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RESEARCH PAPER

The affinity, intrinsic activity and selectivity of a structurally novel EP_2 receptor agonist at human prostanoid receptors

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BACKGROUND AND PURPOSE

Prostanoid EP_2 receptor agonists exhibit several activities including ocular hypotension, tocolysis and anti-inflammatory activity. This report describes the affinity and selectivity of a structurally novel, non-prostanoid EP_2 receptor agonist, PGN-9856, and its therapeutic potential.

EXPERIMENTAL APPROACH

The pharmacology of a series of non-prostanoid EP_2 receptor agonists was determined according to functional and radioligand binding studies, mostly using human recombinant prostanoid receptor transfectants. The selectivity of PGN-9856, as the preferred compound, was subsequently determined by using a diverse variety of non-prostanoid target proteins. The therapeutic potential of PGN-9856 was addressed by determining its activity in relevant primate cell, tissue and disease models.

KEY RESULTS

PGN-9856 was a selective and high affinity (pKi ≥ 8.3) ligand at human recombinant EP_2 receptors. In addition to high affinity binding, it was a potent and full EP_2 receptor agonist with a high level of selectivity at EP_1, EP_3, EP_4, DP, FP, IP and TP receptors. In cells overexpressing human recombinant EP_2 receptors, PGN-9856 displayed a potency ($pEC_{50} \geq 8.5$) and a maximal response (increase in cAMP) comparable to that of the endogenous agonist PGE_2. PGN-9856 exhibited no appreciable affinity (up 10 μM) for a range of 53 other receptors, ion channels and enzymes. Finally, PGN-9856 exhibited tocolytic, anti-inflammatory and long-acting ocular hypotensive properties consistent with its potent EP_2 receptor agonist properties.

CONCLUSIONS AND IMPLICATIONS

PGN-9856 is a potent, selective and efficacious prostanoid EP_2 receptor agonist with diverse potential therapeutic applications: tocolytic, anti-inflammatory and notably anti-glaucoma.

Abbreviations

EFS, electrical field stimulation; FLIPR, fluorometric imaging plate reader; IOP, intraocular pressure; SNP, sodium nitroprusside

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Introduction

The actions of the five naturally occurring prostanoid metabolites of arachidonic acid, namely, PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and TxA_2, are mediated via interaction with specific, plasma membrane GPCRs. Five major subdivisions of the prostanoid receptor family termed DP, EP, FP, IP and TP have been defined on the basis of their pharmacological sensitivity and molecular identity (Coleman *et al*., 1994; Woodward *et al*., 2011). EP receptors have been pharmacologically classified further into EP_1, EP_2, EP_3 and EP_4 subtypes, on the basis of their relative sensitivities to a range of naturally occurring and synthetic agonists and antagonists. Increased understanding of the specific roles of each of the EP receptor subtypes is expected to lead to new opportunities for drug discovery. Of particular interest in the present context is the EP_2 receptor subtype. Evolving research suggests that selective EP_2 receptor agonists have the potential for therapeutic utility in several diseases, notably those requiring remedial anti-inflammatory activity. Therefore, the discovery and optimization of 'drug-like' EP_2 receptor agonists remain attractive propositions.

The prostanoid EP receptor pharmacology approaches adopted in the present studies and the structure–activity relationships that emerged, resulted in the identification of simple, low MW, non-prostanoid EP_2 receptor agonists. These became the focus of a medicinal chemistry-driven lead optimization programme, during which about 250 novel molecules were synthesized and tested. Evaluation of compounds on key target human cells and tissues was at the core of the screening process to ensure that agonist efficacy was appropriately optimized. This programme resulted in the identification of several potential development candidates. The primary focus here is to describe the affinity, selectivity and potency of one such EP_2 receptor agonist, PGN-9856, which became the potential candidate for preclinical development based on chemical structural considerations. In addition to tocolysis and ocular hypotension, particular attention was given to anti-inflammatory activity, where the broad potential of EP_2 agonists as modulators of inflammation continues to be of interest (Armstrong, 1995; Kay *et al*., 2006; Woodward *et al*., 2011; Zaslona *et al*., 2014; Wang *et al*., 2016).

Methods

Ca^{2+} signalling studies by fluorometric imaging plate reader (FLIPR)

The FLIPR method using stable cell transfectants overexpressing human prostanoid DP_1, $EP_{1–4}$, FP, IP and TP receptors has been previously described (Matias *et al*., 2004). Responses of G_s and G_i protein coupled prostanoid receptors were measured as a Ca^{2+} signal, by employing chimeric G-protein cDNAs. Ca^{2+} signalling studies were performed using a FLIPR system (Molecular Devices, Sunnyvale, CA, USA) in a 96-well format. Briefly, cells were seeded at a density of 5×10^4 cells per well in Biocoat poly-D-lysine-coated blackwall, clear-bottom 96-well plates (BD Biosciences, Franklin Lakes, NJ) and allowed to attach overnight in an incubator at 37°C. The cells were then washed twice with HBSS-HEPES buffer (Hanks' balanced salt solution without bicarbonate and phenol red, 20 mM HEPES, pH 7.4). After 60 min exposure to Fluo-4AM (Invitrogen, Carlsbad, CA, USA) to achieve dye loading at a final concentration of 2×10^{-6} M, the plates were washed four times with HBSS-HEPES buffer. A 7-point serial dilution of the standard agonist for each corresponding receptor subtype was administered to give final concentrations from 10^{-11} to 10^{-5} M in 10-fold serial dilution increments for cells expressing human recombinant DP_1, EP_1, EP_2, EP_3, EP_4, FP and IP receptors. The dose range for the standard agonist for human recombinant TP receptors was from 10^{-12} to 10^{-6} M. HBSS-HEPES buffer was used as the negative control. Cells were excited with an argon laser at 488 nm, and emission was measured through a 510 to 570 nm emission filter. Standard agonists were as follows: DP = BW 245C, EP_1–EP_4 = PGE_2, FP = 17-phenyl-$PGF_{2\alpha}$, IP = carbaprostacyclin and TP = U-46619. To obtain concentration–response curves, compounds were tested in triplicate in each plate over the desired concentration range in at least three separate experiments to give $n = 3$. The raw data were processed using a non-linear regression curve fit, first by Activity Base to calculate the percentage activity of each data point relative to the positive control (=10^{-6} M of the standard agonist), then exported to GraphPad Prism to calculate the average EC_{50} value for each compound.

Membrane preparation for radioligand binding assays

The ability of compounds to bind to human EP_2 receptors and their selectivity against other EP receptors was demonstrated in radioligand competition displacement binding experiments using cell lines stably transfected with the human EP receptors.

Membranes were prepared from cells stably transfected with human EP receptor cDNA (nucleotide accession numbers: EP_1 receptor (NM_000955), EP_2 receptor (NM_000956), EP_3 receptor (NM_000957) and EP_4 receptor (NM_000958).

In brief, cells were cultured to confluency, scraped from culture flasks and centrifuged (800× *g* for 8 min, at 4°C). Cells were twice washed in ice-cold homogenization buffer containing (mM) 10 Tris–HCl, 1 EDTA.2Na, 250 sucrose, 1 PMSF, 0.3 indomethacin, pH 7.4, then homogenized and re-centrifuged as before. The supernatant was stored on ice and pellets re-homogenized and re- centrifuged. Supernatants were pooled and centrifuged at 40 000× *g* for 10 min at 4°C. The resultant membrane pellets were stored at −80°C until use.

