RESEARCH PAPER

Investigation of the prostacyclin (IP) receptor antagonist RO1138452 on isolated blood vessel and platelet preparations

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Background and purpose: The current study examined the utility of the recently described prostacyclin (prostanoid IP) receptor antagonist RO1138452 (2-(4-(4-isopropoxybenzyl)-phenylamino) imidazoline) as a tool for classifying prostanoid receptors.

Experimental approach: pA_2 values were determined on isolated smooth muscle and platelet preparations.

Key results: RO1138452 antagonized relaxation of human pulmonary artery, guinea-pig aorta and rabbit mesenteric artery induced by the selective IP agonist cicaprost. Schild plots had slopes close to unity, generating pA_2 values of 8.20, 8.39 and 8.12 respectively. Non-surmountable antagonism was sometimes found with the higher concentrations of RO1138452, attributable to the EP_3 contractile action of cicaprost. RO1138452 did not block relaxation of guinea-pig trachea induced by the EP_2 -selective agonist butaprost. In contrast, there was a modest inhibition of butaprost-induced relaxation of human pulmonary artery by RO1138452, implying activation of both EP_2 and IP receptors by butaprost. RO1138452 did not affect relaxation induced by PGE_2 (EP_4 agonist) and substance P (NK_1 /endothelium-dependent agonist) in rabbit mesenteric artery. In human and rat platelet-rich plasmas, RO1138452 antagonized cicaprost-induced inhibition of platelet aggregation in a surmountable manner; pA_2 values may have been affected by binding of RO1138452 to plasma protein. RO1138452 did not affect the inhibitory actions of PGD_2 (DP_1 agonist) and NECA (adenosine A_{2A} agonist) in human platelets.

Conclusions and implications: The data indicate that RO1138452 is a potent and selective IP receptor antagonist. RO1138452 represents an important addition to our armoury of prostanoid receptor antagonists and a potential clinical agent in situations where prostacyclin has a pathophysiological function.

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Abbreviations: CI, confidence interval; EMR, equi-effective molar ratio; L-NAME, *N*-nitro-*L*-arginine methyl ester; NECA, 5'-*N*-ethylcarboxamidoadenosine; PGE_2 , prostaglandin E_2 ; $pIC_{50\%}$, negative logarithm of concentration producing 50% inhibition of the induced response; PRP, platelet-rich plasma

Introduction

Prostacyclin was discovered and its structure elucidated some 30 years ago (Bunting *et al.*, 1976; Moncada *et al.*, 1976;

Whittaker *et al.*, 1976). While many hundreds of prostacyclin analogues have since been synthesized (see Wise and Jones, 2000), specific antagonists for the prostacyclin (prostanoid IP) receptor have been slow to emerge. Recently, Clark *et al.* (2004) reported on a series of 2-(phenylamino)-imidazolines that competitively antagonized cyclic AMP accumulation induced by the prostacyclin analog carbacyclin in SH-SY5Y neuroblastoma cells. The optimum agent, RO1138452 (compound 25d) had a pK_i of 8.7 in a competitive binding assay and a pK_b of 8.8 for inhibition of carbacyclin-induced adenylate cyclase activation in CHO-K1 cells overexpressing the human IP receptor (Bley *et al.*, 2006). RO1138452 had minimal binding affinity for

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prostanoid EP₁, EP₃, EP₄ and TP receptors. The high specificity of RO1138452 extended to α_{1B} -adrenoceptors, muscarinic M₁, M₂, M₃ and M₄ receptors, and 5HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C} and 5-HT₄ receptors ($pK_i \leq 6.1$); exceptions were the α_{2A} -adrenoceptor ($pK_i = 6.5$), the PAF receptor ($pK_i = 7.9$) and the imidazoline I₂ receptor ($pK_i = 8.3$).

In this study, the utility of RO1138452 as an IP receptor antagonist has been investigated on isolated vascular smooth muscle and platelet preparations, corresponding to the well-known vasodilator and platelet-inhibitory actions of prostacyclin. Antagonist affinities (pA_2 values) were estimated by classical Schild analysis (Arunlakshana and Schild, 1959). Cicaprost, a 6a-carba analog of prostacyclin, was our first choice of IP agonist, based on its high potency and chemical stability (Sturzebecher *et al.*, 1986) and its higher IP receptor selectivity compared to other readily available prostacyclin analogs (carbacyclin, iloprost) (Dong *et al.*, 1986; Lawrence *et al.*, 1992). Some experiments were performed with the potent IP agonist TEI-9063 (Negishi *et al.*, 1991; Jones and Chan, 2001), which also has considerable EP₁ agonist activity (Jones *et al.*, 1997). As RO1138452 was destined for use as an analgesic in man, two human IP preparations were studied, the pulmonary artery ring (Hadhazy *et al.*, 1983; Haye-Legrand *et al.*, 1987; Jones *et al.*, 1997) and platelet-rich plasma (PRP) (Moncada *et al.*, 1976; Andersen *et al.*, 1980; Armstrong *et al.*, 1986). IP systems in guinea-pig aorta, rabbit mesenteric artery and rat platelets were also investigated. These preparations contain other inhibitory prostanoid receptors thereby affording a way of assessing the selectivity of RO1138452. Accordingly, prostaglandin D₂ (PGD₂) was used as a DP₁ agonist on human platelets, butaprost as an EP₂ agonist on guinea-pig trachea and human pulmonary artery, and prostaglandin E₂ (PGE₂) as an EP₄ agonist on rabbit mesenteric artery. Finally, the effect of RO1138452 on endothelium-dependent relaxation induced by substance P (NK₁ agonist) in rabbit mesenteric artery was examined.

