Binding and activity of the prostacyclin receptor (IP) agonists, treprostinil and iloprost, at human prostanoid receptors: Treprostinil is a potent DP$_1$ and EP$_2$ agonist

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A B S T R A C T

The prostacyclin analogues, iloprost and treprostinil are extensively used in treating pulmonary hypertension. Their binding profile and corresponding biochemical cellular responses on human prostanoid receptors expressed in cell lines, have now been compared. Iloprost had high binding affinity for EP$_1$ and IP receptors (K_i 1.1 and 3.9 nM, respectively), low affinity for FP, EP$_3$ or EP$_4$ receptors, and very low affinity for EP$_2$, DP$_1$ or TP receptors. By contrast, treprostinil had high affinity for the DP$_1$, EP$_2$ and IP receptors (K_i 4.4, 3.6 and 32 nM, respectively), low affinity for EP$_1$ and EP$_4$ receptors and even lower affinity for EP$_3$, FP and TP receptors. In functional assays, iloprost had similar high activity in elevating cyclic AMP levels in cells expressing the human IP receptor and stimulating calcium influx in cells expressing EP$_1$ receptors (EC$_{50}$ 0.37 and 0.3 nM, respectively) with the rank order of activity on the other receptors comparable to the binding assays. As with binding studies, treprostinil elevated cyclic AMP with a similar high potency in cells expressing DP$_1$, IP and EP$_2$ receptors (EC$_{50}$ 0.6, 1.9 and 6.2 nM, respectively), but had low activity at the other receptors. Activation of IP, DP$_1$ and EP$_2$ receptors, as with treprostinil, can all result in vasodilatation of human pulmonary arteries. However, activation of EP$_1$ receptors can provoke vasoconstriction, and hence may offset the IP-receptor mediated vasodilator effects of iloprost. Treprostinil may therefore differ from iloprost in its overall beneficial pulmonary vasorelaxant profile and other pharmacological actions, especially in diseases where the IP receptor is down-regulated.

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1. Introduction

The endogenous prostanoid, prostacyclin, is of substantial therapeutic benefit in the treatment of the highly debilitating disease, pulmonary hypertension [1–4]. Prostacyclin itself is however chemically unstable at physiological temperatures and pH, and rapidly decomposes to a relatively inactive breakdown product as reviewed by Whittle and colleagues [5,6]. Therefore, the early clinical use of prostacyclin, as the chemically synthesised material epoprostenol, necessitated the use of a high pH formulation and ice packs for its prolonged intravenous use. The development of chemically stable prostacyclin analogues such as iloprost, treprostinil and beraprost obviated the requirement for such a formulation [6]. These agents have been used clinically for different indications, including pulmonary hypertension, peripheral vascular disease as well as Raynaud's phenomenon and digital ulcers associated with scleroderma [7–13]. In particular, iloprost and treprostinil are currently used extensively in Europe and the US for the treatment of pulmonary arterial hypertension [14–18].

As with most other mediators, prostaglandins such as prostacyclin elicit their molecular, pharmacological and biochemical effects through binding and activation of specific receptor sites [19]. It was initially established by pharmacological techniques that there was a range of specific receptors for the naturally occurring prostanoids (see [20]) and these receptors have been subsequently cloned and expressed [19,21]. The original classification of the different prostanoid receptors [20,22,23] has remained essentially intact since the early proposals [24]. Thus, the receptors are identified as the IP, EP$_1$, EP$_2$, EP$_3$, EP$_4$, DP (now DP$_1$, see below), FP and TP receptor [23–25]. The IP, EP$_2$, EP$_4$ and DP$_1$ receptors are classically known to be G$_s$-coupled receptors linked to cyclic AMP (cAMP) generation, while EP$_1$, FP and TP receptors couple to calcium mobilisation pathways through G$_q$, G$_i$ and as yet unidentified G proteins [19,25]. There are several splice variants of EP$_3$ which can couple negatively or positively to G$_i$ or G$_s$, respectively [19].

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The natural ligand for the IP receptor is prostacyclin (PGI$_2$), with prostaglandin E$_2$ (PGE$_2$) for the EP receptors, PGF$_{2\alpha}$ for the FP receptors and thromboxane A$_2$ for the TP receptor [24]. A recent pharmacological study has suggested evidence for a second IP receptor on human airway epithelial cells that mediates the inhibition of cytokine release [26]. This is not thought to be a splice variant although its occurrence elsewhere has not been described. The original classification of the DP receptor with prostaglandin D$_2$ (PGD$_2$) as the natural ligand has now been designated as DP$_1$ [24]. This takes into account the more recently identified DP$_2$ receptor or CRTh$_2$ receptor, that while recognising PGD$_2$, is more closely associated with chemo-attractant molecules and has no significant homology with the other prostanoid receptors [24].

Despite their extensive clinical use over the past decade, there is relatively little direct comparative pharmacology of iloprost and treprostinil in experimental systems and models. It is generally assumed that both are potent agonists at the prostacyclin IP receptor and that such agonist activity predominantly underlies their respective responses, including their potent vasodilator effects in the pulmonary vasculature, at least under physiological conditions [27–29]. Indeed, based on this premise, novel agents that are highly selective agonists at the IP receptor such as the non-prostanoid moiety, selexipag, are being developed for clinical utilities including pulmonary hypertension [30,31]. However, the situation is more complex, since the prostacyclins appear to have functionally relevant effects at other prostanoid receptors as reviewed by Clapp and Patel [32].

Although the receptor binding profile of iloprost, including its high affinity for the IP as well as the EP$_1$, and EP$_3$ receptor, has been reported for both murine and human prostanoid receptors [21,33], there has been no reported comparable evaluation of treprostinil. Because of the multiple pathophysiological processes involved in pulmonary hypertension, there is a need to understand more about the respective pharmacology of these two extensively used prostacyclins. Thus, the current study investigates the binding profile of treprostinil on human prostanoid receptors, individually expressed in separate cell lines, and has directly compared this profile to that of iloprost in the same studies. In addition, the cellular responses of either an elevation of intracellular cyclic AMP or calcium levels as appropriate, as a consequence of activation of the individual human prostanoid receptors by either iloprost or treprostinil, have also been evaluated.

2. Methods and materials

2.1. In vitro radio-ligand binding assays

Evaluation of the affinities of treprostinil and iloprost for each prostanoid receptor was determined in radioligand binding assays using standard techniques. Cell lines, conditions and materials used are documented in Table 1 and broadly follow protocols

previously described [21,34,35]. Briefly, cells from each cell line stably expressing the recombinant human prostanoid receptor were spun down at 4 °C and the cell pellet suspended in a 50 mM Tris/HCl (pH 7.4) buffer containing 5 mM EDTA, 20 mm NaCl, 5 mM KCl, 5 mM MgCl$_2$, 1.5 mM CaCl$_2$, 10 μg/ml trypsin inhibitor, 1 μg/ml leupeptin and 75 μg/ml phenylmethylsulphonyl fluoride.