Radioligand displacement binding at prostanoid EP_2, EP_3 and EP_4 receptors

For radioligand displacement binding assays, membranes expressing human EP_2, EP_4 or EP_3 receptors were incubated in Millipore $Multiscreen_{HTS}$-HV (0.45 μm) plates (EMD Millipore, Billerica, MA, USA) containing assay buffer, radiolabelled $[^3H]PGE_2$ and test compounds (0.1 to 10 000 nM). Incubations were performed at suitable temperatures and for suitable times to allow equilibrium to be reached, as described in Table 1. Non-specific binding was determined in the presence of 10 μM PGE_2. Bound and free radiolabel was separated by vacuum manifold filtration using appropriate wash buffers, and bound radiolabel was

Table 1

Experimental conditions for radioligand displacement binding at recombinant $EP_{2–4}$ prostanoid receptors

Receptor		EP_2	EP_3	EP_4
Protein per well (μg)		8	5	8
Final [^{3}H-PGE_2] (nM)		3	2.5	1
Buffer	Assay	10 mM MES pH 6.0; 10 mM $MgCl_2$; 1 mM EDTA	10 mM MES pH 6.0; 10 mM $MgCl_2$; 1 mM EDTA, 100 μM GTP-γ-S	10 mM MES pH 6.0; 10 mM $MgCl_2$; 1 mM EDTA, 3 μM indomethacin
–	Wash	10 mM MES pH 6.0; 10 mM $MgCl_2$	10 mM MES pH 6.0; 10 mM $MgCl_2$	10 mM MES pH 6.0; 1 mM EDTA
Temperature (°C)		30	30	30
Incubation time (min)		60	60	60

MES, 2-(*N*-morpholino) ethanesulphonic acid.

determined by scintillation counting. Constituents of each of the buffers are detailed in Table 1. The affinity or pK_i of each compound for each receptor was calculated from the concentration causing 50% radioligand displacement (IC_{50}) using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

$$K_i = IC_{50}/\left(1 + \left(\left[\left[^3H\right]PGE_2\right]/K_D\right)\right.$$

Radioligand displacement binding at prostanoid EP_1 receptors

Membranes (protein 250 μg per well) expressing EP_1 receptors were incubated in deep-welled, 96-well plates containing assay buffer (10 mM Tris–HCl; 150 mM NaCl, 3 μM Indomethacin, 0.3% BSA), radiolabelled [^{3}H] PGE_2 4 nM and test compounds (0.1 to 10 000 nM) at pH 7.6. Incubations were performed at 37°C for 60 min until equilibrium was reached. Non-specific binding was determined in the presence of 10 μM PGE_2. After 60 min incubation at 37°C, the assay was terminated by filtration through a combination of GF-C and GF-B filters, pre-soaked in 1% polyethyleneimine, using a Brandel cell harvester, and subsequently washed three times using wash buffer. Radioactivity retained on the filters was determined by liquid scintillation counting. The affinity or pK_i of each compound for each receptor was calculated from the concentration causing 50% radioligand displacement (IC_{50}) using the Cheng–Prusoff equation as previously described.

Radioligand displacement binding at prostanoid DP receptors

Membranes were prepared from 1321 NI astrocytoma cells (Sigma-Aldrich, Gillingham, Dorset, UK)) stably expressing human recombinant DP_1 receptors (Woodward et al., 2007, Wang et al., 2016). For assays, membranes (protein 15 μg per well) expressing the human DP_1 receptor were incubated in Millipore $Multiscreen_{HTS}$-HV (0.45 μm) plates containing assay buffer (50 mM Tris, 5 mM $MgCl_2$, 10 μg·mL^{-1} saponin and 10 mM indomethacin), [^{3}H] PGD_2 3.2 nM and the test compounds (0.1 to 10 000 nM) at pH 7.4. Incubations were performed at 25°C for 60 min to allow equilibrium to be reached. Non-specific binding was determined in the presence of 10 μM PGD_2. Bound and free radiolabel were separated by vacuum manifold filtration using an appropriate wash buffer, and bound radiolabel was determined by scintillation counting. The affinity or pK_i of each compound was calculated from the concentration causing 50% radioligand displacement (IC_{50}) using the Cheng–Prusoff equation as previously described.

Radioligand displacement binding at the prostanoid IP receptors

Platelet membranes from human blood were prepared as follows. Peripheral venous blood samples were obtained from healthy volunteers with informed consent and approval from the local ethics committee (East and North Herts Local Research Ethics Committee). Platelets were isolated from whole venous blood by Optiprep™ (1.063g/ml) density centrifugation at 350× *g* for 15min at 20°C. The platelet containing band and platelet-rich plasma were carefully harvested using a Pasteur pipette and centrifuged at 1690× *g* for 15min at 4°C. The resulting pellet was resuspended in 5 mM Tris (pH 7.4) and homogenized using a glass/Teflon homogenizer. The homogenate was centrifuged at 30 000× *g* for 20 min at 4°C, and the resultant pellet was resuspended in 50 mM Tris (pH 7.4). The protein concentration was determined, and membranes were stored at −80°C until required.

The binding assay was as follows. Membranes (50 μg protein per well) were incubated in 96-well Millipore $Multiscreen_{HTS}$-FB (1 μm) plates, pre-coated with 0.5% polyethylenimine. Each well contained assay buffer (50 mM TRIS and 10 mM $MgCl_2$ at pH 7.4), [^{3}H] iloprost 10 nM and test compounds (0.1 to 10 000 nM). Incubations were performed at a 25°C temperature for 15 min to allow equilibrium. Non-specific binding was determined in the presence of 10 μM iloprost. Bound and free radiolabels were separated by vacuum manifold filtration using 50 mM TRIS wash buffer at pH 7.4, and bound radiolabel was determined by scintillation counting. The affinity or pK_i of each compound was calculated from the concentration causing 50% radioligand displacement (IC_{50}) using the Cheng–Prusoff equation as previously described.

In addition to human recombinant EP receptors, the affinity of one of the compounds, PGN-9856, was determined at a range of 45 receptors, 4 ion channels, 2

transporters and 2 kinases. This work was carried by Cerep SA (Celle-Lévesqcault, Poitiers, France) and is reported as supplementary data.

Assessment of agonist activity at recombinant EP_2 and EP_4 receptors (cAMP assay)

HEK cells stably expressing human EP_2 or EP_4 receptors (Wang *et al.*, 2016) were routinely cultured at 37°C in a humidified 5% CO_2 incubator. The culture medium used was DMEM supplemented with 10% FBS, 100 U·mL^{-1} penicillin, 100 ng·mL^{-1} streptomycin, 2.5 μg·mL^{-1} fungizone, 2 mM glutamine and 250 μg·mL^{-1} geneticin. In addition, 200 μg·mL^{-1} zeocin was included in the EP_2 cell media, while 200 μg·mL^{-1} hygromycin was included in the EP_4 cell media.

For assay, cells were prepared at a density of 50 000 cells per well in 96-well poly-L-lysine-coated plates and allowed to grow to confluence (3–4 days) prior to use. Culture media were rinsed off using DMEM and replaced with DMEM containing the PDE inhibitor IBMX (1 mM) and the cycloxygenase inhibitor indomethacin (3 μM). This was allowed to incubate for 1 h before the cells were stimulated with PGE_2 or PGN compounds (in duplicate) for 15 min at final concentrations ranging from 0.0001 to 10 μM. The assay was terminated by the addition of 25 μL hydrochloric acid (1 N). Plates were then frozen for a minimum of 12 h or until required for radioligand displacement assay.