The results indicate that RO1138452 is a potent and selective antagonist of IP receptors from several species and should be a useful tool for characterizing prostanoid receptors in general and elucidating the function of prostacyclin in both health and disease.

Methods

Isolated smooth muscle preparations

All procedures on experimental animals were performed under license issued by the Government of the Hong Kong SAR. The project work was endorsed by the Animal Research Ethics Committee and the Human Research Ethics Committee of the Medical Faculty, Chinese University of Hong Kong. Pulmonary arterial vessels (3 mm in diameter) were obtained from a nominally healthy area of a lung lobe resected from patients undergoing surgery for carcinoma of the lung at the Prince of Wales Hospital, Shatin, Hong Kong. Thoracic aorta/trachea and mesenteric artery were obtained, respectively, from male Dunkin-Hartley guinea-pigs (400–450 g) and male New Zealand White rabbits (2.5–3.5 kg) bred in-house under standard conditions; euthanasia was by exposure to CO₂.

Contiguous ring preparations (3 mm in length) were set up in conventional 10 ml tissue baths for recording of isometric tension (Grass FT03 transducers linked to an AD Instruments PowerLab pre-amplifier-digitizer/Macintosh computer system). Endothelium was removed from some preparations by gentle rotation of a wooden cocktail stick within the lumen. Resting tension was set at 1.0 g for pulmonary and mesenteric arteries and trachea and 0.8 g for aorta. The bathing fluid was Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.18 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM glucose) aerated with 95% O₂/5% CO₂, maintained at 37°C and containing 1 μ M indomethacin to inhibit cyclo-oxygenase activity. At the start of the experiment, each preparation was challenged with 40 mM KCl two or three times to ensure reproducibility of response.

Platelet preparations

Blood (20 ml) was collected from the antecubital vein of human volunteers and immediately mixed with 2.6 ml of acid-citrate-dextrose (ACD) solution. Adult male rats (Sprague-Dawley, 250–300 g) were anaesthetized with diethyl ether by inhalation. A mid-line laparotomy was performed and blood (usually 8–10 ml) was collected via a heparinized needle inserted into the lower abdominal aorta; 0.1 vol. of ACD was added. PRP was obtained by centrifugation at 150 and 500 g, respectively, for 20 min. The PRP was stored under an atmosphere of 95% O₂/5% CO₂ at room temperature.

Experimental design

The basic protocol for assessing RO1138452 antagonism in the smooth muscle experiments consisted of constructing concentration–response relationships for the relaxant agonist in the presence of sequentially increasing concentrations of RO1138452. On each test preparation, four agonist dosing sequences (S1–S4) corresponding to RO1138452 concentrations in the ratio 0:1:5:25 were applied. On contemporaneous control preparations, S1 corresponded to no treatment and S2, S3 and S4 to appropriate vehicles for RO1138452. Each dosing sequence (S_n) comprised the following additions: antagonist (if present) at time 0, tone-inducer at 30 min, cumulative relaxant agonist starting at 45–50 min (6–10 min for each concentration); washout was followed by a rest period of 30–40 min.

In each platelet experiment, concentration–response relationships for the inhibitory agonist (cicaprost, TEI-9063, PGD₂, 5'-N-ethylcarboxamidoadenosine (NECA)) were obtained in the presence of vehicle and two concentrations (ratio 1:5) of RO1138452. The three treatments were arranged in a balanced randomized manner in both the human and rat series. For each measurement, PRP (250 μ l), Krebs-Henseleit solution (175 μ l), and 0.9% NaCl solution containing RO1138452/vehicle (50 μ l) were placed in a 1 ml silanized glass cuvette and transferred to a heating block held at 37°C. After 5 min, the inhibitory agonist was added in 25 μ l saline (final volume = 500 μ l) and the cuvette was placed in the recording well of a Chronolog platelet aggregometer (magnetic stirring at 900 r.p.m.). ADP (6 μ M)

was added after 2 min and light transmission was recorded for a further 1.5–2 min.

Data analysis and statistical procedures

Relaxation of smooth muscle was measured as a percentage of the steady response to the tone-inducer. Inhibition of platelet aggregation was measured as a percentage of the increase in light transmission to 6 μ M ADP attained 80 or 90 s after its addition and measured from the signal level associated with full shape change. Agonist log concentration–response curves were constructed and fitted with variable-slope sigmoidal curves using GraphPad Prism software; the upper asymptote was constrained to 100%. In each series of smooth muscle experiments, responses to the tone-inducer and log concentration–response curve parameters for S1–S4 were analyzed by repeated-measures 2-factor ANOVA (first factor = control/test, repeated-measures factor = cicaprost concentration) accompanied by planned orthogonal contrasts (planned contrasts; Glass and Hopkins, 1995) using SuperANOVA software. To assess the parallelism of the linear portions of the cicaprost curves for S1–S4, the fold increase in cicaprost concentration on progressing from 20 to 80% relaxation was analyzed by one-factor ANOVA accompanied by post-test for linear trend (GraphPad Prism). Dose-ratios were calculated at the 50% inhibition level (from negative logarithm of concentration producing 50% inhibition of the induced response ($pIC_{50\%}$) values) for agonists capable of inducing complete relaxation/inhibition of aggregation. For PGE₂ and substance P as agonists on rabbit mesenteric artery and NECA as agonist on human PRP, dose-ratios were measured at the 40% inhibition level, approximating to a half-maximal response. In the smooth muscle experiments, dose-ratios for a test preparation were corrected for changes in agonist sensitivity of the corresponding control preparation. pA_2 values were obtained either by direct substitution into the Schild equation [$\log(\text{dose-ratio} - 1) = pA_2 - \log[\text{antagonist}]$] or from the corresponding Schild plot using linear regression analysis (GraphPad Prism) (Arunlakshana and Schild, 1959). Values are reported as mean $\pm$ s.e.m. unless otherwise specified. The significance limit was set at $P = 0.05$ and all tests were two-tailed.