Cell lysis was performed by ultra sonication (3 min at 4 °C) using a Vibro cell 72405, followed by centrifugation (Beckman Avanti J30I) of the resulting homogenate at 4 °C (50,000 × g for 15 min). The membrane pellet was resuspended in fresh Tris buffer containing 10% glycerol and stored as aliquots at −70 °C until used in the binding studies. Proteins levels were determined using the Bradford method and the optimised quantity of protein used in the binding studies was 16 μg for the TP receptor, 20 μg for the EP$_2$, EP$_3$, EP$_4$ and FP receptors, 40 μg for the IP receptor and 60 μg per sample for the EP$_1$ and DP$_1$ receptors. Incubations were carried out using nanomolar concentrations of the appropriate [^{3}H] radioligand (Table 1) in the absence or presence of various concentrations of the prostacyclin analogue (final solvent concentration was kept constant). Total binding was determined in the presence of vehicle. Non-specific binding was determined in the presence of 650–5000-fold excess of the corresponding non-labelled ligand. Following a 60–120 min incubation of ligands at room temperature (Table 1), samples were filtered rapidly under vacuum through glass fibre filters, dried, and then counted for radioactivity in a scintillation counter.

The specific ligand binding was calculated as the difference between total binding measured in the presence of radioligand alone and nonspecific binding determined in the presence of an excess of unlabelled ligand, as performed in the laboratory at Cerep (Le bois l'Evêque, France). Specific binding for ligands reached equilibrium after 30–40 min of incubation at room temperature, was stable for greater than 2 h and was determined to be saturable. Results are expressed as a percent of the control specific binding obtained.

Competition curves for each data-set were generated by non-linear regression analysis of the data (Prism 4.03; GraphPad, San Diego, USA) using a four parameter logistic (Hill) equation:

$$Y = D + \frac{(A - D)}{(1 + 10^{(X - \log \text{ IC}_{50}) \times \text{nH}})} \tag{1}$$

where Y = specific binding, D = minimum specific binding, A = maximum specific binding, IC$_{50}$ = the concentration that inhibits half of the control specific binding and nH = Hill factor. The inhibition constants (K_i) were calculated using the Cheng Prusoff equation:

$$K_i = \frac{\text{IC}_{50}}{1 + (L/K_D)} \tag{2}$$

Table 1
Experimental conditions for prostanoid receptor radioligand binding assays. h = human; K_d = dissociation constant; RT = room temperature; HEK-293 = human embryonic kidney 293 cells; CHO = Chinese hamster ovary; 1321N1 = human glial brain astrocytoma.

Prostanoid receptor	Expression system/accession no.	Ligand	Concentration (nM)	K_d (nM)	Nonspecific (μM)	Incubation time @RT (min)
IP (h)	HEK-293/NM_000960	[^{3}H] iloprost	10	8	Iloprost (10)	60
EP$_1$ (h)	HEK-293/NM_000955	[^{3}H] PGE$_2$	1.5	1.5	PGE$_2$ (10)	120
EP$_2$ (h)	HEK-293/NM_000956	[^{3}H] PGE$_2$	3.0	3.0	PGE$_2$ (10)	120
EP$_3$ (h)	HEK-293/NM_198714	[^{3}H] PGE$_2$	0.5	0.8	PGE$_2$ (1)	120
EP$_4$ (h)	CHO/NM_000958	[^{3}H] PGE$_2$	0.5	0.3	PGE$_2$ (10)	120
DP$_1$ (h)	1321N1/NM_000953.1	[^{3}H] PGD$_2$	1.5	1.2	BW245C (1)	60
FP (h)	HEK-293/NM_000959	[^{3}H] PGF$_{2\alpha}$	2	3.8	Cloprostenol (10)	60
TP (h) (TXA$_2$)	HEK-293/U11271	[^{3}H] SQ 29548	5	4	U44069 (10)	60

where L = concentration of radioligand in the assay, and K_D = affinity of the radioligand for the receptor. Scatchard analysis was used to determine K_D from a plot of specific binding/free radioligand concentration *versus* specific binding giving a slope equivalent to $-1/K_D$ and are given in Table 1 (see Figure S1 of Supplementary Information for examples of Scatchard plots).

2.2. Receptor activation assays

2.2.1. Cyclic AMP assay

HEK 293 (expressing EP_2, EP_4) CHO (EP_3, IP) or 1321N1 (DP_1) cells were lifted with a non-enzymatic cell stripper and re-suspended in assay buffer at the desired cell density for each cell-line. Cyclic AMP was assayed in suspension of cells using a CisBio HTRF cAMPHiRange Kit (Cisbio US, Bedford, MA, USA) according to the manufacturer's protocol. Cells were incubated with the prostacyclin analogues for 20 min at 37 °C. The reaction was terminated by sequentially adding D2-labelled cyclic AMP and cryptate-labelled anti-cyclic AMP antibody contained in lysis buffer. The plate was incubated at room temperature for 60 min before reading of fluorescent emissions at 620 nm and 668 nm with excitation at 314 nm were made on a microplate reader (Molecular Devices, Sunnyvale, CA, USA). These experiments were performed in the laboratory at Multispan (Hayward, CA, USA). Data were converted from a cyclic AMP standard curve and expressed as cyclic AMP (nM).

2.2.2. Calcium mobilization

HEK293 cells expressing FP, TP or EP_1 receptors were seeded in 384-well plates at appropriate densities and cultured overnight. The calcium flux assay was conducted according to the manufacturer's protocol using the FLIPR Calcium 4 Assay Kit (R8142; Molecular Devices). Loading buffer, containing the calcium-sensitive dye, was added to the cells and incubated for 60 min at 37 °C. The plate was then transferred to a FlexStation® 3 benchtop multi-mode microplate reader (Molecular Devices), where compounds were automatically injected into each well. Intracellular calcium, monitored as changes in fluorescent, was recorded for 90 s with a single compound application occurring after 19 s. These experiments were performed in the laboratory at Multispan (Hayward, CA, USA). Assay results (5–10 determinations per analogue concentration) were plotted as relative fluorescence units (RFU).

2.3. Materials

Treprostinil was provided in powder form by United Therapeutics Corporation (Research Triangle Park, NC, USA). Iloprost (50:50 R/S isomer), BW245C, prostaglandin E_2 (PGE_2) and PGD_2 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Cloprostenol, U-44069 and buffer reagents and materials were purchased from Sigma–Aldrich (Lyon, France). Treprostinil was dissolved in DMSO at a stock concentration of 10 mM and iloprost was dissolved in methylacetate at a concentration 13.9 mM. For concentration–response experiments, the highest agonist concentration used was 10 μM with serial 1:10 dilutions.

In binding assays, stable cells expressing respective human prostanoid receptors were used by Cerep (Table 1). The radioligands used in these studies (Table 1) were obtained from Perkin Elmer NEN (Courtaboeuf, Cedex 191945, France), or for iloprost, from Isobio (Fleurus, Belgium). Likewise for functional assays conducted in the laboratories of Multispan, stable cell lines expressing human receptors were: EP_1 (GenBank accession number NM_000955.2; Cat# C1201a) in HEK293T, EP_2 (GenBank Accession Number NM_000956.3; Cat# C1202) in HEK293T, EP_3 (GenBank Accession Number NM_000957; Cat# C1203-1a), in

CHO-K1, EP_4 (GenBank Accession Number NM_000958; Cat# C1204) in HEK293T, FP (GenBank Accession Number NM_000959; Cat# C1205) in HEK293T, IP (GenBank Accession Number NM_000960; Cat# C1206-1) in CHO-K1, DP_1 (GenBank Accession Number NM_000953; Cat# C1200) in HEK293T and TP (TXA_2R; GenBank Accession Number NM_001060.4; Cat# C1365) in HEK293T were from Multispan.