The cAMP radioligand displacement assay was as follows. Plates were thawed quickly at 37°C, and neutralized with 25 μL sodium hydroxide (1 N). Samples of supernatant (30 μL) were transferred to 96-well Millipore Multiscreen$_{HTS}$-FB (1 μm) plates coated with 0.1% polyethylenimine. These samples were diluted by addition of 90 μL cAMP assay buffer (50 mM Tris, 5 mM EDTA, pH 7.0). A cAMP standard curve (from 10^{-11} to 10^{-5} M) was constructed. A 15 μL of 3′:5′-cAMP-dependent protein kinase (final concentration 8 μg per well) and 15 μL [^{3}H]-cAMP (final concentration 2 nM per well) were added to each well. Plates were incubated on ice for 2 h, before bound and free radiolabels were separated by vacuum filtration harvesting on a Millipore manifold, using ice-cold water as the termination buffer. Filter plates were allowed to dry overnight, before addition of 50 μL Microscint 0. Radioactivity was determined using the Microbeta Trilux scintillation counter. cAMP accumulation was determined from the standard curve.

Isolation of monocytes and lymphocytes from human peripheral blood

Human peripheral blood mononuclear cells were obtained from samples of whole venous blood taken from healthy volunteers with informed consent for use in biomedical research and ethical approval (East and North Herts Local Research Ethics Committee). Monocytes were isolated from whole blood by Ficoll–Hypaque (1.077 g·mL^{-1}), density gradient centrifugation and adherence to plastic for 1 h at 37°C to separate lymphocytes (non-adherent cells) from monocytes (adherent cells). The non-adherent lymphocyte fraction was used in the lymphocyte assay, and the adherent monocytes were recovered by scraping and subsequently used in the monocyte assay.

The viability of lymphocytes and monocytes was determined by Trypan blue exclusion. The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Dorset, England) containing 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin (referred to as complete medium). To eliminate any potential effects of endogenous PGE_2, the cyclooxygenase inhibitor, indomethacin (3 μM), was included in the culture medium.

IL-2 release by anti-CD3 activated lymphocytes

Prior to the lymphocyte assay, 96-well plates were coated with anti-CD3 monoclonal antibody (clone: OKT3) at 25 ng·mL^{-1} in PBS for 3 h at 37°C. The plates were then washed three times with PBS before the initiation of the assay. Test compounds (final concentration range 10^{-11} to 10^{-4} M) were first added to corresponding duplicate wells according to the experimental design, followed by the addition of lymphocytes (2×10^6 cell per mL, 100 μL per well) in complete medium. The plates were incubated for 24 h at 37°C with 5% CO_2 in air, and the supernatants were recovered and stored at −20°C prior to measurement of IL-2 by ELISA, as described below.

TNF-α production by LPS-stimulated monocytes

Freshly isolated, viable monocytes were plated onto 96-well plates at 1×10^5 cells per well and pretreated for 1 h at 37°C/5% CO_2 with PGE_2 (included as a positive control) or test compound (final concentration range 10^{-10} to 10^{-4} M) in triplicate wells. Vehicle controls were included where appropriate. Monocytes were then stimulated with 100 ng·mL^{-1} LPS from *Escherichia coli* for a further 24 h. Cell-free culture supernatants were pooled and stored at −20°C prior to measurement of TNF-α by ELISA. Briefly, The cytokines IL-2 and TNF-α from culture supernatants were quantified by standard sandwich ELISA (DuoSet® cytokine ELISA Development System, R&D Systems, Minneapolis, MA, USA) according to the manufacturer's instructions. Pooled data from independent experiments performed on cells obtained from three or more donors are expressed as mean ± SEM.

TNF-α release from human peripheral blood

The assay was developed based on previously described methodology (Beck *et al.*, 2002). Peripheral blood samples were obtained from healthy volunteers with full informed consent and ethical approval. Blood samples were collected into tubes containing tripotassium EDTA as an anticoagulant. The samples were kept at 4°C and used within 2 h of collection.

The test compounds were prepared as stock solutions in DMSO (10^{-2} M) and subsequent dilutions were made in complete cell culture medium (containing RPMI1640, 10% FCS, penicillin and streptomycin). The final 1:10 dilution of the test compounds was made in whole blood by adding 50 μL of compound solution to 450 μL of whole blood in a 48-well plate. To eliminate the potential effect of endogenous PGE_2, the cyclooxygenase inhibitor, indomethacin (3 μM final concentration), was included in all treatments. The plate was gently shaken for 1 min at 300 r.p.m. in a 37°C incubator and incubated for a further 14 min before the addition of

LPS solution (50 μL) to a final concentration of 100 ng·mL^{-1}. The plate was then placed on the shaker for a further 1 min at 300 r.p.m. to ensure an even distribution of the test compounds and LPS. The total incubation time for this assay was 4 h. At the end of the experiment, the blood samples were collected and centrifuged at 16 060× *g* for 2 min in a microcentrifuge and the plasma samples collected, stored at −20°C prior to analysis of TNF-α levels by ELISA.

ELISA analysis of TNF-α levels in the plasma was performed using an ELISA kit supplied by R&D Systems (Quantikine human TNF-α ELISA kit, R&D Systems Europe) according to the manufacturer's instruction. The plasma samples were thawed at 4°C overnight and centrifuged at 16 060× *g* for 2 min before being added to the ELISA plate.

Relaxation of human myometrium

Sections of non-pregnant myometrium were prepared from samples of surgically removed uterus, with informed consent for use as biomedical research biomaterial and ethical approval (East and North Herts Local Research Ethics Committee). All human tissue sourcing adhered to the relevant sections of the Helsinki Declaration. Longitudinal myometrial muscle strips (2 mm wide by 10 mm long) were then cut and suspended between stainless steel hooks in organ chambers containing oxygenated (95% O_2/ 5% CO_2) Krebs solution at 37°C. The composition of the Krebs solution was as follows: NaCl (118.2 mM), KCl (4.69 mM), $MgSO_4.7H_2O$ (1.18 mM), KH_2PO_4 (1.19 mM), glucose (11.1 mM), $NaHCO_3$ (25.0 mM), $CaCl_2.6H_2O$ (2.5 mM) and indomethacin (3 μM).

Tissues were placed under a tension equivalent to 25 mN and left overnight at room temperature. The following day the incubation temperature was raised to and maintained at 37°C. The tissues were washed and placed under a tension of 15 mN, then allowed to equilibrate for a period of at least 30 min. Responses were recorded using isometric transducers coupled to a computer via a MacLab interface. After 60 min, the muscle sections of the human myometrium were stimulated electrically (15 ms pulse width, for 10 s every 100 s at 15 V and 5 Hz) using parallel platinum wire electrodes and a Multistim D330 pulse stimulator. Upon electrical stimulation, the strips of human myometrial smooth muscle responded with a rapid contraction. Once the response to electrical stimulation had stabilized, the strips were exposed to increasing concentrations of test compounds (1×10^{-10} to 1×10^{-5} M) and incubated for approximately 10 min with each concentration. At the end of the experiment, the standard EP_2 receptor agonist, butaprost (1×10^{-5} M), was applied followed by sodium nitroprusside (SNP; 1×10^{-4} M), which was used to produce a standard relaxant response. To determine the potencies of the compounds, the concentration of each test compound required to produce a half-maximal effect (EC_{50}) was calculated. Experimental protocols and designs were devised, and statistical analyses were performed in adherence to BJP guidelines.