Chemicals

The following compounds were purchased: indomethacin, *N*-nitro-*L*-arginine methyl ester (*L*-NAME), NECA, phenylephrine hydrochloride, sodium nitroprusside and substance P from Sigma-Aldrich Chemicals, St Louis, MO, USA; butaprost, 11-deoxy PGE₁, PGD₂, PGE₂ and U-46619 (15S-hydroxy-11 α ,9 α -epoxymethano-prosta-5Z,13E-dienoic acid) from Cayman Chemicals, Ann Arbor, MI, USA; SC-51322 (8-chlorodibenz[b,f][1,4]oxazepine-10(11H)-carboxylic acid 2-[3-2-(furanylmethyl)thio]-1-oxopropyl]hydrazide) from Bio-Mol Research Laboratories, Plymouth, Meeting, PA, USA. The following compounds were gifts: AH-13205 (*trans*-2-(4-(1-hydroxyhexyl)phenyl)-5-oxocyclopentaneheptanoic acid) from Glaxo Group Research, UK; cicaprost from Schering AG, Germany; TEI-9063 (17 α ,20-dimethyl- $\Delta^{6,6a}$ -6a-carba-PGI₁) from Teijin Institute, Japan. RO1138452 {2-(4-(4-

isopropoxy)-benzyl)-phenylamino) imidazoline} was prepared in the Department of Medicinal Chemistry, Roche Palo Alto, CA, USA; it was dissolved in ethanol to give a 10 mM stock solution; the concentration of ethanol corresponding to 1 μ M RO1138452 was 0.02 mM. Other prostanoid ligands were prepared in absolute ethanol at 5 or 10 mM. All substocks were prepared in 0.9% NaCl solution, except for SC-51322, which was added directly to the bathing fluid from a 1 mM stock solution in ethanol as it is prone to crystallization from concentrated aqueous solution.

Results

Estimation of the IP antagonist potency of RO1138452 on vascular smooth muscle preparations

Concentration–response relationships for the IP agonist cicaprost were obtained in the presence of increasing concentrations of RO1138452 for the purpose of calculating dose-ratios and constructing a Schild plot. Each test preparation was exposed to four cumulative dosing sequences of cicaprost (S1–S4) for which the ratio of RO1138452 concentrations was 0:1:5:25; the control preparation received no treatment in S1 and the corresponding vehicles for RO1138452 in S2–S4.

Preliminary experiments established that stable tone (~60% of the tissue maximum) and good sensitivity to cicaprost-induced relaxation could be obtained with 3 and 4 nM of the TP agonist U-46619 in human pulmonary artery and guinea-pig aorta, respectively, and 1 μ M of the α_1 -adrenoceptor agonist phenylephrine in rabbit mesenteric artery. Induced tone increased slightly between S1 and S4 on human pulmonary artery and rabbit mesenteric artery, but increased by 50–100% in guinea-pig aorta over the same time period (see later). In each series of experiments, RO1138452 at each concentration level (see Table 1) did not affect the induced tone ($P > 0.05$ for comparison of control and test means by repeated-measures 2-factor ANOVA accompanied by planned contrasts). Furthermore, the sensitivities of control and test preparations to cicaprost in S1 (no treatment) were not significantly different ($P > 0.05$ for comparison of $pIC_{50\%}$ values by repeated-measures 2-factor ANOVA/planned contrasts).

On human pulmonary artery ($n = 6$), treatment with 10, 50 and 250 nM RO1138452 progressively shifted the cicaprost log concentration–response curve to the right (Figure 1a). The block was always surmountable, except for 250 nM RO1138452 in one experiment (62% maximal relaxation). On control preparations, the sensitivity to cicaprost decreased marginally through S1 to S4 ($pIC_{50\%} = 9.00 \pm 0.09$, 8.96 ± 0.10 , 8.98 ± 0.07 , 8.91 ± 0.06 , respectively; $n = 6$; $P = 0.045$, linear trend analysis). The cicaprost curves became shallower with increasing RO1138452 concentration: to increase relaxation from 20 to 80%, the cicaprost concentration had to be increased by 7.2 ± 1.3 , 10.7 ± 1.9 , 11.7 ± 0.9 and 14.1 ± 2.6 fold in S1–S4 ($P < 0.05$, linear trend analysis); there was no significant trend in the corresponding values for control preparations (6.7 ± 1.1 , 6.6 ± 0.8 , 7.5 ± 1.4 , 8.0 ± 1.6 ; $P > 0.05$). A Schild plot (Figure 2a) was generated using dose-ratios measured at the 50% relaxation level and

Table 1 Profile of the IP receptor antagonist RO1138452 on smooth muscle and platelets