2.4. Data analysis

In binding studies, IC_{50} values were obtained from each individual concentration–response curve for specific binding (n = 6) and used to determine the affinity constant, K_i.

Concentration-dependent relationships for each prostacyclin analogue stimulating elevations in either intracellular cyclic AMP or calcium (mean $\pm$ S.E.M. of n determinants per concentration as indicated) as appropriate, were constructed using a variable slope sigmoidal fitting routine in GraphPad Prism 4.03 (San Diego, CA, USA). The EC_{50} value, the concentration of agonist causing 50% of the maximal response (E_{max}), was determined from individual fits to each data-set and expressed as mean $\pm$ S.E.M. Statistical analysis was performed using GraphPad with significance assessed using a Student's t-test or ANOVA with correction for multiple comparisons. A P value <0.05 was considered significant.

3. Results

3.1. Radioligand binding data

The data obtained from the competition binding assays with the tritiated ligands in the presence of either iloprost (10^{-11} to 10^{-5} M) or treprostinil (10^{-11} to 10^{-5} M) for the eight recombinant human prostanoid receptors studied, the IP, EP_1, EP_2, EP_3, EP_4, DP_1, FP and TP receptor, are shown in Fig. 1. Both iloprost and treprostinil yielded concentration-dependent reductions in specific binding for each of the receptor types over the range of concentrations evaluated. However, neither prostacyclin analogue yielded a full specific binding curve for the TP receptor because of the high concentrations (>10 μM) that would have been required to reach full displacement of radioligand (Fig. 1). The derived affinity constant, the K_i value, for either iloprost or treprostinil at each prostanoid receptor, is given in Table 2. To aid comparison of this data to that obtained from earlier human prostanoid receptor assays, the K_i values reported for iloprost from the work of Abramovitz and colleagues [21], are also presented in Table 2.

The data from the current study shown in Table 2 indicate that iloprost has high binding affinities for the IP and EP_1 receptors, though this was significantly (P = 0.002) greater for the EP_1 receptor, as indicated by the lower K_i value. Its affinity for the FP, EP_3 and EP_4 receptors was some two log orders lower and was even lower for the DP_1, EP_2 and TP receptors (Table 2).

In general, the overall binding profile to the prostanoid receptors obtained in the current work with iloprost was similar to that previously reported for iloprost against human prostanoid receptors (see Table 2; data from Ref. [21]). Comparison of the K_i values in Table 2 indicates that the order of affinity for iloprost in the current work was $EP_1 > IP \gg FP > EP_3 = EP_4 > DP_1 > EP_2 > TP$, while that reported previously by Abramovitz and colleagues [21] was $EP_1 = IP > EP_3 > EP_4 > FP > DP_1 > EP_2 > TP$. Thus, the main difference found between the two studies utilising iloprost was the ranking of the K_i for the FP receptor.

The prostanoid receptor binding profile for treprostinil differed from that observed with iloprost (Table 2). Treprostinil had a high and similar affinity for the DP_1 and EP_2 receptor, which was some 10-fold ($P < 0.01$, one way ANOVA) greater than that for the IP receptor. It had a much lower affinity for the EP_1 receptor, weaker
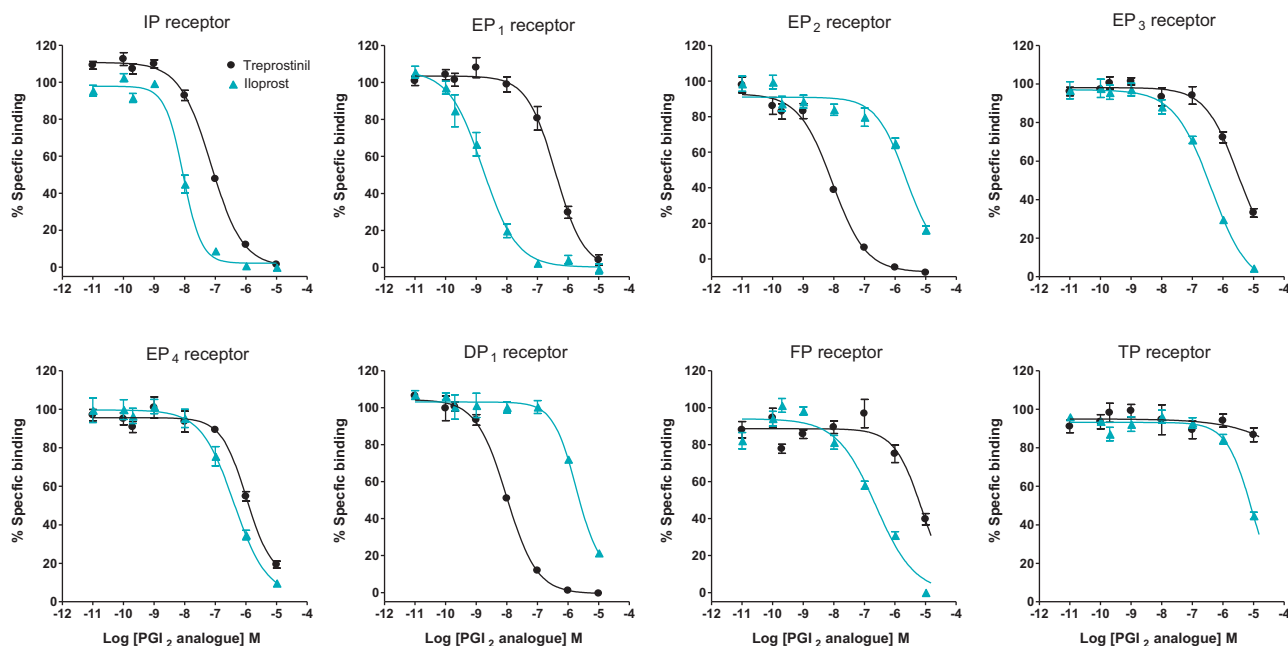
Fig. 1. Competition binding assays for different recombinant human prostanoid receptors. Receptors were stably expressed in HEK-293 (IP, EP_1, EP_2, EP_3, TP, FP), CHO (EP_4) or 1321N1 (DP_1) cell lines. The total specific and non-specific binding was determined for each [3H] ligand as per methods and equilibrium competition binding assays performed in the presence of 0.01–10,000 nM of either iloprost or treprostinil. Data are shown as mean ± S.E.M. of 6 individual determinations performed on two separate occasions. Statistical analysis using 2-way ANOVA indicated that differences in binding affinity curves existed between treprostinil and iloprost for the IP, EP_1, EP_2 EP_3 DP_1, FP ($P < 0.001$) but not EP_4 ($P = 0.08$) receptor.

affinity for the EP_4 receptor, and very weak affinity for EP_3, FP and TP receptors (Table 2). Thus, the rank order of affinity of treprostinil for the human prostanoid receptors based on the derived K_i values was $DP_1 = EP_2 > IP > EP_1 > EP_4 > EP_3 > FP > TP$.