Intraocular pressure (IOP) measurement

All animal care and experimental protocols adhered to the US National Research Council's 'Guide for the Care and Use of Laboratory Animals', ARVO guidelines and BJP guidelines for experiments involving animals and animal tissues. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). The animals used in these studies were kept for life as permanent residents of a USA-based colony. Animal welfare was overseen by a select committee and professionally managed by an environmental enrichment specialist. The environmental enrichment included visual, auditory, dietary, tactile and olfactory stimuli, with rotation to prevent boredom. These present studies employed monkeys as the most reliable species for clinical translation and this model has been widely used for glaucoma research for three decades (Lee *et al.*, 1985; Woodward *et al.*, 2003; Prassana *et al.*, 2011; Toris *et al.*, 2017).

The six female cynomolgus monkeys (2 to 4 kg body weight) used in the study had a mean age of 10.2 ± 6.4 SD and an age range of 4.5 to 17.7 years. They were housed in pairs. All animals had received laser photocoagulation treatment to the trabecular meshwork of the left eye to elevate IOP. It should be noted that bilateral laser treatment of the eyes is strictly forbidden by animal experimentation regulating authorities. The monocular lasering procedure was performed as required to maintain elevated IOP, the degree of which varies widely between individuals. The reduction of vision is restricted to the peripheral vision in one eye, which is apparent only if the animal is approached quietly from behind and in the direction of the lasered eye. This visual impairment has no discernible effect on the normal behaviour or disposition of the monkeys. Although cynomolgus monkeys menstruate, it is known that menstruation has no effect on IOP in primates (Green *et al.*, 1984).

The study animals were randomly selected from the glaucomatous monkey colony with each animal being used evaluate the effects of PF-04217329 and PGN 9856i in turn, with an intervening washout period of 1 month. Using special treats and toys, the animals had been trained to accept IOP measurements with minimal stress. For IOP recordings, the animals were placed in custom-designed restraining chairs. One drop of 25 μL proparacaine was applied to the hypertensive eye at 30 s before each IOP determination (Model 30 pneumatonometer, Reichert Technologies, Buffalo, NY, USA). The trained animals remained conscious during pneumatonometry. Two baseline IOP measurements of the lasered eye (ocular hypertensive eye) were taken 30 min and immediately before drug administration. Prior to administration, the drug solution was vortexed for approximately 10 s. Drugs were administered as a 25 μL drop to the surface of the ocular hypertensive eye immediately following the second baseline reading. Subsequent to dosing, further IOP readings were taken at 2, 4, 6, 24 and 48 h. Only one dose was administered per compound.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018). The *n* value for each group of ocular hypertensive monkeys was set as 6, since this has long proved adequate to identify clinically sufficient and statistically significant reductions in IOP (Lee *et al.*, 1985; Woodward *et al.*, 2003). Although the experiments were conducted according to the design and analysis in pharmacology

recommendations (Curtis *et al.*, 2018), there are certain design constraints peculiar to the model. Only one eye is rendered ocular hypertensive for animal welfare reasons. Thus, drug versus vehicle (left vs. right eye) paired comparisons would be meaningless. Statistical analysis compared the pre-treatment IOP values (mm Hg) with post-treatment values, according to a two-tailed paired Student's *t*-test. The *P* value was set at $P < 0.01$ to be considered statistically significant. The experimenter was not informed of the identity of the solutions provided until the experiment was finished and the data analysed. All animals were typically provided with a washout period of at least 14 days between each drug treatment. In these experiments, PF-04217329 was given 28 days before PGN 9856i.

Materials

The compounds PGN-9856, PGN-9856-isopropyl ester, PGN-9858, PGN-9862, PGN-9863, CP-533536 and PF-04217329 were synthesized by Target Molecules (Southampton, England). Their structures are shown in Figure 1. Butaprost, PGD_2, PGE_2, iloprost, carbaprostacyclin, 17-phenyl-$PGF_{2\alpha}$, U-46619 and BW 245C were purchased from Cayman Chemical (Kalamazoo, MI, USA). The anti-CD3 monoclonal antibody (clone: OKT3) was supplied by Janssen-Cilag Ltd. (High Wycombe, UK); LPS from *Escherichia coli* by Sigma Aldrich Ltd. (Poole, UK), and the proparacaine was supplied by Allergan Inc. (Irvine, CA). For ocular studies, PGN-9856i and PF-04217329 were prepared as 0.1% (1 mg·mL^{-1}) suspensions in 10 mM Tris/1% polysorbate 80.

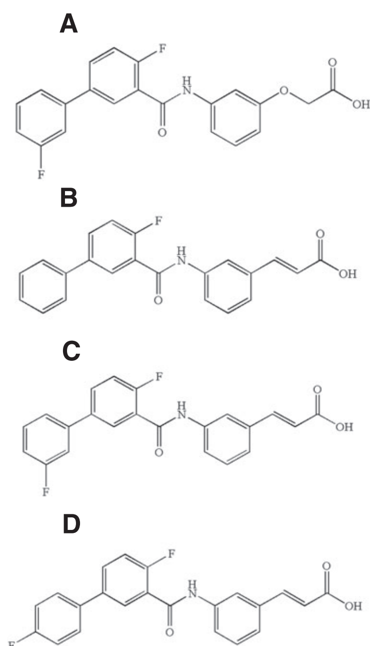

Figure 1

Structures of compounds used in these experiments: PGN-9856, PGN-9858, PGN-9862 and PGN-9863.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

Ca^{2+} signalling (FLIPR assay)

The pEC_{50} values for PGN 9856, 9858, 9862 and 9863 agonist activity (intracellular Ca^{2+} release) at human recombinant DP_1, EP_1, EP_2, EP_3, EP_4, FP, IP and TP receptors are shown in Table 2. All four compounds exhibited a high degree of selectivity for the EP_2 receptor, the only measurable off-target activity was for PGN-9862 at the EP_4 receptor. The rank order of potency at the EP_2 receptor was PGN-9856 > PGN-9862 > PGN-9863 ≥ PGN-9858.

Binding affinity at human prostanoid receptors

The affinity of PGN-9856 at prostanoid receptors was determined by radioligand binding competition experiments. It

Table 2

Agonist potency (as pEC_{50}) of compounds for intracellular Ca^{2+} release following stimulation of human recombinant DP_1, $EP_{1–4}$, FP, IP and TP receptors

Compound	DP_1	EP_1	EP_2	EP_3	EP_4	FP	IP	TP
PGN-9856	<5	<5	8.7	<5	<5	<5	<5	<5
PGN-9858	<5	<5	7.1	<5	<5	<5	<5	<5
PGN-9862	<5	<5	8.2	<5	5.3	<5	<5	<5
PGN-9863	<5	<5	7.3	<5	<5	<5	<5	<5

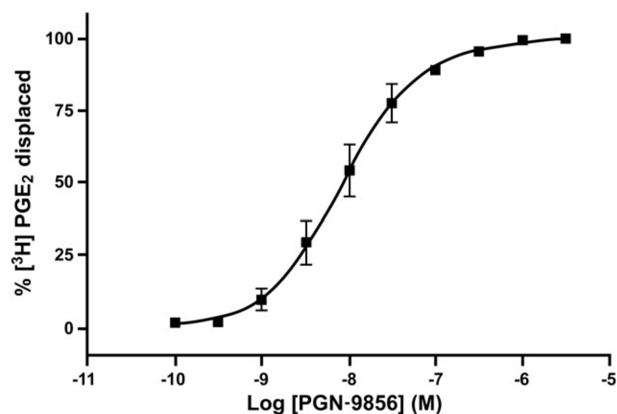

Figure 2

Concentration-dependent displacement of the binding of radio-labelled PGE_2 to human recombinant EP_2 receptors by PGN-9856. Data are shown as mean ± SEM for n = 3–4 experiments.

