at the recombinant IP receptor (data not shown). Neither compounds inhibited platelet aggregation, independent of stimulus (ADP or adrenaline), as would be expected of an agonist at the native IP receptor (data not shown).

Selectivity of RO1138452 or RO3244794

Receptor selectivity profiles for RO1138452 and RO3244794 were determined by evaluating the effects of the IP receptor ligands on specific binding of standard radioligands to more than 50 receptors. Full displacement curves were constructed with those receptor subtypes at which RO1138452 and RO3244794 (10 μM) inhibited specific radioligand binding by >70 and $>30\%$, respectively, in an initial screen: α_{1B} and α_{2A} adrenoceptor, I_2, muscarinic (M_1–M_5), platelet activating factor (PAF) and 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} and 5-HT_4. Potency (IC_{50}) and affinity (pK_i) estimates of RO1138452 and reference standards at these receptor subtypes are indicated in Table 1. Except for the I_2 and PAF receptor, RO1138452 displayed relatively low affinities at all other receptor subtypes tested. RO1138452 did not exhibit any significant potency at non-IP prostanoid receptors (EP_1, EP_3, FP and TP; data not shown). As a result of intrinsically lower IP receptor affinity, the threshold for displacement by RO3244794 (10 μM) at the receptor subtypes in the selectivity panel was lowered to 30%. In this experiment, RO3244794 displaced 30% or more of the radioligands for the following receptor subtypes: adenosine (A_3), EP_1, EP_3, EP_4 and TP. RO3244794 displayed relatively weak affinities at these four receptor subtypes (Table 1), with no activity at I_2 or PAF receptors.

We also determined RO1138452 and RO3244794 to be weak inhibitors of COX-1 and COX-2 enzymes. RO1138452 (10 μM) produced less than 10% inhibition of ram COX-1, but

Table 1 Receptor selectivity of RO1138452 and RO3244794

Receptors	*RO1138452*		*RO3244794*		*Reference compounds*	
	IC_{50} (nM)	pK_i	IC_{50} (nM)	pK_i		pK_i
α_{1B} (rat)	3280	5.87	—	—	Spiperone	8.68
α_{2A} (human)	724	6.49	—	—	Yohimbine	8.38
I_2 (rat)	7	8.33	—	—	Idazoxan	8.68
M_1 (human)	2570	5.66	—	—	Pirenzepine	7.89
M_2 (human)	2220	5.88	—	—	Methoctramine	7.76
M_4 (human)	1450	6.14	—	—	4-DAMP	9.19
M_5 (human)	3110	5.81	—	—	4-DAMP	9.01
PAF (rabbit)	52.9	7.9	—	—	C16-PAF	10.4
5-HT_{1A} (human)	8580	5.37	—	—	8-OH-DPAT	8.78
5-HT_{1B} (rat)	1130	6.11	—	—	Serotonin	7.87
5-HT_{2A} (rat)	3040	5.71	—	—	Ketanserin	8.84
5-HT_{2C} (pig)	1190	6.11	—	—	Serotonin	7.63
5-HT_4 (guinea-pig)	8910	5.35	—	—	Serotonin	7.19
A_3 (human)	—	—	6860	5.32	IB-MECA	8.62
EP_1 (human)	—	—	—	—	17-phenyl-PGE_2	7.85
EP_3 (human)	—	—	3980	5.38	PGE_2	8.37
EP_4 (human)	—	—	4190	5.74	17-phenyl-PGE_2	7.85
TP (human)	—	—	12,600	5.09	U-44069	6.01

Receptor profiling was carried out at Cerep (Celle L'Evescault, France). Inhibition curves were generated for RO1138452 and RO3244794 at receptor subtypes that displayed significant inhibition of specific radioligand binding (>70% for RO1138452 and >30% for RO3244794) at 10 μM of compound concentration. — indicates inactivity at the receptor subtype.