Preparation	Receptor ^a	Agonist	RO1138452		
			Concentration (nM)	pA ₂	Slope of Schild regression
Human pulmonary artery	IP	Cicaprost	10–250	8.20 ^b	1.16 ± 0.08
	EP ₂	Butaprost	1000	See text	—
Guinea-pig aorta	IP	Cicaprost	4–100	8.39 ^b	1.09 ± 0.14
	EP ₂	Butaprost	1000	< 6.0	—
Rabbit mesenteric artery	IP	Cicaprost	40–1000	8.12 ^b	1.07 ± 0.05
	EP ₄	PGE ₂	1000	< 6.0	—
	NK ₁	Substance P	1000	< 6.0	—
Human platelet-rich plasma	IP	Cicaprost	200, 1000	7.38, 8.21	—
	DP ₁	PGD ₂	1000	< 6.0	—
	A _{2A}	NECA	1000	< 6.0	—
Rat platelet-rich plasma	IP	Cicaprost	200, 1000	7.46, 7.73	—
	IP	TEI-9063	200, 1000	7.35, 7.78	—

^aNominally under study.^bpA₂ derived from linear regression on Schild plot (mean ± s.e.m. for slope); other values from Schild equation. pA₂ < 6.0 derived from a dose-ratio < 2.0.

corrected for changes in sensitivity of corresponding control preparations; the single nonsurmountable value (filled symbol) was not included in the linear regression analysis. The variation explainable by regression (r^2) was 94% ($P < 0.001$) and the slope of the line (1.16 ± 0.08) was not significantly different from unity (95% confidence interval (CI) 0.99–1.32). The pA₂ for RO1138452 derived by constraining the slope to unity was 8.20 (Table 1). Removal of RO1138452 (1 μ M) from the tissue bath (four double-washes at 15 min intervals) returned the cicaprost sensitivity almost to the S1 value after 1.5–2 h.

Initial investigation showed that relaxation of human pulmonary artery induced by the prostacyclin analogue TEI-9063 ($\text{pIC}_{50\%} = 8.42 \pm 0.04$, $n = 6$) was blocked by RO1138452 (10–250 nM) with similar affinity to the cicaprost/RO1138452 interaction. However, reversal of the inhibitory action of TEI-9063 following washout was slower than for cicaprost, resulting in suppression of U-46619-induced tone in subsequent sequences. A complete analysis of the TEI-9063/RO1138452 interaction by the Schild procedure was therefore abandoned.

The effects of 4, 20 and 100 nM RO1138452 on relaxation of guinea-pig aorta induced by cicaprost are shown in Figure 1b. The block produced by 100 nM RO1138452 was clearly nonsurmountable on four of five preparations (maximum relaxation = 80–84%). Cicaprost induced full relaxation in S1–S4 on control preparations and the corresponding $\text{pIC}_{50\%}$ value (8.81 ± 0.07 , 8.81 ± 0.04 , 8.76 ± 0.08 , 8.79 ± 0.06 , $n = 5$) did not change significantly ($P > 0.05$, linear trend analysis). However, we were concerned that part of the nonsurmountability might be related to the marked increase in U-46619-induced tone over the course of the experiment. A second series of experiments was, therefore, performed in which test preparations were exposed to 100 nM RO1138452 in S2; the antagonism was nonsurmountable in only one of four preparations (81% maximal relaxation). In the same experiments, 500 nM RO1138452 applied in S2 caused a further right-shift of the log concentration–response curve for cicaprost, but no further suppression of the cicaprost maximum (79–94%, $n = 4$) compared to the 100 nM RO1138452 (series 1) treatment. It

is noteworthy that the sensitivity to cicaprost for 500 nM RO1138452 (series 2) experiments was significantly higher than for 100 nM RO1138452 (series 2) and 4–100 nM RO1138452 (series 1) experiments; $\text{pIC}_{50\%} = 9.12 \pm 0.04$, ($n = 8$), 8.82 ± 0.08 ($n = 8$), 8.78 ± 0.05 ($n = 10$) ($P < 0.01$ and $P < 0.001$, respectively, 1-factor ANOVA/planned contrasts applied to combined control/test S1 values). Again, all dose-ratio data were included in the Schild plot (Figure 2b), but only those values derived from curves showing surmountable characteristics were used in the regression analysis ($r^2 = 83\%$, $P < 0.001$). The Schild slope (1.09 ± 0.14) was not significantly different from unity (95% CI 0.78–1.40); the pA₂ of RO1138452 was estimated as 8.39.

Surmountable block was obtained with 40 and 200 nM RO1138452 on rabbit mesenteric artery (Figure 1c). In the presence of 1000 nM RO1138452, transient contraction was observed after addition of the third, fourth and fifth doses of cicaprost on two of the five preparations. Reversal to relaxation was prominent on the first of these preparations, whereas 50% relaxation was not achieved on the second preparation (dose-ratio > 320; value excluded from the regression analysis). The sensitivity to cicaprost on control preparations increased slightly through S1 to S4; $\text{pIC}_{50\%} = 8.40 \pm 0.10$, 8.44 ± 0.09 , 8.49 ± 0.10 , 8.56 ± 0.06 , respectively ($n = 5$; $P < 0.001$, linear trend). The Schild regression (Figure 2c) gave r^2 of 92% ($P < 0.001$) and a slope of 1.07 ± 0.09 , which was not significantly different from unity (95% CI 0.87–1.27). Constraining the regression slope to unity afforded a pA₂ of 8.12 for RO1138452.