In the current work, the K_i values at the IP receptor showed a 10-fold difference ($P < 0.001$, unpaired t-test) in affinity between iloprost and treprostinil. The major difference between the overall binding profile of iloprost and treprostinil for G_s-coupled receptors was the high affinity of treprostinil for the DP_1 and EP_2 receptor. This was reflected by the 230-fold and 325-fold lower K_i value obtained in the current study for the DP_1 and EP_2 receptor respectively with treprostinil compared with iloprost. Treprostinil had a higher K_i than iloprost at the EP_4 receptor, though overall the

specific binding curves were not significantly different ($P = 0.08$, 2-way ANOVA). These binding studies also indicted that treprostinil had a 200-fold lower affinity for the EP_1 receptor than did iloprost, as well as a much lower affinity for the FP and TP receptor (Table 2).

3.2. Prostanoid receptor activation studies

Studies on the effect of iloprost or treprostinil over a wide concentration range (10^{-12} to 10^{-5} M) on functional responses in cells expressing each prostanoid receptor were conducted. The concentration–response curve for each prostacyclin analogue against each prostanoid receptor is shown in Fig. 2, the responses being determined, depending on the receptor under investigation, as an elevation of intracellular cyclic AMP or calcium influx (Fig. 2). Typical sigmoid curves were obtained for all but one of the prostanoid receptors with either analogue (Fig. 2). The exception was iloprost at the DP_1 receptor, which unlike in the binding study, showed an atypical sigmoidal relationship with a shallow slope, the response at 10 μM being comparable to the maximal response to treprostinil, achieved at 10 nM (Fig. 2). From the concentration–response data obtained for each prostanoid receptor, the EC_{50} was calculated and shown in Table 3.

The rank order of iloprost potency for evoking a response in cells expressing each particular prostanoid human receptor was $EP_1 = IP > EP_3 > FP > EP_4 > TP > DP_1 = EP_2$, which is broadly similar to the ranking observed in the binding studies. Thus, iloprost had high activity at both the IP and the EP_1 receptor in the expression system used and indeed had a similar EC_{50} value for activity (sub nanomolar) at either receptor. Furthermore, iloprost was 75-fold less active at the EP_3 receptor than at the IP receptor, 500–1000-fold less active at the FP and EP_4 receptor and had EC_{50} values in the micromolar range for activity at the EP_2, DP_1, and TP receptors (Table 3).

As with the radioligand binding studies, iloprost had higher activity in evoking a functional response in cells expressing the IP receptor than did treprostinil, having a 5-fold ($P < 0.01$, unpaired

Table 2
Prostanoid receptor binding profiles for treprostinil and iloprost. Specific binding was determined using displacement radioligand binding in cell membranes over expressing recombinant human prostanoid receptors. Values of the inhibition constant, K_i are shown as the mean ± S.E.M. of 6 individual determinations obtained on two separate occasions. The K_i for iloprost at the EP_1 receptor was significantly ($P < 0.002$) greater than that for the IP receptor, and its K_i for IP receptor was significantly ($P < 0.001$) greater than the K_i of treprostinil at this receptor. For comparison, the table also contains K_i values for iloprost obtained from historical binding data published by Abramovitz et al. for human prostanoid receptors expressed in HEK 293 (EBNA) cells [21]. NC = not calculable.

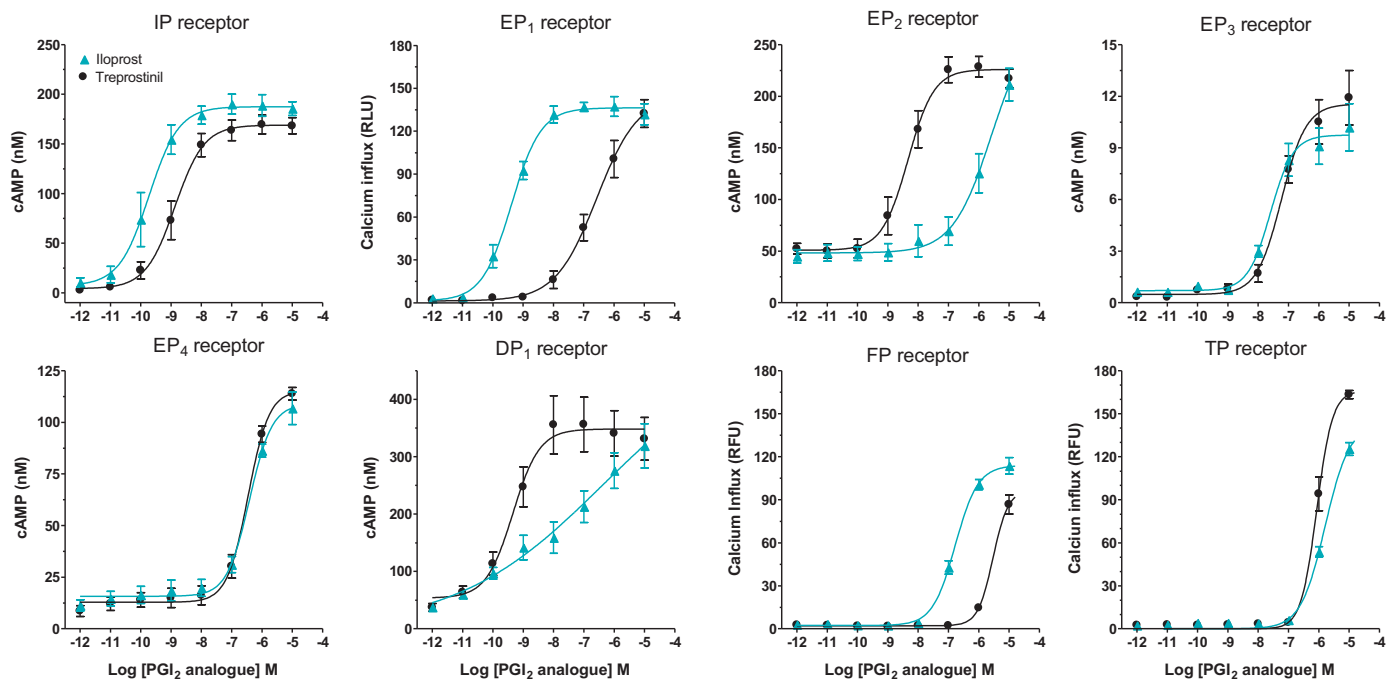| Receptor	Radioligand binding assay		Abramovitz et al. [21]
	Treprostinil K_i (nM)	Iloprost K_i (nM)	Iloprost K_i (nM)
IP	32.1 ± 0.2	3.9 ± 0.6	11 ± 1
EP_1	212 ± 56	1.1 ± 0.3	11 ± 1
EP_2	3.6 ± 0.3	1172 ± 159	1870 ± 176
EP_3	2505 ± 263	208 ± 26	56 ± 6
EP_4	826 ± 116	212 ± 27	284 ± 9
DP_1	4.4 ± 0.4	1016 ± 63	1035 ± 171
FP	4680 ± 927	131 ± 17	619 ± 159
TP	NC	3778 ± 375	6487 ± 29

Fig. 2. Receptor activation assays in cells stably expressing human prostanoid receptors. Concentration-dependent increases in intracellular cyclic AMP (IP, EP$_2$, EP$_3$, EP$_4$, DP$_1$ receptors) or calcium (EP$_1$, FP, TP) were measured upon treatment with either treprostinil or iloprost (0.001–10,000 nM) for 1 h. Data are shown as mean ± S.E.M. of 5–10 determinations performed on 2–3 separate occasions. Curves have been generated from fitting data to a variable slope sigmoidal function. Statistical analysis using 2-way ANOVA indicated that differences in concentration response curves existed between treprostinil and iloprost for the IP, EP$_1$, EP$_2$ DP$_1$, FP, TP ($P < 0.001$) but not EP$_3$ and EP$_4$ ($P > 0.9$) receptor.

t-test) lower EC$_{50}$ value (Table 3) and a concentration–response curve significantly shifted ($P < 0.001$, 2-way ANOVA) to the left (Fig. 2).