Table 2 Pharmacokinetic parameters of RO1138452 and RO3244794 in rats

Characteristics	*RO1138452*	*RO3244794*
Molecular mass (g mol^{-1})	309.41	451.43
Polar surface area	42.57	72.19
Plasma $T_{1/2}$ (h; i.v.)	1.3	3.4
Plasma protein binding (%)	95.3	99.86
Vdβ (l kg^{-1})	12.7	1.64
Oral bioavailability (%)	0.70	50.8

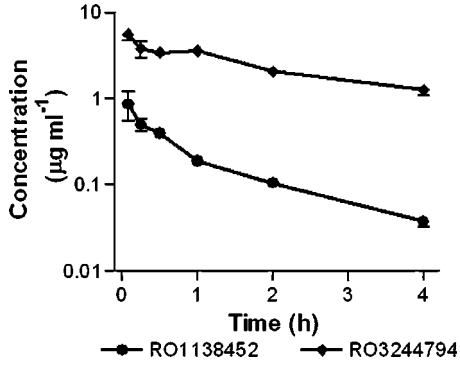

Figure 4 Total plasma concentrations of RO1138452 and RO3244794 following intravenous administration of 5 mg kg^{-1} to male rats, in saline or Tris Base, respectively. Points indicate mean (±s.d.).

inhibited the activity of human COX-2 by 29%. Likewise RO3244794 (10 μM) did not inhibit human COX-1 activity, but produced comparable inhibition of human COX-2 (27%).

Pharmacokinetics

RO1138452 and RO3244794 displayed dissimilar pharmacokinetic properties (Table 2). RO1138452 showed a shorter plasma half-life, lower plasma protein binding, a larger volume of distribution (Vdβ) and a lower total plasma concentration than RO3244794 following i.v. administration to rats. However, despite the difference in total plasma concentration following equivalent i.v. doses of the two compounds (Figure 4), the free plasma levels could be similar if the higher plasma protein binding of RO3244794 is taken into account. For example, 1 h after administration of RO1138452 and RO3244794 (5 mg kg^{-1}, i.v.) to rats, the total plasma concentrations were 0.189 and 3.57 μg ml^{-1}, respectively, whereas the free plasma concentrations were calculated to be 0.009 and 0.005 μg ml^{-1} (28 and 11 nM), respectively. RO3244794 also has greater oral bioavailability than RO1138452 in rats: 50.8 *versus* 0.69%, respectively.

Tests of nociception, hyperalgesia and inflammation

Intraperitoneal injection of an irritant, such as acetic acid, provokes prostanoid-dependent chemical nociception characterized by abdominal constrictions (Berkenkopf & Weichman, 1988). To determine if IP receptors mediate the response, the two selective IP receptor antagonists were evaluated for their effects on acetic acid-induced abdominal constrictions in rats. In vehicle-treated rats, acetic acid induced 12±1.7 constrictions during the 15-min observation period (Figure 5a). RO1138452 (1–10 mg kg^{-1}, i.v., $n=6$–8) significantly ($P<0.05$) inhibited constrictions by 65±10% at 10 mg kg^{-1}, i.v. (see Figure 5a). In a separate study (Figure 5b), acetic acid induced 9.8±0.8 constrictions and RO3244794 (1–30 mg kg^{-1}, i.v., $n=8$) significantly ($P<0.01$) and dose-dependently decreased the number of constrictions by 100% at the highest dose level, with an ED_{50} value of 3.8±1.03 mg kg^{-1}.