Selectivity of RO1138452 on smooth muscle preparations

On the histamine (1 μ M)-contracted guinea-pig trachea, PGE₂ (1–444 nM) induced 40–60% maximal relaxation, reflecting a balance between EP₁ contraction and EP₂ relaxation. In the presence of the EP₁ receptor antagonist SC-51322 (1 μ M), PGE₂ induced $\geq 90\%$ relaxation and was about three times more potent than the selective EP₂ agonist butaprost (Figure 3a). RO1138452 (1 μ M) did not block relaxation induced by butaprost ($\text{pIC}_{50\%} = 6.83 \pm 0.11$ and 6.74 ± 0.10 for S1 and S2, respectively, $n = 4$); the corrected dose-ratio

Figure 1 Antagonism by RO1138452 of relaxation induced by cicaprost in (a) human pulmonary artery ($n=6$), (b) guinea-pig aorta (series 1, $n=5$) and (c) rabbit mesenteric artery ($n=5$). Indomethacin ($1 \mu\text{M}$) was present in the bathing fluid. Vertical bars represent s.e.m.

was 1.29 ± 0.09 . Furthermore, established relaxations to butaprost (430 or 1430 nM) in S2 on control preparations were not affected by $1 \mu\text{M}$ RO1138452 ($n=4$); an example is shown in Figure 3c.

Figure 2 Schild plots for the cicaprost/RO1138452 interaction in (a) human pulmonary artery, (b) guinea-pig aorta and (c) rabbit mesenteric artery. Unfilled and filled data points derive from surmountable (S) and non-surmountable (non-S) antagonism profiles, respectively; only data corresponding to surmountable antagonism were included in the linear regression analyses.

Figure 3 Effect of RO1138452 on log-concentration–response curves for butaprost on test preparations of (a) guinea-pig trachea and (b) human pulmonary artery; tone was induced with 1 μM histamine and 3 nM U-46619, respectively. (a) Values are means ± s.e.m. ($n = 4$). The experimental tracings show the effects of RO1138452 on established relaxations to butaprost on control preparations of (c) guinea-pig trachea and (d) human pulmonary artery. Indomethacin (1 μM) was present for both tissues; 1 μM SC-51322 was also present for guinea-pig trachea.

The human pulmonary artery showed a sensitivity to butaprost similar to that shown by guinea-pig trachea. However, RO1138452 at 1 μM produced a modest block of butaprost-induced relaxation ($n = 2$, Figure 3b). Sensitivity to butaprost was well maintained on the control preparations (S1/S2 dose-ratios = 1.09 and 1.03). On the control preparation in the second experiment, the established response to butaprost (775 nM) in S2 was partially reversed by 1 μM RO1138452 over a period of 20 min (Figure 3d). For comparison, established relaxation (~80%) of human pulmonary artery induced by 5 nM cicaprost was reversed to the initial tone level by 100 nM RO1138452 over a period of 30–40 min ($n = 3$, data not shown).

Relaxation of test preparations of rabbit mesenteric artery induced by PGE₂ (0.1–4.4 nM) was not antagonized by 1 μM RO1138452; pIC_{40%} values for S1 and S2 were 9.22 ± 0.08 and 9.31 ± 0.13 , respectively ($n = 4$) (Figure 4a). The corrected dose-ratio for 1 μM RO1138452 was 0.86 ± 0.12 . Above 10 nM, PGE₂ showed contractile activity, resulting in a bell-shaped log concentration–response curve. A similar profile was found for 11-deoxy PGE₁ (1–144 nM; receptor selectivity: EP₄ ≥ EP₂ = EP₃ > EP₁), while butaprost (30–1430 nM) and AH-13205 (0.1–14.4 μM; EP₂ ≥ EP₁ = EP₃ > EP₄) induced relaxation only (Figure 4a). Equi-effective molar ratios (EMR) measured at the 40% relaxation level for PGE₂, 11-deoxy PGE₁, butaprost and AH-13205 were 1.0, 7.5, 160 and 3400, respectively.

Effect of RO1138452 on endothelium-dependent relaxation

In preliminary experiments on rabbit mesenteric artery, substance P (0.1–14.4 nM) induced consistent relaxations that were abolished by endothelium removal; cicaprost (1–14 nM) responses were unaffected by this procedure. Omitting indomethacin from the bathing fluid did not alter the profile of substance P (data not shown). In subsequent experiments, all preparations were exposed to a priming sequence of sodium nitroprusside (10–440 nM, pIC_{40%} ~ 7.3). The NOS inhibitor L-NAME at 100 μM in S2 abolished

relaxation induced by substance P over its lower concentration range and suppressed the maximum response by 52% ($n = 5$; Figure 4b). In separate experiments ($n = 5$), 1 μM RO1138452 did not antagonize relaxation induced by substance P (Figure 4b); pIC_{40%} values for substance P were 8.90 ± 0.20 and 8.93 ± 0.22 for S1 and S2, respectively, and the corresponding maxima were 86 ± 3 and $85 \pm 3\%$. The corrected dose-ratio for 1 μM RO1138452 treatment on substance P was 1.10 ± 0.23 .