Table 3

Affinity (pK_i) of PGN-9856 for human recombinant EP_{1-4} and DP receptors and native IP receptors

Compound	EP_2 (n = 3)	EP_1 (n = 2)	EP_3 (n = 2–3)	EP_4 (n = 3)	DP (n = 2)	IP (n = 2)
PGN-9856	8.5 ± 0.1	<5	<5	5.9 ± 0.1	<5	<5
PGE_2	8.2 ± 0.1	7.3	8.9 ± 0.1	8.8 ± 0.1	PGD_2 = 8.6	Iloprost = 7.8

Data are shown as mean ± SEM for stated number of experiments.

was found to bind with high affinity to the human EP_2 receptor expressed in a recombinant cell line (Figure 2). The results (Table 3) also show that the compound exhibits negligible affinity for EP_1, EP_3, DP and IP receptors and has 180-fold selectivity for EP_2 over EP_4 receptors.

Specificity of lead compound PGN 9856

Broad spectrum profiling of PGN-9856 was completed (Cerep, Celle-Lévescault, Poitiers, France) to determine its specificity against a range of 45 receptors (including the Tx TP receptor), 4 ion channels, 2 transporters and 2 kinases. Based on screening data at a concentration of 10 μM, PGN-9856 caused less than 50% inhibition of binding at 30°C (correlating to a pKi < 5) at all targets tested. The mean values for the effects of PGN-9856 in binding assays are summarized in Table S1, and the mean values for the effects of PGN-9856 in enzyme assays are summarized in Table S2. These screening data are provided in the Supporting Information.

Agonist potency at human recombinant EP_2 receptors

In HEK cells overexpressing the human recombinant EP_2 receptor, PGE_2 causes a concentration-dependent elevation of cAMP levels (Figure 3). This is a result of EP_2 receptor stimulation as no response to PGE_2 is seen in naïve HEK cells. PGN-9856 behaved as a potent and full agonist in this test system, with agonist potency and maximal response comparable to that of PGE_2 (Figure 3). The pEC_{50} values obtained were 9.9 ± 0.1 and 9.7 ± 0.1 for PGN-9856 and PGE_2 respectively.

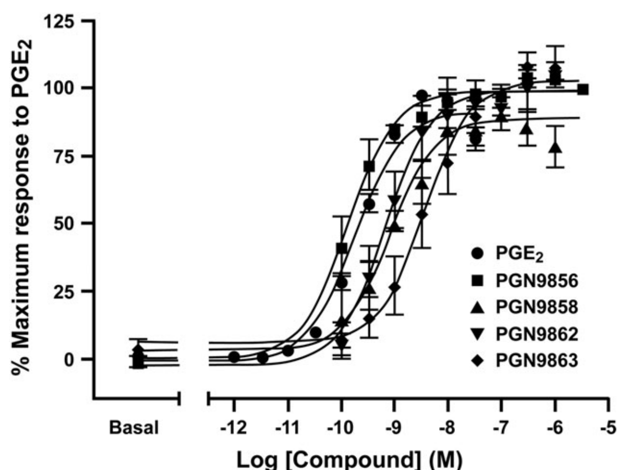

Figure 3

Agonist potency (cAMP response) of PGN-9856, PGN-9858, PGN - 9862, PGN-9863 and PGE_2 at human recombinant EP_2 receptors. Data are expressed relative to the response to 3 x 10^{-8}M PGE_2 (set at 100%). Values shown are means ± SEM from 3 individual experiments.

Agonist potency at recombinant EP_4 receptors (cAMP)

In HEK cells overexpressing human recombinant EP_4 receptors, PGE_2 causes a concentration-dependent elevation of cAMP levels. This would be a result of EP_4 receptor stimulation since no response to PGE_2 was seen in naïve HEK cells. In the cAMP assay, in contrast to the FLIPR assay, PGN-9856 was a weak partial agonist at the recombinant EP_4 receptor with a pEC_{50} of 6.1 ± 0.1, compared to 10.5 ± 0.1 for PGE_2 (data not shown).

Inhibition of TNF-α release from human peripheral blood monocytes

In human peripheral blood monocytes stimulated with LPS, the standard selective EP_2 receptor agonist, butaprost, causes a concentration-dependent inhibition of TNF-α release (Figure 4). Similarly, all four molecules under investigation behaved as highly potent agonists in this test system with agonist potencies greater than that of butaprost (Figure 4A). In addition, all four leading compounds behaved as full agonists, with a maximum response comparable to that of butaprost. PGE_2 was also tested in this system and behaved as a full agonist with a pEC_{50} = 7.5 ± 0.1. The pEC_{50} for butaprost = 6.9 ± 0.1 and for PGN-9856 = 7.8 ± 0.2.

Inhibition of IL-2 release from human peripheral blood lymphocytes

In human lymphocytes stimulated with anti-CD3, the standard EP_2 receptor agonist, butaprost, causes a concentration-dependent inhibition of IL-2 release (Figure 4B). Similarly, all four compounds behaved as highly potent agonists in this test system, with potencies greater than that of butaprost (Figure 4B). In addition, they were also full agonists with a maximum response comparable to that of butaprost (Figure 4B) in this system. PGE_2 was a full agonist (pEC_{50} = 7.7, n = 5). The pEC_{50} values for PGN-9856 and butaprost were 7.8 ± 0.1 and 6.8 ± 0.2 respectively.

Inhibition of TNF-α release from human peripheral blood

Incubation of samples of whole blood with LPS (100 ng·mL^{-1}) released TNF-α, at levels varying between donors. The mean value following LPS stimulation was 1485 ± 477 pg·mL^{-1}. Samples in the absence of LPS stimulation produced very little TNF-α (50.3 ± 37.0 pg·mL^{-1}). PGN-9856 was tested