Injection of carrageenan into the rat hind paw elicits a persistent inflammatory response characterized, in part, by mechanical hyperalgesia (Vinegar *et al*., 1976). To determine the involvement of the IP receptor in carrageenan-induced mechanical hyperalgesia, the effects of selective IP receptor antagonists administered 2 h after carrageenan treatment and 1 h before testing were assessed. In vehicle-treated, naïve rats

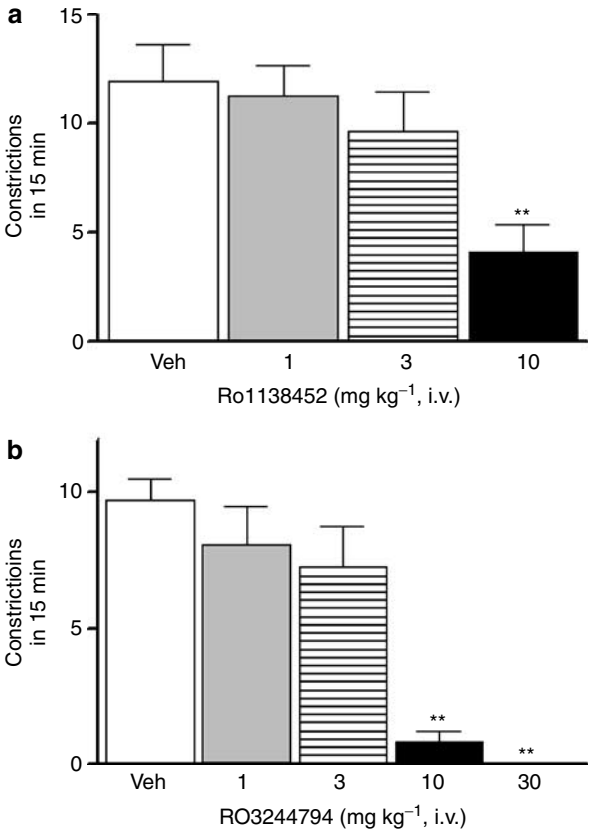

Figure 5 Effects of (a) RO1183452 (1, 3 and 10 mg kg^{-1}, i.v.) and (b) RO3244794 (1, 3, 10 and 30 mg kg^{-1}, i.v.) or vehicles (water or buffered 90% propylene glycol (1 ml kg^{-1}, i.v.), respectively) on 1% acetic acid-induced abdominal constrictions in conscious rats ($n=6$–8). The data are expressed as the mean ($\pm$s.e.m.) number of abdominal constrictions during a 15-min observation period. ** indicates $P<0.01$, nonparametric method (Kruskal–Wallis test) followed by Wilcoxon's *post hoc* tests, compared to vehicle.