Estimation of the IP antagonist potency of RO1138452 on isolated platelet preparations

ADP at 6 μM consistently induced irreversible aggregation in platelet-rich plasma (PRP) from man and rat; RO1138452 at 1000 nM did not affect these responses, nor the reversible wave induced by 3 μM ADP in human PRP (Figure 5c). Cicaprost inhibited the 6 μM ADP response in human PRP with pIC_{50%} of 9.52 ± 0.10 ($n = 4$, Figure 5a). Treatment with RO1138452 at 200 and 1000 nM produced a surmountable block of the cicaprost-induced inhibition; dose-ratios were 6.3 ± 1.4 and 164 ± 16 ($n = 4$), respectively. The corresponding pA₂ values (7.38 ± 0.12 and 8.21 ± 0.04 , Table 1) were significantly different ($P < 0.001$, unpaired *t*-test). Cicaprost and TEI-9063 inhibited aggregation in rat PRP with pIC_{50%} values of 8.72 ± 0.04 ($n = 5$) and 8.60 ± 0.10 ($n = 5$), respectively. RO1138452 at 200 and 1000 nM afforded dose-ratios of 6.9 ± 0.6 and 60 ± 12 with cicaprost as agonist and 5.7 ± 0.8 and 66 ± 10 with TEI-9063 as agonist; the corresponding pA₂ values were $7.46 \pm 0.04/7.73 \pm 0.10$ and $7.35 \pm 0.08/7.78 \pm 0.09$. For both agonists, pA₂ values corresponding to the two RO1138452 concentrations were significantly different ($P < 0.05$ and < 0.01 , respectively, unpaired *t*-test).

Selectivity of RO1138452 on human platelets

PGD₂ (1–20 nM), which activates DP₁ receptors on human platelets (Miller and Gorman, 1979; Monneret *et al.*, 2003), inhibited ADP-induced aggregation with pIC_{50%} of 8.34 ± 0.07

Figure 4 Rabbit mesenteric artery precontracted with phenylephrine. (a) Effect of RO1138452 on relaxation induced by PGE₂. The activities of butaprost (S3) and AH-13205 (S4) on control preparations are also shown (all $n=4$). (b) Effect of L-NAME and RO1138452 on relaxation induced by substance P (separate series of experiments, both $n=5$); the curves for no treatment/RO1138452 are virtually superimposable. Indomethacin (1 μM) was present in the bathing fluid. Vertical bars represent s.e.m.

($n=4$). RO1138452 at 1 μM did not affect the action of PGD₂ (dose-ratio = 1.10 ± 0.04 ; $n=4$, Figure 5b). Moreover, 1 μM RO1138452 did not affect the inhibitory action of the adenosine A_{2A} receptor agonist NECA ($pIC_{40\%} = 7.05 \pm 0.22$, $n=4$; dose ratio = 1.27 ± 0.23 , $n=4$, Figure 5b).

In human PRP, the EP₃ agonist sulprostone (20 nM) converted the reversible aggregation wave induced by 3 μM ADP into irreversible aggregation (Figure 5c); RO1138452 at 1 μM did not affect this enhancement ($n=3$).

Discussion

Characteristics of IP receptor antagonism by RO1138452

The current investigation has shown that RO1138452 is a potent, competitive antagonist at prostanoid IP receptors

Figure 5 Human PRP: aggregation induced by ADP. Effect of RO1138452 on inhibition of aggregation induced by (a) cicaprost and (b) PGD₂ and NECA (all $n=4$). Vertical bars represent s.e.m. The experimental tracing (c) shows that 1 μM RO1138452 does not affect either reversible aggregation induced by ADP or its enhancement by sulprostone; RO1138452 and sulprostone were added 7 and 2 min, respectively, before ADP.

present in isolated preparations of human pulmonary artery, guinea-pig aorta and rabbit mesenteric artery. With cicaprost as agonist, pA₂ values for RO1138452 were 8.20, 8.39 and 8.12, respectively, in these three tissues, with Schild plot slopes not significantly different from unity. The affinity estimate for RO1138452 on human pulmonary artery is

somewhat lower than those reported from competitive binding studies ($[^3\text{H}]$ -iloprost as radioligand) on native IP receptors in human platelet membranes (pK_i 9.3) and human recombinant IP receptors expressed in CHO cell membranes (pK_i 8.7) (Bley *et al.*, 2006). The current value was also lower than the pK_i (9.0) estimated from inhibition of carbacyclin-stimulated AC activity in CHO cells expressing recombinant human IP receptors (Bley *et al.*, 2006). It is possible that these differences reflect restricted penetration of RO1138452 to IP receptors within a solid tissue as opposed to unhindered access to IP receptors on the surfaces of dispersed cells and cell membranes.

In estimating pA_2 values for RO1138452, dose-ratios derived from cicaprost log concentration–response curves exhibiting nonsurmountability were excluded from the linear regression analyses. The nonsurmountability was associated with the higher concentrations of RO1138452 and may indicate the emergence of a noncompetitive mechanism. However, the observation that cicaprost induced transient contraction of rabbit mesenteric artery in the presence of $1\text{ }\mu\text{M}$ RO1138452 suggests that the deviations from ideality arise from the agonist rather than the antagonist. Essentially, cicaprost at concentrations sufficient to overcome the RO1138452 block may activate an excitatory prostanoid system leading to a functional antagonism of its own relaxant action. The presence of contractile EP_3 systems in the human pulmonary artery (Qian *et al.*, 1994), guinea-pig aorta (Jones *et al.*, 1998) and rabbit mesenteric artery (Jones and Chan, 2001) strongly suggests that the functional antagonism operates through EP_3 receptors.