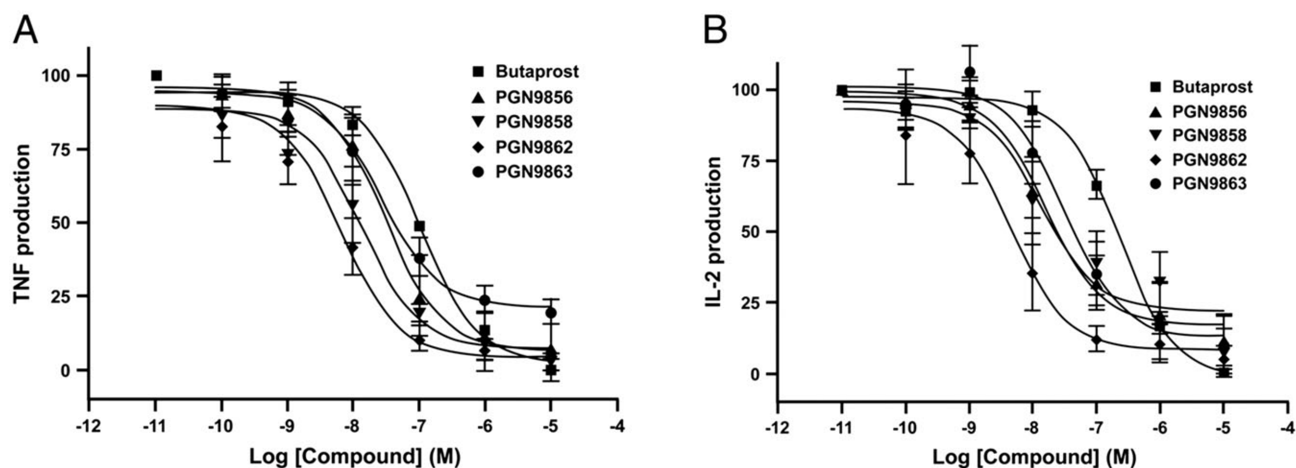

Figure 4

(A) Effect of PGN-9856, PGN-9858, PGN-9862, PGN-9863 and the standard EP_2 receptor agonist, butaprost, on TNF-α release from human LPS-stimulated peripheral blood monocytes Data are mean ± SEM from 4–5 individual experiments. (B) Activity of PGN-9856, PGN-9858, PGN-9862, PGN-9863 and the standard EP_2 receptor agonist, butaprost, on IL-2 release from in human lymphocytes stimulated by anti-CD3 antibody. Data are mean ± SEM from 3–4 individual experiments.

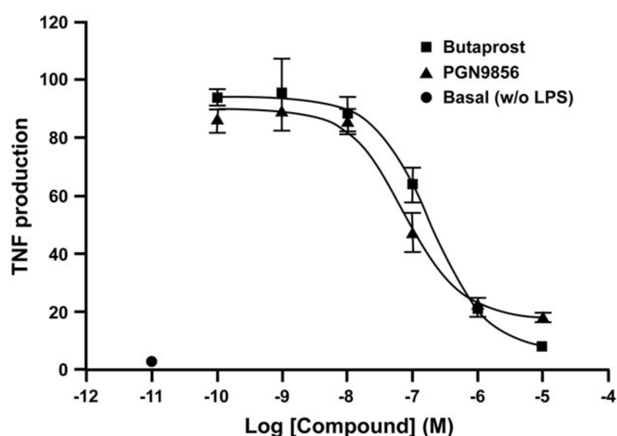

Figure 5

Comparison of the effects of PGN-9856 and butaprost on inhibition of TNF-α release in LPS-stimulated whole blood. Basal release (no LPS) is indicated. Data are given as mean ± SEM from four donors.

between 10^{-10} and 10^{-5} M in the whole blood assay. As Figure 5 shows, this compound inhibited TNF-α production in a concentration-dependent manner with a pEC_{50} estimate of 7.1 (7.1 ± 0.1, *n* = 4). Maximal inhibition achieved by this compound at 10^{-5} M was 81.6% of LPS control or 85.7 ± 2.5 when normalized to the response produced by the standard EP_2 receptor agonist, butaprost.

Relaxation of human myometrium

Electrical field stimulation (EFS) caused frequency-dependent contractions of all myometrial tissues tested. At a sub-maximal frequency of 5 Hz, EFS also caused contractions of all the tissues tested. PGN-9856 concentration-dependently inhibited the contractile response of uterine smooth muscle to EFS with a potency greater or equal to that of the standard EP_2 receptor agonist, butaprost, and a comparable maximum response (Figure 6A). The pEC_{50} for butaprost was 7.5 ± 0.2, and the % inhibitory response to SNP (100 μM) was 83 ± 5. The pEC_{50} for PGN-9856 was 8.7 ± 0.5, and the % inhibitory response to SNP (100 μM) was 87 ± 7. A representative physiography trace comparing PGN-9856 and butaprost is shown in Figure 6B.

IOP studies in monkeys

For IOP studies, PGN-9856 and CP-533536 were derivatized to the isopropyl ester prodrugs, as this derivative has been found to be particularly useful for increasing ocular bioavailability (Bito and Baroody, 1981; Bito and Baroody, 1987). A comparison of the effects of PGN-9856-ispropyl ester (PGN-9856i) and CP-533536 isopropyl ester (PF-04217329) on monkey IOP is shown in Figure 7. Both compounds were administered as a single 0.1% (1 $mg \cdot mL^{-1}$) dose. Over the first 24 h of the experimental time course, both compounds produced a similarly profound decrease in IOP. The study was extended to 48 h, and at this time point, the activities diverged. The activity of PF-04217329 had returned to baseline at 48 h post-dosing, whereas the activity of PGN-9856i was essentially fully maintained at the 48 h time point.

Discussion

Although it has long been established that PGE_2 acts primarily via a family of four receptor subtypes, EP_1, EP_2, EP_3 and EP_4, the initial identification of these different receptors was based upon rank order of agonist potency and, in some cases, antagonist activities. Such subclassification of EP receptors was made extremely difficult by the lack of truly selective ligands, and full acceptance of the proposed ligand-based classification was only eventually achieved through the molecular identification of the four receptor subtypes

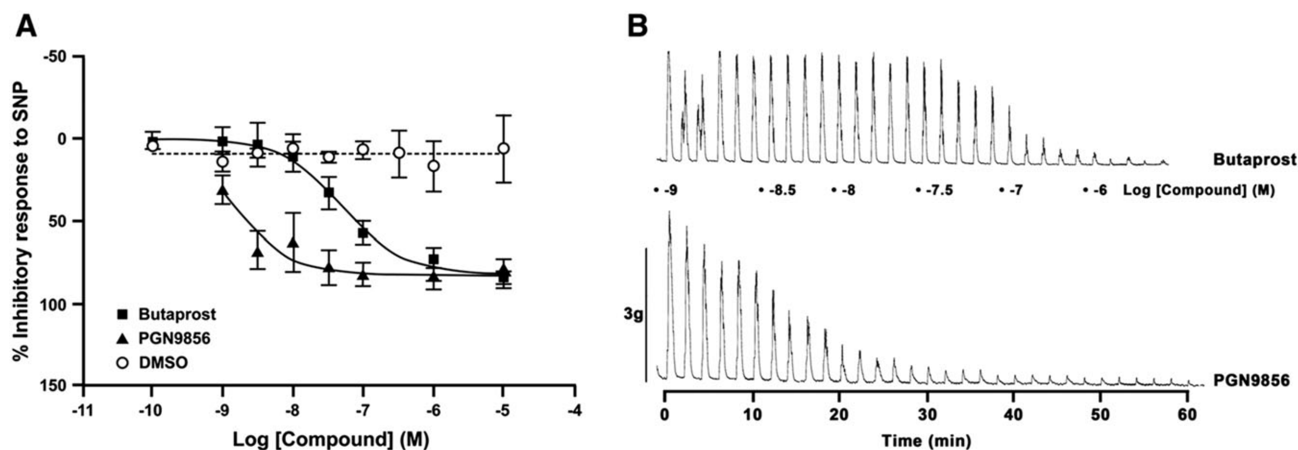

Figure 6

(A) Inhibitory effects of PGN-9856 ($n = 3$) and the standard EP_2 receptor agonist butaprost ($n = 7$) and DMSO vehicle control ($n = 7$) on electrically stimulated human isolated non-pregnant myometrium. Data shown are mean ± SEM % of the inhibition induced by sodium nitroprusside (SNP) 10^{-4}M. (B) Original recordings of responses of human myometrial smooth muscle to electrical stimulation at 5 Hz for 10 sec every 100 sec and the inhibition of these responses produced by increasing cumulative concentrations of butaprost (upper trace) and PGN9856 (lower trace). Dots indicate approximately when drug concentrations were administered in 0.5 log unit increments at approximately 10 min intervals, beginning with 10^{-9} M (-9). Vertical bar indicates calibration of tension developed (g) for both traces.