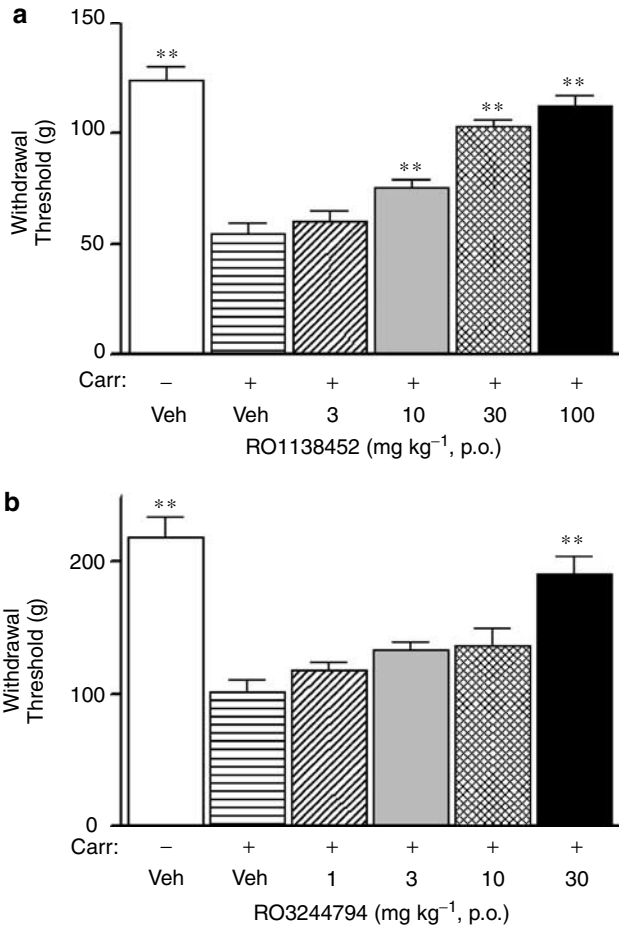

Figure 6 Effects of (a) RO1138452 (3, 10, 30 and 100 mg kg^{-1}, p.o.) and (b) RO3244794 (1, 3, 10 and 30 mg kg^{-1}, p.o.) or vehicles (water (2 ml kg^{-1}, p.o.) or 10% DMSO and 50% propylene glycol (1 ml kg^{-1}, p.o.), respectively) on carrageenan-induced mechanical hyperalgesia of the hind paw in conscious rats ($n=10$). The data are expressed as (mean $\pm$ s.e.m.) paw withdrawal threshold in grams. ** indicates $P<0.01$, ANOVA and Dunnett's *post hoc* tests, compared to vehicle.

(not treated with carrageenan), the paw withdrawal threshold was 125$\pm$6 g, whereas the withdrawal threshold in rats pretreated with vehicle and carrageenan was 55$\pm$5 g with the lower threshold reflecting mechanical hyperalgesia (Figure 6a). RO1138452 (3–100 mg kg^{-1}, p.o., $n=10$) significantly ($P<0.01$) increased the withdrawal threshold in a dose-dependent manner to 113$\pm$5 g, (84$\pm$8% inhibition of hyperalgesia) with an ED_{50} value of 18.3$\pm$1.9 mg kg^{-1} (Figure 6a). In a separate study (Figure 6b), in which carrageenan pretreatment reduced the paw withdrawal threshold from 220$\pm$15 g in naïve control rats to 104.5$\pm$4.9 g, RO3244794 (1–30 mg kg^{-1}, p.o., $n=10$) significantly ($P<0.01$) increased the paw withdrawal threshold in a dose-dependent manner to a maximum of 192$\pm$13.5 g (76$\pm$12% inhibition of hyperalgesia), with an ED_{50} value of 14$\pm$3.6 mg kg^{-1}. This response did not result from sedation, since a higher dose (100 mg kg^{-1}, p.o.) does not affect locomotor activity in rats (data not shown).

Injection of carrageenan into the rat hind paw also induces the formation of edema, which, in part, is attenuated by COX enzyme inhibitors (Vinegar *et al*., 1976). To determine the role of the IP receptor in prostanoid-dependent component of edema formation, the effects of the two selective IP receptor antagonists were evaluated on carrageenan-induced edema, with the compounds being administered immediately before injection of carrageenan. In vehicle treated rats, carrageenan stimulated 0.71$\pm$0.04 ml (Figure 7a) of edema. Indomethacin (10 mg kg^{-1}, p.o.) significantly ($P<0.01$) reduced the edema to 0.37$\pm$0.03 ml, a 100% reduction of the prostanoid-dependent edema formation. Under the same conditions, RO1138452 (3–100 mg kg^{-1}, p.o. $n=10$) significantly ($P<0.01$) decreased edema to 0.48$\pm$0.03 ml, a 77$\pm$27% reduction of prostanoid-dependent edema at the highest dose level tested (Figure 7a). In a separate study (Figure 7b), carrageenan-stimulated edema in vehicle treated rats was 0.56$\pm$0.03 g. Indomethacin (5 mg kg^{-1}, p.o.) significantly ($P<0.01$) reduced the edema to 0.32$\pm$0.04 g. RO3244794 (0.3–10 mg kg^{-1}, p.o., $n=10$) significantly ($P<0.01$) decreased edema formation in a dose-dependent manner to 0.33$\pm$0.03 g, a 99$\pm$7% reduction of prostanoid-dependent edema. This value was not significantly different from that produced by indomethacin. A higher dose (30 mg kg^{-1}) did not further reduce edema formation (data not shown).