By contrast to the profile of iloprost, the rank order for evoking a response with treprostinil in cells expressing each separate receptor was DP$_1 \geq$ IP $>$ EP$_2 >$ EP$_3 >$ EP$_4 >$ EP$_1 >$ TP $>$ FP, again in general agreement with the rank order for the radioligand binding studies. Thus, treprostinil had high potency in activating DP$_1$ and EP$_2$ receptors as well as the IP receptor. From comparison of the EC$_{50}$ values, it was some 36-fold less active at the EP$_3$ receptor, 95-fold less active at the EP$_4$ and 150-fold less active at the EP$_1$ site than at the IP receptor. As can be seen from Table 3, treprostinil had little activity at the FP or TP receptor sites.

Table 3

Receptor activation assays in cells stably expressing human prostanoid receptors. For IP, EP$_2$, EP$_3$, EP$_4$ and DP$_1$ receptor activation assays, concentration-dependent intracellular cyclic AMP accumulation was measured upon treatment with either treprostinil or iloprost. For FP, TP and EP$_1$ receptor activation assays, concentration-dependent increases in intracellular calcium were measured upon prostacyclin analogue treatment. The concentration of agonist causing 50% of the maximal response, the EC$_{50}$ value, were determined from the concentration–response curves (5–10 determinations per drug concentration performed on to 2–3 separate occasions) and shown as the mean ± S.E.M. The EC$_{50}$ values for iloprost at the IP and EP$_1$ receptor were not significantly different ($P = 0.6$, unpaired t-test); the EC$_{50}$ values at the DP$_1$ receptor for iloprost and treprostinil were significantly different ($P < 0.02$, unpaired t-test).

Receptor	Treprostinil EC$_{50}$ (nM)	Iloprost EC$_{50}$ (nM)
IP	1.9 ± 0.4	0.37 ± 0.10
EP$_1$	285 ± 143	0.3 ± 0.1
EP$_2$	6.2 ± 1.2	2094 ± 560
EP$_3$	68.9 ± 7	27.5 ± 0.5
EP$_4$	181 ± 37	389 ± 86
DP$_1$	0.6 ± 0.1	2059 ± 765
FP	>3500	191 ± 44
TP	919 ± 110	1417 ± 141

4. Discussion

The current study has compared the activity of two clinically used prostacyclin analogues, iloprost and treprostinil, in receptor binding assays and in biochemical functional responses using cells stably expressing individual human prostanoid receptors. The prostanoid receptors investigated were those classified as IP, EP$_1$, EP$_2$, EP$_3$, EP$_4$, DP$_1$, FP and TP [23,24]. Substantial differences in the profile of activity between these prostacylins have now been identified, the key findings being that unlike iloprost, treprostinil is a potent agonist at both the DP$_1$ and EP$_2$ receptor, while having little activity at the EP$_1$ receptor.

Previous work has reported on the binding of iloprost to these human prostanoid receptors [21], and it was reassuring that the K_i values and rank order of affinity derived from the current work is comparable. A K_i value of 11 nM for iloprost at the human IP receptor in that previous work, and 4 nM in the current study, are also similar to the K_i of 4 nM for iloprost at this receptor in another report [36]. Moreover, studies on the binding of iloprost to murine IP receptors gave a K_i value of 11 nM [33]. As described previously for both murine and human prostanoid receptors [21,33], iloprost also had high affinity for the human EP$_1$ receptor. Indeed, in the current work, the K_i value for the EP$_1$ receptor was even lower (1 nM) than for the IP receptor. Likewise, other radioligand binding studies have reported high affinity binding with iloprost for the human EP$_1$ receptor, with a K_i not significantly different from the natural ligand, PGE$_2$ [37].

Iloprost had a relatively low affinity for the human FP or EP$_4$ receptor, and even lower affinity for the EP$_2$, DP$_1$ or TP receptor in the current study, comparable to that found previously in radioligand binding studies on both murine and human prostanoid receptors [21,33]. In the former two studies however, iloprost did have significant affinity for the murine or human prostanoid EP$_3$ binding site, but this was less pronounced in the current work using the human EP$_3$ receptor. As the EP$_3$ receptor is known to exhibit a range of splice variants for both murine and human

receptors [19], this may have some bearing on differences in the K_i values obtained in these assays.

Findings on the relative affinities for the different prostanoid receptors in the binding assay were generally translated to activity in the biochemical functional assays utilised in the present work. Thus, iloprost had high activity in stimulating cyclic AMP levels in the cells expressing the human IP receptor or in stimulating calcium influx in cells expressing the EP_1 receptor; indeed the EC_{50} values for these responses were the same ($\sim$0.35 nM, Table 2). Earlier pharmacological studies using a range of isolated smooth muscle bioassay preparations also concluded that iloprost has potent activity at both the IP and EP_1 receptor [25,38,39].

In the present biochemical functional assays, iloprost also activated the human EP_3 receptor to elevate intracellular cyclic AMP levels, although the EC_{50} value was some 75-fold higher than that required to activate the response in cells expressing the IP receptor. Iloprost was less active on the cells expressing the FP or EP_4 receptor, and very much less active in eliciting a response in cells expressing the TP, EP_2 or DP_1 receptors. Earlier work in cells expressing either the human EP_2 or EP_4 receptor has also shown iloprost be a very weak agonist in terms of its ability to elevate cyclic AMP in such cells [40]. Recent studies in HEK-293 cells over-expressing EP_2 receptors also showed iloprost failing to elevate intracellular cyclic AMP [26]. However, iloprost had some activity in cells over-expressing the EP_4 receptor, and partial agonist activity in cells over-expressing the DP_1 receptor, with Wilson and colleagues [26] concluding that the latter receptor may be activated at high concentrations of iloprost. In the current study and in all previous work, iloprost likewise had very low activity on the DP_1 receptor expressed in a number of different cell systems including human platelets and COS-M6 cells [21,41,42].

In the present work, treprostinil exhibited a very different profile in the radioligand binding assays for the human prostanoid receptors when compared to iloprost. Thus, unlike iloprost, treprostinil had a high affinity for both EP_2 and DP_1 receptors in the binding assay, which was surprisingly, some 10-fold greater than that for the IP receptor. On the other hand, treprostinil had a 200-fold lower affinity for the EP_1 receptor compared with iloprost, and the affinity for the EP_3 and FP receptors was in the low to mid micromolar range as opposed to the nanomolar range for iloprost. Affinity for the EP_4 receptor was low for treprostinil and iloprost, and both had minimal affinity for the human TP receptor.