Ligand binding data on recombinant EP_3 receptors support our contention that high concentrations of cicaprost could activate (contractile) EP_3 receptors. For example, the highly potent EP_3 agonist sulprostone has K_i values of 0.35 and 0.60 nM for human and mouse EP_3 receptors, respectively; the corresponding values for cicaprost are 255 and 170 nM (Kiriya *et al.*, 1997; Abramovitz *et al.*, 2000). Estimating the potency of cicaprost relative to sulprostone on functional EP_3 systems has been more difficult, mainly due to interference from inhibitory IP systems. This scenario occurs in the guinea-pig vas deferens, the archetypal EP_3 preparation (Coleman *et al.*, 1987; Lawrence *et al.*, 1992), in which activation of EP_3 receptors on sympathetic varicosities suppresses transmitter release and thereby inhibits twitch responses elicited by electrical field stimulation. IP receptor agonists (cicaprost, TEI-9063, iloprost) exert an opposite effect through promotion of transmitter release; the threshold concentration of cicaprost for this enhancement is about 1 nM (Tam *et al.*, 1997). However, we have recently found that vas deferens preparations from some guinea-pigs showed weak IP receptor-mediated enhancement (RL Jones and LW Lau, unpublished observations). Cicaprost induced only slight enhancement at concentrations of 3–30 nM and markedly inhibited twitch responses at 30–300 nM. Cicaprost was about 100 times less potent than sulprostone on these anomalous preparations. As sulprostone induces threshold contraction of guinea-pig aorta at concentrations of 0.3–1 nM (Jones *et al.*, 1998; Shum *et al.*, 2003), cicaprost would be expected to induce similar effects at concentrations of 30–100 nM and above. These values correspond to the cicaprost

concentrations required to overcome the block produced by 100 nM RO1138452, where nonsurmountable antagonism was a prominent feature (Figures 1b and 2b). Surmountable antagonism was also seen with this concentration of RO1138452 in some aorta experiments. Such variability is not surprising given that the equilibrium point of the (EP_3 /IP) functional antagonism will depend on the relative sensitivities and receptor reserves of the two opposing (prostanoid) systems; the system with the higher receptor reserve is expected to overcome even intense stimulation of the other system (van den Brink, 1973). Furthermore, the interaction between the EP_3 and tone-induction systems must be taken into account. EP_3 agonists are usually weak contractile agents on vascular smooth muscle, but synergize well with strong agents (e.g. TP receptor and α_1 -adrenoceptor agonists and K^+) (Jones *et al.*, 2002; Hung *et al.*, 2006). Thus, in human pulmonary artery, EP_3 /TP synergism involving cicaprost and U-46619 (as tone inducer) could well mean that cicaprost has a contractile input at concentrations below the predicted value (~ 700 nM) for direct contraction. In which case, we have an explanation for the flattening of the cicaprost curves over the lower range of cicaprost concentration (30–300 nM) in the presence of RO1138452 on human pulmonary artery.

RO1138452 antagonized IP agonist action in a surmountable manner in human and rat platelets. However, the pA_2 values for RO1138452 on the platelets preparations (7.4–7.8) were smaller than the corresponding values on the smooth muscle preparations and also smaller than the affinity of RO1138452 ($\text{pK}_i=8.7$) for the human recombinant IP receptor (Bley *et al.*, 2006). Binding of RO1138452 to proteins present in PRP is a likely cause of these differences. Equilibrium binding experiments using $[^{14}\text{C}]$ -RO1138452 show that about 95% of the antagonist is bound to plasma proteins in undiluted plasma from man, dog and rat (AR Tabatabaei, Roche Palo Alto, personal communication). In our platelet experiments, the binding may be slightly lower since the plasma protein concentration was approximately half of that of native plasma. The pA_2 values were also significantly greater at 1.0 compared to $0.2\text{ }\mu\text{M}$ RO1138452 in both tissues. Saturation of the binding site pool with increasing RO1138452 concentration would explain this difference. EP_3 receptor agonists enhance aggregation in human platelets (Matthews and Jones, 1993); RO1138452 at $1\text{ }\mu\text{M}$ did not block this effect in the current study. We believe that functional antagonism involving an EP_3 system is unlikely to account for the pA_2 differences as the sensitivity of the EP_3 system in the human platelet (threshold concentration of sulprostone = 5–10 nM) is considerably less than that of the IP system. Thus, the concentrations of cicaprost required to overcome $1\text{ }\mu\text{M}$ RO1138452 (10–150 nM, Figure 5a) may be subthreshold for activation of the EP_3 system.

Selectivity/specificity of RO1138452

The selectivity of RO1138452 for IP receptors relative to other inhibitory prostanoid receptors is high. Firstly, inhibition of human platelet aggregation induced by PGD_2 was unaffected by RO1138452 at $1\text{ }\mu\text{M}$, indicating its low affinity