Intra-articular injection of mIOA produces a chronic osteoarthritic-like condition in rats, presumably associated with joint discomfort (Bove *et al*., 2003). Since RO3244794 showed superior pharmacokinetics with generally better *in vivo*

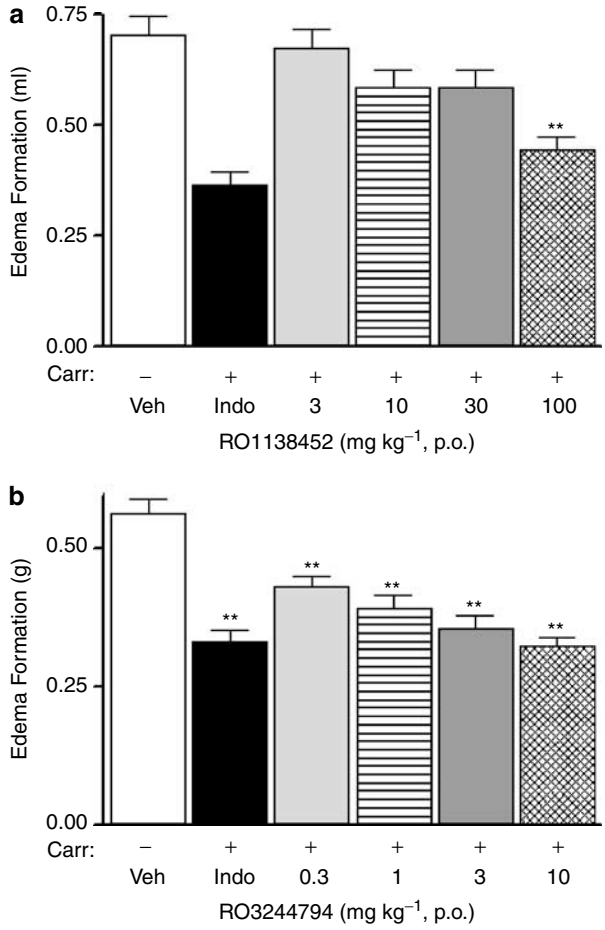

Figure 7 Effects of (a) RO1138452 (3, 10, 30 and 100 mg kg^{-1}, p.o.) and (b) RO3244794 (0.3, 1, 3 and 10 mg kg^{-1}, p.o.) and indomethacin (Indo, 5 mg kg^{-1}, p.o.) or their vehicles (water (10 ml kg^{-1}, p.o.) or 0.5% carboxymethylcellulose, 0.4% polysorbate 80, 0.9% benzyl alcohol in saline (1 ml kg^{-1}, p.o.), respectively) on carrageenan-induced edema formation of the treated hind paw in conscious rats ($n=10$). The data are expressed as the mean ($\pm$s.e.m.) difference between the treated and untreated hind paw (a) volume displacement (ml) or (b) paw weight (g). ** indicates $P<0.01$, nonparametric method (Kruskal–Wallis test) followed by Wilcoxon's *post hoc* tests, compared to vehicle.