The rank order of activity of treprostinil in evoking changes in either cyclic AMP or intracellular calcium levels in the cells expressing the individual human prostanoid receptors was comparable to that found in the radioligand binding assays. Thus, treprostinil elevated cyclic AMP with a similar high potency in cells expressing either the IP or DP_1 receptor, and its activity on cells with the EP_2 receptor was also high. Other work assessing prostanoid receptor antagonists in murine alveolar macrophages has suggested that treprostinil acts on EP_2 receptors to inhibit phagocytosis and cytokine release [43]. In the current work, treprostinil was less active on cells expressing the human EP_3 or the EP_4 receptor, and poorly active on the EP_1 receptor, with very low activity on the TP and FP receptors.

As with the binding studies, the high activity of iloprost at the EP_1 receptor site along with the finding that treprostinil had high affinity and potent activity at the DP_1 and EP_2 sites, are the key differences in the profiles of these two prostacyclin analogues. Interestingly, from a phylogenic perspective, the EP_2, DP_1 and IP receptor are the most highly related receptors within one of two subgroups of prostanoid receptors [41,44]. Such potent activity of treprostinil at the DP_1 receptor provides a novel aspect to interpreting pharmacological activity of this prostacyclin analogue, as activation of the DP_1 receptor will lead to both

vasodilatation and inhibition of human platelet aggregation, as does IP receptor activation [45,46].

In terms of pharmacological responses that could underlie the therapeutic benefit of these prostacyclin analogues in the clinical treatment of pulmonary hypertension, studies on human pulmonary vascular tissue are clearly important. It is known from studies utilising pharmacological agonists and antagonists that the prostanoid receptors involved in the relaxation of human pulmonary venous preparations in vitro are the DP_1 and IP receptors, and to a lesser extent the EP_4 receptor [47,48]. In human pulmonary artery preparations however, the IP receptor appears to be the predominant receptor involved in relaxation [47]. Additional studies have indicated that the prostanoid receptors involved in the contraction of human isolated pulmonary veins were the EP_1 and TP receptor [49]. Indeed, EP_1 receptors are expressed in human pulmonary veins, as demonstrated by immunohistochemistry [48]. Earlier pharmacological work had also suggested that EP_3 receptor agonists had potent contractile activity on the human isolated pulmonary artery [50].

It is not yet known whether the high affinity and potency of iloprost for the EP_1 receptor will lead to vasoconstriction and oppose the vasodilatation evoked through IP receptor activation in arteries or veins. This will depend on factors such as the relative density and distribution of the EP_1 and IP receptor in these tissues, especially human pulmonary vasculature. There is however, some evidence that activation of the EP_3 receptor, which like EP_1 receptor activation elicits vasoconstriction, can offset the vasodilator response to IP receptor activation by iloprost in rat small pulmonary arteries in vitro [51]. In other studies, EP_3 or EP_1 receptor activation has been suggested to limit the relaxant activity of prostacyclin analogues in guinea-pig aorta [52] or rabbit iliac artery [53]. Moreover, the vasorelaxant actions of both iloprost and treprostinil in rat tail artery was enhanced to a small but significant degree by an antagonist at the EP_3 receptor, suggesting a functional antagonism with IP receptors in this tissue [54].

Apart from the potential opposing functional interactions between the vasodilator and vasoconstrictor response following prostanoid receptor activation, there is the possibility of additive or synergistic effects through simultaneous activation of the different G_s-coupled prostanoid receptors, which theoretically could enhance the therapeutic efficacy of the prostacyclins. Iloprost has relatively poor affinity for the EP_4 receptor that can evoke vasodilatation in human vascular tissue [48,55], and even less affinity for the DP_1 and EP_2 receptors, that along with the IP receptor, are primarily involved in the pulmonary vasodilator response to prostanoids [56]. Therefore, additive or synergistic effects of iloprost at prostanoid receptors evoking vasodilatation, is unlikely. In contrast, the high affinity and activity of treprostinil at the human DP_1 and EP_2 receptors in addition to the IP receptor could synergise to potently evoke a vasodilator response, while the minimal activity of treprostinil at EP_1 receptors would not be expected to produce an opposing vasoconstriction. This profile suggests that treprostinil could have a comparatively preferential vasodilator profile in vascular tissue, particularly in the human pulmonary circulation.

The difference in the pharmacological profile between iloprost and treprostinil in some models may hence reflect activity at multiple prostanoid receptor sites. Thus in human pulmonary arterial smooth muscle cells, treprostinil evoked a full dose-dependent elevation of intracellular cyclic AMP, whereas iloprost was less potent and reached a far lower maximal response [57]. Whether this reflected (a) activation by treprostinil of multiple prostanoid receptors coupled to G_s compared with iloprost (b) that iloprost was only a partial agonist at these sites, (c) that the

response to iloprost at the IP receptor was limited by concurrent EP_1 and EP_3 receptor activation or (d) a combination of the above, is not known.

The disparity of the profile between iloprost and treprostinil at the various prostanoid receptors will have importance when determining the overall pharmacological events that they initiate, especially when used to treat disease. This could also contribute to any differences in the degree of side-effects of these prostacyclins in clinical use, including those exerted on the gastro-intestinal tract. Under physiological conditions, both analogues are potent agonists at the IP receptor, which may dominate the nature of the overall pharmacological responses in vascular tissue. However, it has been demonstrated clearly in two studies using human pulmonary tissue, that in idiopathic pulmonary arterial hypertension, the expression of the IP receptor is down-regulated when compared to control tissue, as detected by both immunoblotting and immunohistochemical techniques [29,58]. Under such conditions of low IP receptor density or stimulus-coupling activity, the pharmacological responses of either iloprost or treprostinil through IP receptors could potentially be compromised. Indeed, in a rat model of pulmonary hypertension where almost complete down-regulation of the IP receptor was observed, it was suggested that iloprost may act through another vasodilator receptor, the EP_4 receptor, as this was not similarly down-regulated [58]. The expression of the EP_4 receptor has been detected in human pulmonary vein using immunohistochemical techniques [48]. However, the relatively poor affinity and activity of iloprost at the human EP_4 receptor suggests that activation of this receptor is unlikely to occur in the therapeutic dosing range of iloprost, the upper plasma concentrations achieved with intravenous administration in humans for example, being less than 1 nM [59].

Should expression of IP receptors be sufficiently down-regulated in pulmonary vascular disease to reduce efficacy at the IP receptor, treprostinil could have the capacity to act on the other key vasodilator prostanoid receptors in the lung, namely the DP_1 receptor and the EP_2 receptor. As treprostinil has high affinity and activity at these latter prostanoid receptors, such positive interactions should be achieved within the same clinical dose range that affects IP receptors, with plasma concentrations of treprostinil in patients treated by intravenous or subcutaneous routes ranging from 2.5 to 25 nM [60]. This would require that unlike the IP receptor, the DP_1 and EP_2 prostanoid receptors were not similarly down-regulated in human pulmonary vascular disease. Interestingly, EP_2 receptor expression in pulmonary arterial smooth muscle cells did not appear to be affected by monocrotaline treatment that produced experimental pulmonary hypertension in rats [58], though its effects on DP_1 expression were not monitored.

The importance of the differential prostanoid receptor agonist profile of iloprost and treprostinil will therefore become clearer with further knowledge of the pathology of this disease, particularly as regards to changes in IP and other prostanoid receptor expression or desensitisation and their coupled functional activity in the pulmonary vasculature. Moreover, consideration of pharmacological actions other than the vasoactive properties of the prostacyclins is warranted. Thus, the degree of involvement of IP receptor or other receptor activation in the processes limiting the characteristic exaggerated vascular smooth muscle proliferation in pulmonary hypertension requires careful evaluation [28,29]. All such information may guide the eventual selection, based on its pharmacological profile, of a particular prostacyclin analogue or IP agonist for the various aetiologies that comprise the spectrum seen in pulmonary hypertensive patients.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bcp.2012.03.012.