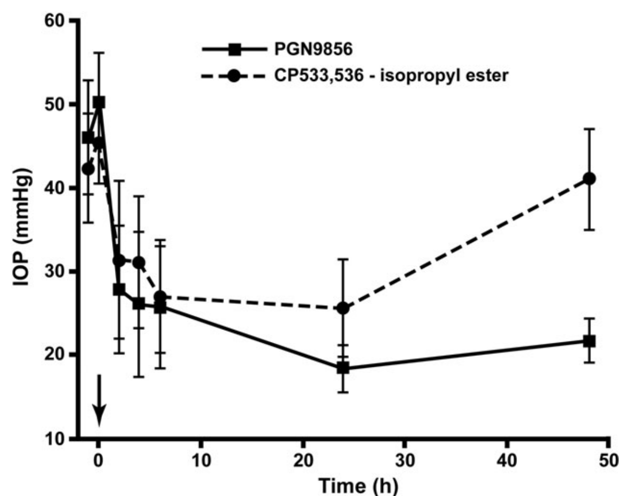

Figure 7

Comparison of the effects of PGN-9856-isopropyl ester (PGN-9856i) and PF-04217329 on the intraocular pressure of laser-induced ocular hypertensive monkeys. PGN-9856i produced a significant reduction in intraocular pressure at the 6, 24, and 48 h post-dosing time points, compared to the immediate pre-dosing time point (time 0). PF-04217329 also produced identically significant reductions in IOP at all time points except at 48 h, when the effect on intraocular pressure had essentially returned to baseline and no statistically significant effect was apparent. Data are means ± SEM, $n = 6$.

(Coleman *et al.*, 1994; Woodward *et al.*, 2011). Despite this pharmacological definition and the development of recombinant receptor-assisted technologies, the development of potentially useful drugs based on agonist activity at one or more of the EP receptor subtypes has been hampered by the difficulty in developing truly subtype selective agonists. Moreover, close structural analogues based on the C20 prostanoid backbone have inherent problems associated with bioavailability, stability and metabolism. In addition, the 'cost of goods' of such compounds renders their development prohibitively expensive as systemically administered medication. In particular, there has been a need for 'druggable' EP_2 receptor ligands. The compound described in this paper appears to meet this need.

The majority of receptor-selective prostanoid EP_2 agonists had prostanoid-like structures. until recently. The first selective agonists at EP_2 receptors were AH-13205 (Nials *et al.*, 1993) and butaprost (Gardiner, 1986). These were both structural analogues of PGE_2. Neither compound was particularly potent, although both appeared to be selective for the EP_2 receptor relative to other prostanoid receptors. Butaprost is a particular problem in that contains a methyl ester group that is subject to facile and tissue-dependent hydrolysis, leading to the suggestion that the free acid hydrolysis product of butaprost is actually the EP_2 receptor agonist (Abramovitz *et al.*, 2000). 19-R (OH) PGE_2 is unique in being a naturally occurring selective EP_2 agonist (Woodward *et al.*, 1993) but exhibits the chemical instability issues inherent in PGE_2. ONO-AE1-259 has proven a useful compound in pharmacological characterization of EP_2 receptor pharmacology in complex systems (Cao *et al.*, 2002; Clarke *et al.*, 2004; Jones and Chan, 2005) but is still another analogue of PGE_2. The first example of a non-prostanoid agonist was CP-533536, a compound which appears to have been initially considered for development as a treatment for bone fractures by virtue of its potent bone anabolic activity (Paralkar *et al.*, 2003). More recently, its isopropyl ester has been demonstrated to be an effective ocular hypotensive agent and was considered as a potential treatment for glaucoma (Prassana *et al.*, 2011). Anabolic and ocular hypotensive activities of EP_2 agonists only represent a small part of the potential therapeutic spectrum. There are a range of additional possible therapeutic opportunities.

The potential therapeutic uses of selective EP_2 agonists also include treatment of bronchoconstriction, pre-term labour, systemic hypertension by stimulating natriuresis, neuroprotection and inflammation (Woodward *et al.*, 2011). Prostanoid EP_2 receptors inhibit T-cell proliferation and regulate antigen-presenting cell function (Nataraj *et al.*, 2001), inhibit TNF-α release from bone marrow-derived dendritic cells (Vassiliou *et al.*, 2003), inhibit major histocompatibility complex class II expression in dendritic cells (Harizi *et al.*, 2003), suppress IFN-α release by NK cells (Walker and Rotondo, 2004), inhibit Th1 and Th2 polarized antigen-specific T-cell responses (Okano *et al.*, 2006) and augment the signalling and function associated with the anti-inflammatory cytokine IL-10 (Cheon *et al.*, 2006. Thus, inhibition of cytokine release was a prominent feature of these present studies with PGN-9856. All the EP_2 agonist compounds studied in this series potently inhibited IL-2 and TNF-α release. The effect of PGN-9856 on TNF-α production was also studied in an *in vitro* system that closely resembles the *in vivo* environment in terms of the concentrations of plasma proteins; namely, an assay was developed using human whole blood. When stimulated by LPS at a sub-maximal concentration, cells in the whole blood produce a considerable amount of TNF-α. This response was effectively blocked by the standard EP_2 receptor agonist, butaprost and the non-prostanoid PGN-9856, which behaved as a potent agonist in this assay and produced a concentration-dependent inhibition of LPS-stimulated TNF-α production. In human whole blood in the presence of physiological concentrations of human plasma proteins, the potency of PGN-9856 is reduced by less than fivefold relative to that observed in the monocyte assays. Thus, the presence of plasma proteins has a negligible impact on the potency of PGN-9856, which suggests that these compounds should retain significant efficacy and anti-inflammatory activity *in vivo*. This systemic efficacy is likely to translate to all other therapeutic situations, except glaucoma where the major target tissues, the trabecular meshwork and Schlemm's canal are avascular.