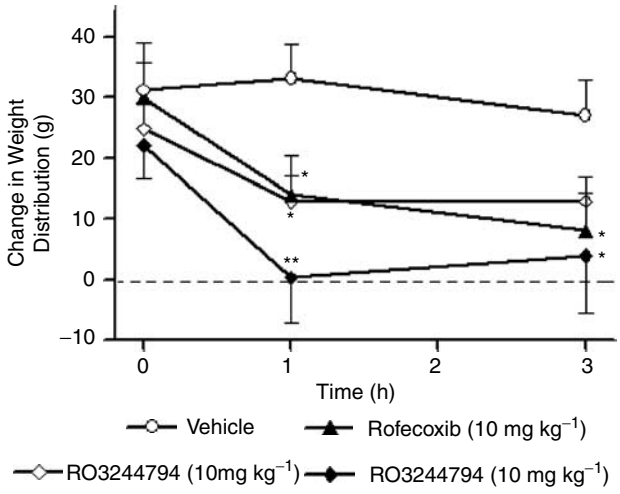

Figure 8 Effects of RO3244794 (1 and 10 mg kg^{-1}, p.o.) or the positive control, rofecoxib (10 mg kg^{-1}, p.o.) or vehicle (0.5% carboxymethylcellulose, 0.4% polysorbate 80, 0.9% benzyl alcohol in saline (1 ml kg^{-1}, p.o.)) on weight distribution change (g) in rats with mIOA-induced osteoarthritis determined before (0 h) or 1 and 3 h after dosing. The data are expressed as the mean ($\pm$s.e.m.) change between the osteoarthritic and control hind paws. * indicates $P<0.05$ and ** indicates $P<0.01$, ANOVA and Dunnett's *post hoc* tests, compared to vehicle.

potency and efficacy as compared with RO1138452, it was further characterized in the mIOA model of osteoarthritis in rats. RO3244794 (1 and 10 mg kg^{-1}, p.o., $n=10$) significantly ($P<0.05$) reduced the difference in weight distribution between the osteoarthritic and control hind paws, reflecting amelioration of joint discomfort (Figure 8). At 1 mg kg^{-1}, the difference in weight distribution was reduced from 24.8 ± 4.4 to 12.8 ± 4.2 g ($P<0.05$) at 1 h following dosing compared with vehicle, which increased the weight distribution slightly from 31.2 ± 7.8 to 33.1 ± 5.7 g during the same 1 h. At 10 mg kg^{-1}, the difference in weight distribution decreased from 22.1 ± 5.4 to 0.34 ± 7.5 g ($P<0.01$) at 1 h following dosing, compared with the vehicle control; this constituted a $99\pm23\%$ reduction. Activity seen with both doses of RO3244794 at 1 h was sustained to at least 3 h. Further, the effects achieved with both doses of RO3244794 were similar in magnitude to that obtained with the selective COX-2 inhibitor, rofecoxib (10 mg kg^{-1}), the positive control.

Discussion and conclusions

In the present series of studies, we have provided the first detailed pharmacological characterization of RO1138452 and RO3244794, two potent, selective and structurally distinct antagonists of human and rat IP prostanoid receptors. As a consequence, all five subclasses of prostanoid receptors now have selective antagonists identified. RO324474 shares the structural motif of a carboxylic acid group with all of the endogenous prostanoid agonists, and may thus bind to the same arginine residue on the seventh transmembrane region of the IP receptor as does PGI_2 (Stitham *et al*., 2002). In contrast, it is possible that the guanidine-containing RO1138452 binds to a nearby aspartate residue, also on the seventh transmembrane region (Clark *et al*., 2004).

Although the imidazoline, RO1138452, displays a higher affinity for the human IP receptor ($pK_i=9.3$ in platelets) than the propionic acid, RO3244794 ($pK_i=7.7$), both compounds are competitive antagonists, lack agonistic activity at the IP receptor and behave comparably as functional antagonists, with pK_i values of 9.0 and 8.5, respectively. There seems to be a modest difference between the binding and functional affinity estimates for RO3244794, but not RO1138452. This suggests that the difference is not due to some assay artefact, but rather raises the possibility of peculiar differences in mode of action of the two antagonists. That is, there could be differences in either the on-rate or off-rate of the antagonists or in the ability of the individual antagonists to reach equilibrium; such differences could be attributed to the structural dissimilarity of the compounds. With respect to selectivity, the most significant non-IP receptor interactions of RO1138452 are with the I_2 binding site ($pK_i=8.33$) and the PAF receptor ($pK_i=7.90$); see Table 1. By contrast, RO3244794 interacts with measurable affinity (pK_i 5.09–5.74) at only four of the 51 receptors evaluated. Although it is possible that activities of RO1138452

at either I_2 or PAF receptors may have contributed in some manner to the antihyperalgesic and anti-inflammatory responses observed *in vivo* (discussed below), the similar profiles of the two antagonists in the rat models would argue against this and toward a common mechanism of action.