References

[1] Rubin LJ, Groves BM, Reeves JT, Frosolono M, Handel F, Cato AE. Prostacyclin-induced acute pulmonary vasodilation in primary pulmonary hypertension. Circulation 1982;66:334–8.
[2] Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, et al. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. The Primary Pulmonary Hypertension Study Group. N Engl J Med 1996;334:296–302.
[3] Barst R. How has epoprostenol changed the outcome for patients with pulmonary arterial hypertension. Int J Clin Pract Suppl 2010;23–32.
[4] Safdar Z. Treatment of pulmonary arterial hypertension: the role of prostacyclin and prostaglandin analogs. Respir Med 2011;105:818–27.
[5] Whittle BJR, Moncada S, Vane JR. Biological activities of some metabolites and analogues of prostacyclin. In: De Las Heras FG, Vega S, editors. Medicinal chemistry advances. Oxford: Pergamon Press; 1981. p. 141–58.
[6] Whittle BJR, Moncada S. Antithrombotic assessment and clinical potential of prostacyclin analogues. In: Ellis GP, West GB, editors. Progress in medical chemistry. North Holland: Elsevier Science Publishers; 1984. p. 237–79.
[7] Olschewski H, Rose F, Schermuly R, Ghofrani HA, Enke B, Olschewski A, et al. Prostacyclin and its analogues in the treatment of pulmonary hypertension. Pharmacol Ther 2004;102:139–53.
[8] Murakami M, Watanabe M, Furukawa H, Nakahara H. The prostacyclin analogue beraprost sodium prevents occlusion of bypass grafts in patients with lower extremity arterial occlusive disease: a 20-year retrospective study. Ann Vasc Surg 2005;19:838–42.
[9] Berman S, Quick R, Yoder P, Voigt S, Strootman D, Wade M. Treprostinil sodium (Remodulin), a prostacyclin analog, in the treatment of critical limb ischemia: open-label study. Vascular 2006;14:142–8.
[10] Fernandez B, Strootman D. The prostacyclin analog, treprostinil sodium, provides symptom relief in severe Buerger's disease—a case report and review of literature. Angiology 2006;57:99–102.
[11] Kawald A, Burmester GR, Huscher D, Sunderkotter C, Riemekasten G. Low versus high-dose iloprost therapy over 21 days in patients with secondary Raynaud's phenomenon and systemic sclerosis: a randomized, open, single-center study. J Rheumatol 2008;35:1830–7.
[12] Moriya H, Ishioka K, Honda K, Oka M, Maesato K, Ikee R, et al. Beraprost sodium, an orally active prostaglandin I_2 analog, improves renal anemia in hemodialysis patients with peripheral arterial disease. Ther Apher Dial 2010;14:472–6.
[13] Piaggesi A, Vallini V, Iacopi E, Tedeschi A, Scatena A, Goretti C, et al. Iloprost in the management of peripheral arterial disease in patients with diabetes mellitus. Minerva Cardioangiol 2011;59:101–8.
[14] Hoeper MM, Schwarze M, Ehlerding S, Adler-Schuermeyer A, Spiekerkoetter E, Niedermeyer J, et al. Long-term treatment of primary pulmonary hypertension with aerosolized iloprost, a prostacyclin analogue. N Engl J Med 2000;342:1866–70.
[15] Simonneau G, Barst RJ, Galie N, Naeije R, Rich S, Bourge RC, et al. Continuous subcutaneous infusion of treprostinil, a prostacyclin analogue, in patients with pulmonary arterial hypertension: a double-blind, randomized, placebo-controlled trial. Am J Respir Crit Care Med 2002;165:800–4.
[16] Gomberg-Maitland M, Olschewski H. Prostacyclin therapies for the treatment of pulmonary arterial hypertension. Eur Respir J 2008;31:891–901.
[17] Skoro-Sajer N, Lang I, Naeije R. Treprostinil for pulmonary hypertension. Vasc Health Risk Manage 2008;4:507–13.
[18] Vachiery JL. Prostacyclins in pulmonary arterial hypertension: the need for earlier therapy. Adv Ther 2011;28:251–69.
[19] Hirata T, Narumiya S. Prostanoid receptors. Chem Rev 2011;111:6209–30.
[20] Kennedy I, Coleman RA, Humphrey PP, Levy GP, Lumley P. Studies on the characterisation of prostanoid receptors: a proposed classification. Prostaglandins 1982;24:667–89.
[21] Abramovitz M, Adam M, Boie Y, Carriere M, Denis D, Godbout C, et al. The utilization of recombinant prostanoid receptors to determine the affinities and

selectivities of prostaglandins and related analogs. Biochim Biophys Acta 2000;1483:285–93.

[22] Coleman RA, Humphrey PPA, Kennedy I, Lumley P. Prostanoid receptors – the development of a working class classification. Trends Pharmacol Sci 1984;5:303–6.

[23] Coleman RA, Kennedy I, Humphrey PPA, Bunce K, Lumley P. Prostanoids and their receptors. In: Hansch C, Samnes PG, Taylor JB, editors. Comprehensive medicinal chemistry. Oxford: Pergamon Press; 1990. p. 643–714.

[24] Woodward DF, Jones RL, Narumiya S. International union of basic and clinical pharmacology. LXXXIII: Classification of prostanoid receptors, updating 15 years of progress. Pharmacol Rev 2011;63:471–538.

[25] Coleman RA, Smith WL, Narumiya S. International union of pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994;46:205–29.

[26] Wilson SM, Sheddan NA, Newton R, Giembycz MA. Evidence for a second receptor for prostacyclin on human airway epithelial cells that mediates inhibition of CXCL9 and CXCL10 release. Mol Pharmacol 2011;79:586–95.

[27] Lombroso M, Nicosia S, Paoletti R, Whittle BJR, Moncada S, Vane JR. The use of stable prostaglandins to investigate prostacyclin (PGI$_2$)-binding sites and PGI$_2$-sensitive adenylate cyclase in human platelet membranes. Prostaglandins 1984;27:321–33.

[28] Falcetti E, Flavell DM, Staels B, Tinker A, Haworth SG, Clapp LH. IP receptor-dependent activation of PPARγ by stable prostacyclin analogues. Biochem Biophys Res Commun 2007;360:821–7.

[29] Falcetti E, Hall SM, Phillips PG, Patel J, Morrell NW, Haworth SG, et al. Smooth muscle proliferation and role of the prostacyclin (IP) receptor in idiopathic pulmonary arterial hypertension. Am J Respir Crit Care Med 2010;182:1161–70.

[30] Kuwano K, Hashino A, Asaki T, Hamamoto T, Yamada T, Okubo K, et al. 2-[4-[(5,6-Diphenylpyrazin-2-yl)(isopropyl)amino]butoxy]-N-(methylsulfonyl)a-cetamide (NS-304), an orally available and long-acting prostacyclin receptor agonist prodrug. J Pharmacol Exp Ther 2007;322:1181–8.