for DP₁ receptors. Secondly, RO1138452 at 1 μ M did not affect PGE₂-induced relaxation of rabbit mesenteric artery ($pA_2 < 6.0$). The EP₄ receptor subtype is likely to be responsible for the relaxation based on the high potency of PGE₂ and the agonist potency ranking PGE₂ > 11-deoxy PGE₁ $\gg$ butaprost $\gg$ AH-13205 (Coleman *et al.*, 1994; Milne *et al.*, 1995; Lydford *et al.*, 1996; Wilson *et al.*, 2004). Thirdly, RO1138452 at 1 μ M did not affect relaxation of guinea-pig trachea induced by the selective EP₂ agonist butaprost ($pA_2 < 6.0$). This preparation does not appear to contain IP receptors (Dong *et al.*, 1986) and interference from EP₁ receptors mediating contraction was excluded by the presence of the EP₁ antagonist SC-51322 (pA_2 on guinea-pig trachea = 8.45; Hung *et al.*, 2006). In contrast to the guinea-pig trachea findings, RO1138452 at 1 μ M clearly inhibited relaxation of human pulmonary artery elicited by butaprost. However, the antagonism was less than that found with cicaprost as agonist. For example, on the control pulmonary artery preparation shown in Figure 3d, the established (87%) relaxation to butaprost was reversed by only one-third by 1 μ M RO1138452; using a similar protocol cicaprost-induced relaxation was completely reversed by 1 μ M RO1138452. These data support our previous contention (Qian *et al.*, 1994) that both EP₂ and IP receptors may contribute to relaxation induced by butaprost on preparations containing a highly sensitive IP system. Further support comes from the recent report by Tani *et al.* (2002) in which butaprost (free acid) showed moderate agonist potency ($EC_{50} = 25$ nM) in a recombinant human IP receptor/AC assay, whereas its 2-series relative (containing a 5,6-*cis* double bond) had minimal activity ($EC_{50} > 10\,000$ nM). This situation is analogous to the much greater IP agonist potency of PGE₁ compared to PGE₂ on human platelets (Kloetze, 1967; Andersen *et al.*, 1980).

In summary, RO1138452 did not block DP₁, EP₂, EP₃, EP₄ and TP receptors in the functional systems examined, thereby complementing the low binding affinities ($K_i < 6.0$) for EP₁, EP₃, EP₄ and TP receptors observed by Bley *et al.* (2006). At the present time, the effects of RO1138452 on DP₂ and FP receptors are unknown.

Turning to specificity for nonprostanoid receptors, RO1138452 has a high affinity for PAF receptors (Bley *et al.*, 2006). PAF is a potent activator of human platelets (Fouque and Vargaftig, 1984), but does not aggregate rat platelets *in vitro* (Klee *et al.*, 1991). Human platelets can produce PAF on stimulation with thrombin (Touqui *et al.*, 1985). However, specific PAF receptor antagonists had no effect on aggregation of human platelets induced by ADP (Nunez *et al.*, 1986; Handley *et al.*, 1987), suggesting that PAF is unlikely to be involved in any release reaction contributing to the aggregation. RO1138452 also did not affect ADP responses in our human and rat platelet assays. We conclude that any interaction of RO1138452 with PAF receptors is unlikely to have interfered with our measurements of its antagonist potency on human and rat platelets. Bley *et al.* (2006) also reported that RO1138452 has a high affinity for imidazoline I₂ receptors, which are present in many blood vessels (Molderings and Göthert, 1999) and human platelets (Ruiz *et al.*, 2002). We feel that this property of RO1138452 is unlikely to have interfered with either the excitatory

(U-46619, phenylephrine, histamine, ADP) or inhibitory (see Table 1) agonists used in our experiments.

Endothelial-dependent relaxation

Prostacyclin elicits relaxation of vascular smooth muscle primarily through activation of IP receptors located on the smooth muscle cells, and indeed removal of endothelium from rabbit mesenteric artery in the current study did not affect relaxation induced by cicaprost. Prostacyclin also has a role as an endothelium-dependent relaxant factor (EDRF), although a survey of the literature would suggest that nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) are more important in this respect. This is the case in relaxation of rabbit mesenteric artery induced by substance P (NK₁ agonist), first described by Stewart-Lee and Burnstock (1989). Our studies confirmed the endothelium-dependency and also implicated NO as the major contributor at the lower substance P concentrations, with EDHF also likely to be involved at higher substance P concentrations. Substance P-induced relaxation was unaffected by RO1138452 at 1 μ M, indicating its inability to block the NK₁ receptor or inhibit the release and action of the associated EDRFs.

Concluding remarks

The IP receptor antagonist RO1138452 appears to interact in a simple competitive manner with IP receptors in tissues from man, rabbit, guinea-pig and rat. Although there was some evidence of a noncompetitive interaction of RO1138452 with the IP receptor at high concentrations, this effect is most likely due to functional antagonism exerted by cicaprost acting at EP₃ receptors, rather than a true noncompetitive property of RO1138452. The use of the recently described EP₃ receptor antagonist L-798106 (Clarke *et al.*, 2004), which was not available to us during the study, would clarify these observations. From a practical standpoint, RO1138452 at a concentration of 1 μ M (corresponding to a dose-ratio ≥ 100) should be sufficient to abolish maximal or near-maximal responses to prostacyclin or its mimetics on an IP system. Block of other inhibitory prostanoids, for example PGD₂ and PGE₂, is expected to be minimal at this concentration, and nonprostanoid endothelium-dependent relaxation is likely to be unaffected. RO1138452 is thus an important member of the growing armory of selective prostanoid receptor antagonists (see Jones, 2004). Structure-activity initiatives proceeding from RO1138452 are far from exhausted, however, and it should be possible to develop antagonists with greater affinity for the IP receptor and with higher specificity (for example, in relation to α_{2A} , PAF and imidazoline I₂ receptors; Bley *et al.*, 2006).

RO1138452 dramatically inhibited experimental hyperalgesia, edema and osteoarthritis in the rat, indicating that prostacyclin has an important role in these pathological situations (Bley *et al.*, 2006). Our observations of marked IP receptor antagonism by RO1138452 on the two human isolated tissue preparations indicates that this compound will be invaluable in investigating the

contribution of IP receptors in equivalent pathophysiological conditions in man.

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Conflict of Interest

The authors state no conflict of interest.

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