The usefulness of RO1138452 and RO3244794 in assessing the physiological roles of IP receptors is clear, and is not apparently complicated by differential action at any subtypes. Although no heterogeneity in IP receptors is reported, splice variants have been identified for the EP_1, EP_3, FP and TP prostanoid receptors (Pierce & Regan, 1998), pharmacological evidence for a novel IP receptor or splice variant in brain (Takechi *et al*., 1996; Watanabe *et al*., 1999) and kidney (Hebert *et al*., 1995) has been reported, but not clearly substantiated. To date, the only documented IP receptor nucleotide polymorphism (Stitham *et al*., 2002) involves a variant that shows decreased agonist affinities at low pH; this has been postulated to produce reduced activity of PGI_2 during ischemic conditions and hence predispose those with this variant to cardiovascular disease (Stitham *et al*., 2002). In addition, the human IP receptor may in fact form homodimers and homooligomers (Giguere *et al*., 2004), as well as heterodimers with the TP receptor (Wilson *et al*., 2004). Nevertheless, none of the studies conducted in order to characterize RO1138452 or RO3244794 have provided evidence for functionally distinguishable IP receptor subtypes. Not only could all IP agonist-induced responses be almost completely attenuated with adequate concentrations of antagonists (Figure 3), but neither PGI_2 or any of the synthetic IP receptor agonists used in our studies displayed concentration–response curves with multiple phases in either human platelets, neuroblastoma cells or rat sensory neurons (data not shown). No attempt has been made in the current study to determine whether either of the novel receptor antagonists interacts with PPAR receptors, as has been reported for PGI_2 and iloprost (Lim & Dey, 2002).

The *in vivo* studies with RO1138452 and RO3244794 reported here have confirmed that PGI_2-mediated activation of IP receptors plays an important pronociceptive and inflammatory role in four well-characterized rodent models. Acetic acid-induced abdominal constrictions are completely attenuated by RO3244794 (Figure 5), an effect that is quite similar to that seen with a high dose level of a nonselective COX inhibitor (Jett *et al*., 1999). High-dose levels of RO3244794 also provide complete reversal of carrageenan-induced hyperalgesia (Figure 6) and edema (Figure 7), and mIOA-induced joint discomfort (Figure 8). These findings are consistent with data derived from IP receptor knockout mice, which have suggested that PGI_2 working through the IP receptor – and not PGE_2 – is the major mediator of acetic acid-induced visceral nociception (Murata *et al*., 1997; Ueno *et al*., 2001) and carrageenan-induced paw edema (Ueno *et al*., 2000).

It should be noted that the slightly higher ED_{50} values reported for RO1138452 relative to RO3244794 following oral or i.v. administration in our *in vivo* rat assays – despite the higher *in vitro* affinity of the former for the IP receptor – may be due to the inferior pharmacokinetic profile of RO1138452 in rat. In contrast to RO3244794, which has a longer (3.4 h) plasma half-life and higher oral bioavailability, RO1138452 has a plasma half-life of 1.3 h and very low oral bioavailability. In addition, the *in vivo* potency of RO3244794 itself may be somewhat reduced by higher levels of plasma protein binding.

The reduction in spontaneous and evoked pain behaviours by both RO1138452 and RO3244794 is consistent with interruption of a direct excitatory and sensitizing effect of PGI_2 on sensory neurons. However, neither peripheral nor central sites of action can be confirmed solely on the basis of our studies, as intraplantar injection of irritants such as carrageenan also induce upregulation of COX-2 and IP receptor mRNA in the spinal cord (Doi *et al*., 2002). Thus, it is possible that the antihyperalgesic effects of IP receptor antagonists take place either in the periphery or spinal cord or supra-spinal regions. Unfortunately, no definitive data are available concerning the distribution of these two compounds into the central nervous system. Future investigation of compound distribution in the central nervous system coupled with studies conducted using directed local administration could clarify this issue.