[31] Morrison K, Ernst R, Hess P, Studer R, Clozel M. Selexipag: a selective prostacyclin receptor agonist that does not affect rat gastric function. J Pharmacol Exp Ther 2010;335:249–55.

[32] Clapp LH, Patel JM. The mechanistic basis for prostacyclin action in pulmonary hypertension. Int J Respir Care 2010;27–33.

[33] Kiriyama M, Ushikubi F, Kobayashi T, Hirata M, Sugimoto Y, Narumiya S. Ligand binding specificities of the eight types and subtypes of the mouse prostanoid receptors expressed in Chinese hamster ovary cells. Br J Pharmacol 1997;122:217–24.

[34] Wright DH, Metters KM, Abramovitz M, Ford-Hutchinson AW. Characterization of the recombinant human prostanoid DP receptor and identification of L-644,698, a novel selective DP agonist. Br J Pharmacol 1998;123:1317–24.

[35] Sharif NA, Davis TL. Cloned human EP1 prostanoid receptor pharmacology characterized using radioligand binding techniques. J Pharm Pharmacol 2002;54:539–47.

[36] Sharif NA, Crider JY, Xu SX, Williams GW. Affinities, selectivities, potencies, and intrinsic activities of natural and synthetic prostanoids using endogenous receptors: focus on DP class prostanoids. J Pharmacol Exp Ther 2000;293:321–8.

[37] Ungrin MD, Carriere MC, Denis D, Lamontagne S, Sawyer N, Stocco R, et al. Key structural features of prostaglandin E$_2$ and prostanoid analogs involved in binding and activation of the human EP$_1$ prostanoid receptor. Mol Pharmacol 2001;59:1446–56.

[38] Dong YJ, Jones RL, Wilson NH. Prostaglandin E receptor subtypes in smooth muscle: agonist activities of stable prostacyclin analogues. Br J Pharmacol 1986;87:97–107.

[39] Armstrong RA, Lawrence RA, Jones RL, Wilson NH, Collier A. Functional and ligand binding studies suggest heterogeneity of platelet prostacyclin receptors. Br J Pharmacol 1989;97:657–68.

[40] Wilson RJ, Rhodes SA, Wood RL, Shield VJ, Noel LS, Gray DW, et al. Functional pharmacology of human prostanoid EP$_2$ and EP$_4$ receptors. Eur J Pharmacol 2004;501:49–58.

[41] Boie Y, Sawyer N, Slipetz DM, Metters KM, Abramovitz M. Molecular cloning and characterization of the human prostanoid DP receptor. J Biol Chem 1995;270:18910–16.

[42] Sharif NA, Williams GW, Davis TL. Pharmacology and autoradiography of human DP prostanoid receptors using [^{3}H]-BWA868C, a DP receptor-selective antagonist radioligand. Br J Pharmacol 2000;131:1025–38.

[43] Aronoff DM, Peres CM, Serezani CH, Ballinger MN, Carstens JK, Coleman N, et al. Synthetic prostacyclin analogs differentially regulate macrophage function via distinct analog-receptor binding specificities. J Immunol 2007;178:1628–34.

[44] Abramovitz M, Adam M, Boie Y, Grygorczyk R, Rushmore TH, Nguyen T, et al. Human prostanoid receptors: cloning and characterization. Adv Prostaglandin Thromboxane Leukot Res 1995;23:499–504.

[45] Whittle BJR, Hamid S, Lidbury P, Rosam AC. Specificity between anti-aggregatory actions of prostacyclin, prostaglandin E1 and D2 on platelets. In: Westwick J, editor. Mechanisms of stimulus secretion coupling in platelets. New York: Plenum Press; 1985. p. 109–25.

[46] Giles H, Leff P, Bolofo ML, Kelly MG, Robertson AD. The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. Br J Pharmacol 1989;96:291–300.

[47] Walch L, Labat C, Gascard JP, de M, Brink C V, Norel X. Prostanoid receptors involved in the relaxation of human pulmonary vessels. Br J Pharmacol 1999;126:859–66.

[48] Foudi N, Kotelevets L, Louedec L, Leseche G, Henin D, Chastre E, et al. Vasor-elaxation induced by prostaglandin E$_2$ in human pulmonary vein: role of the EP$_4$ receptor subtype. Br J Pharmacol 2008;154:1631–9.

[49] Walch L, de Montpreville V, Brink C, Norel X. Prostanoid EP1 and TP-receptors involved in the contraction of human pulmonary veins. Br J Pharmacol 2001;134:1671–8.

[50] Qian YM, Jones RL, Chan KM, Stock AI, Ho JK. Potent contractile actions of prostanoid EP$_3$ receptor agonists on human isolated pulmonary artery. Br J Pharmacol 1994;113:369–74.

[51] Kuwano K, Hashino A, Noda K, Kosugi K, Kuwabara K. A long-acting and highly selective prostacyclin receptor agonist prodrug, 2-{4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}-N-(methylsulfonyl)acetamide (NS-304), ameliorates rat pulmonary hypertension with unique relaxant responses of its active form, {4-[(5,6-diphenylpyrazin-2-yl)(isopropyl)amino]butoxy}acetic acid (MRE-269), on rat pulmonary artery. J Pharmacol Exp Ther 2008;326:691–9.

[52] Clapp LH, Turcato S, Hall SJ, Baloch M. Evidence that Ca^{2+}-activated K$^+$ channels play a major role in mediating the vascular effects of iloprost and cicaprost. Eur J Pharmacol 1998;356:215–24.

[53] McCormick C, Jones RL, Kennedy S, Wadsworth RM. Activation of prostanoid EP receptors by prostacyclin analogues in rabbit iliac artery: implications for anti-restenotic potential. Eur J Pharmacol 2010;641:160–7.

[54] Orie NN, Clapp LH. Role of prostanoid IP and EP receptors in mediating vasorelaxant responses to PGI2 analogues in rat tail artery: evidence for Gi/o modulation via EP3 receptors. Eur J Pharmacol 2011;654:258–65.

[55] Davis RJ, Murdoch CE, Ali M, Purbrick S, Ravid R, Baxter GS, et al. EP$_4$ prostanoid receptor-mediated vasodilatation of human middle cerebral arteries. Br J Pharmacol 2004;141:580–5.

[56] Norel X. Prostanoid receptors in the human vascular wall. ScientificWorldJournal 2007;7:1359–74.

[57] Clapp LH, Finney PA, Turcato S, Tran S, Rubin LJ, Tinker A. Differential effects of stable prostacyclin analogues on smooth muscle proliferation and cyclic AMP generation in human pulmonary artery. Am J Respir Cell Mol Biol 2002;26:194–201.

[58] Lai YJ, Pullamsetti SS, Dony E, Weissmann N, Butrous G, Banat GA, et al. Role of the prostanoid EP$_4$ receptor in iloprost-mediated vasodilatation in pulmonary hypertension. Am J Respir Crit Care Med 2008;178:188–96.

[59] Krause W, Krais T. Pharmacokinetics and pharmacodynamics of the prostacyclin analogue iloprost in man. Eur J Clin Pharmacol 1986;30:61–8.

[60] McSwain CS, Benza R, Shapiro S, Hill N, Schilz R, Elliott CG, et al. Dose proportionality of treprostinil sodium administered by continuous subcutaneous and intravenous infusion. J Clin Pharmacol 2008;48:19–25.