It has long been known that prostanoids can be generated by most cells in response to mechanical, thermal or chemical injuries or inflammatory insults (Samuelsson *et al*., 1978). Accordingly, the hyperalgesic and pro-inflammatory effects of PGE_2 and PGI_2 – and particularly the former – have been studied widely in numerous models of nociception and inflammation (e.g., Moriyama *et al*., 2005. However, when the sensitizing effects of PGE_2 and PGI_2 on sensory neurons have been compared directly, PGI_2 is equally or more effective as a hyperalgesic or sensitizing agent both *in vitro* and *in vivo* (e.g., Smith *et al*., 1998; Nakae *et al*., 2005a; reviewed in Bley *et al*., 1998; Kobayashi & Narumiya, 2002). To date, given the limited pharmacological tools available, unequivocal characterization of the primary pronociceptive and proinflammatory prostanoid receptor subtype and the endogenous agonist has not been possible. Use of compounds such as RO1138452 or RO3244794 should enable a quantitative determination of the relative contributions of PGI_2 and IP receptors to sensory activation and sensitization. The fact that RO1138452 and RO3244794 display high receptor selectivity (Table 1) and are not COX inhibitors enhances their utility for studies in models of inflammation and pain. In contrast, defining the contribution of EP receptors to nociceptive and inflammatory process on the basis of available antagonists is far more problematic: few antagonists are available and extensive receptor affinity and selectivity data have not been published for any. It should be noted that clinical development of two putative analgesic EP_1 receptor antagonists ZD-6416 (Sakar *et al*., 2003) and ONO-8711 (Omote *et al*., 2001) has been discontinued (Investigational Drugs Database, Thompson Current Drugs, U.K.).

Another exciting future application of IP receptor antagonists will be to define the interplay between PGI_2 and TXA_2 in the cardiovascular system. TXA_2 is a vasoconstrictor and induces platelet aggregation. Aspirin affords cardioprotection through inhibition of TXA_2 formation by virtue of its ability to irreversibly inhibit platelet cyclooxygenase (COX-1). PGI_2 is a vasodilator that inhibits platelet function, and has been thought to provide physiological balance to TXA_2 signalling (reviewed by Coleman *et al*., 1994). Recent studies with transgenic mice suggest that reperfusion-induced damage to cardiomyocytes and injury-induced vascular proliferation and platelet activation are enhanced in mice that are genetically deficient in the IP receptor, but are depressed in mice genetically deficient in the TP receptor or treated with a TP antagonist (Xiao *et al*., 2001; Cheng *et al*., 2002). This

interplay might help explain the putative adverse cardiovascular effects associated recently with selective COX-2 inhibitors, which unlike aspirin and nonsteroidal anti-inflammatory drugs, preferentially inhibit PGI_2 *versus* TXA_2 production (Cheng *et al*., 2002; Egan *et al*., 2004).

In conclusion, RO1138452 and RO3244794 are novel IP receptor antagonists that display a high affinity for human and rodent IP receptors. *In vitro* studies have demonstrated that these compounds are selective for the IP receptor over the EP and other members of the prostanoid receptor family, as well as over many other receptors and enzymes. Both compounds have undergone extensive *in vivo* pharmacological evaluation that shows the analgesic and anti-inflammatory potential of IP receptor antagonists. Give the relatively high receptor affinity and selectivity, these compounds should provide useful insights into the physiology of PGI_2 and the role of IP receptors in cardio-protection, blood flow regulation, pain and inflammation. After this manuscript was submitted, the authors became aware of a publication in press (Nakae *et al*., 2005b) describing the biological activities of two compounds belonging to the same structural series as RO3244794 (Cournoyer *et al*., 2001). These data appear to provide confirmation of some of the *in vitro* findings reported in this paper.

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