Following the success of analogues of $PGF_{2\alpha}$ in the treatment of glaucoma, attention has turned to prostanoid EP_2 receptor agonists. Although effects of AH-13205 (Woodward *et al.*, 1995) on monkey IOP are modest, butaprost (Nilsson *et al.*, 2006) produces an effect greater than that of latanoprost (Stjernschantz *et al.*, 1995) and bimatoprost (Woodward *et al.*, 2003). The difference in ocular hypotensive efficacy between AH-13205 and butaprost is likely to be due to the methyl ester moiety that would render butaprost an ester prodrug of butaprost free acid. It has long been known that prostanoid penetration through the cornea is minimal (Bito and Baroody, 1981), a problem that is remedied by esterification of the carboxylate moiety (Bito and Baroody, 1987). Because of this, all EP_2 receptor agonists with an intended use in ophthalmology have been esterified, such as taprenepag isopropyl (Prassana *et al.*, 2011; Yanochko *et al.*, 2014) and omidenepag isopropyl (Aihara *et al.*, 2017). In order to determine the anti-glaucoma potential of PGN-9856, its effects were evaluated in the monkey model of glaucoma (Lee *et al.*, 1985). PGN-9856i was equipotent to PF-04217329 over a 24 h period post-dosing. At 48 h post-dosing, PGN-9856i remained fully efficacious, unlike PF-04217329. Although exaggerated doses of both compounds were employed in these present studies, it remains to be determined whether the effectiveness of PGN-9856i as an ocular hypotensive agent is a result of favourable ocular bioavailability. A comparison of other therapeutic uses for CP-533536 and PGN-9856, and their ester derivatives beyond glaucoma is not possible at present. PGN-9856 has not been evaluated in bone resorption models and any anti-inflammatory effects of CP-533536 have not been reported. Iritis in human volunteers has been reported (Schachar *et al.*, 2011), which portends a lack of clinically useful anti-inflammatory activity for CP-533536 and PF-04217329.

It follows that, in order to fulfil the many potential therapeutic applications of an EP_2 receptor agonist, it is judicious to consider its pharmacological properties in terms of how it may behave as a drug *per se*. PGN-9856 proved itself as not only a high affinity ligand for recombinant EP_2 receptors, comparable to the natural ligand, PGE_2, but also highly selective with no meaningful activity or affinity for other prostanoid receptors. It exhibited only very modest activity at EP_4 receptors in one functional assay employed, but this was more than 100-fold less than that for EP_2. PGN-9856's affinity for EP_2 receptors translated into agonist activity, producing potent, concentration-related elevation of levels of intracellular cAMP in a recombinant expression system. Moreover, its functional pEC_{50} in this system, like that of PGE_2, is approximately an order of magnitude lower than its affinity for the EP_2 receptor, suggesting that partial receptor occupancy is all that is required to elicit a maximal effect. PGN-9856, therefore, appears to be a high efficacy agonist. While activity in recombinant systems is encouraging for a potentially therapeutically useful agonist, in such systems, receptor expression is often abnormally high and, therefore, has the propensity to exaggerate agonist activity. This may not represent the agonist activity that may occur in native systems. It is encouraging that PGN-9856 is also a full agonist in various native expression systems. In the present study, this was demonstrated in the compound's concentration-related inhibition of cytokine release from both human monocytes and lymphocytes. Similarly, it caused marked inhibition of electrically-induced contractions of human myometrium, a tissue known to contain EP_2 receptors, (Senior *et al.*, 1993). Although PGN-9856's inhibitory potency in human myometrium was not markedly different from that in recombinant systems, in blood cells, its potency was reduced but remained therapeutically meaningful. In the only living animal study, conducted on non-human primate IOP, PGN-9856 exceeded potency and efficacy expectations based on previously reported effects EP_2 agonists. A final consideration pertaining to translational reliability is that all studies were, without exception, performed on primate cells and organs.

In conclusion, PGN-9856, the leading molecule arising from a prostanoid EP_2 receptor lead optimization programme, emerged as a potent, selective and specific EP_2 receptor ligand, which acts as a full agonist at human EP_2 receptors. PGN-9856 represents an exciting potential preclinical and clinical development candidate. It may be particularly useful for treating glaucoma by virtue of its extended duration of action, but anti-inflammatory and tocolytic applications are also indicated.

Author contributions

R.A.C. conceived, co-directed, co-designed and interpreted radioligand binding and all cell studies and co-wrote the manuscript. A.J.W. co-directed, supervised and analysed radioligand binding, second messenger studies and leukocyte studies. K.L.C. co-directed project and directed medicinal chemistry/compound design. C.B.T. co-directed, supervised, analysed and co-interpreted the intraocular pressure studies. S.F. performed and co-analysed intraocular pressure and conceived and executed the conscious monkey training for accepting pnuematonometry. J.W.W. conceived, co-directed and analysed high throughput screening. D.F.W. conceived and co-directed glaucoma studies conceived and co-directed HTS and co-wrote the manuscript.

Conflict of interest

D.F.W. and J.W.W. are inventors of a method of use patent assigned to JeniVision Inc.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Abramovitz M, Adam M, Boie Y, Carriére M, Denis D *et al*. (2000). The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. Biochim Biophys Acta 1483: 285–293.

Aihara M, Lu F, Kawata H, Tanaka Y, Yamamura K *et al*. (2017). Pharmacokinetics, safety and IOP lowering profiles of omidenepag isopropyl, a selective EP2 agonist in healthy Japanese and Caucasian volunteers (phase 1 study). Invest Ophthalmol Vis Sci 58: 2104.

Alexander SPH, Christopoulos A, Davenport AP, Kelly E, Marrion NV, Peters JA *et al*. (2017). The concise guide to pharmacology 2017/18: G protein-coupled receptors. Br J Pharmacol 174: S17–S129.

Armstrong RA (1995). Investigation of the inhibitory effects of PGE_2 and selective EP agonists on chemotaxis of human neutrophils. Br J Pharmacol 116: 2903–2908.

Beck G, Bottomley G, Bradshaw D, Brewster M, Broadhurst M, Devos R *et al*. (2002). (E)-2(R)-[1(S)-(Hydroxycarbamoyl)-4-phenyl-3-butenyl]-2′-isobutyl-2′-(methanesulfonyl)-4-methylvalerohydrazide (Ro 32-7315), a selective and orally active inhibitor of tumor necrosis factor-alpha convertase. J Pharmacol Exp Ther 302: 390–396.

Bito LZ, Baroody RA (1981/1982). The penetration of exogenous prostaglandin and arachidonic acid into, and their distribution within, the mammalian eye. Curr Eye Res 1: 659–669.

Bito LZ, Baroody RA (1987). The ocular pharmacokinetics of eicosanoids and their derivatives 1. Comparison of ocular eicosanoid penetration and distribution following topical application of PGF2alpha, PGF2alpha-1-methyl ester, and PGF2alpha-1-isopropyl ester. Exp Eye Res 44: 217–226.

Cao J, Shayibuzhati M, Tajima T, Kitazawa T, Taneike T (2002). *In vitro* pharmacological characterization of the prostanoid receptor population in the non-pregnant porcine myometrium. Eur J Pharmacol 442: 115–123.

Cheng Y, Prusoff WH (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 22: 3099–3108.

Cheon H, Rho YH, Choi SJ, Lee YH, Song GG, Sohn J *et al*. (2006). Prostaglandin E_2 augments IL-10 signaling and function. J Immunol 177: 1092–1100.

Clarke DL, Belvisi MG, Catley MC, Yacoub MH, Newton R, Giembycz MA (2004). Identification in human airways smooth muscle cells of the prostanoid receptor and signalling pathway through which PGE2 inhibits the release of GM-CSF. Br J Pharmacol 141: 1141–1150.

Coleman RA, Smith WL, Narumiya S (1994). VIII. International Union of Pharmacology Classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 46: 205–229.

Curtis MJ, Alexander S, Cirino G, Docherty JR, George CH, Giembycz MA *et al*. (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. Br J Pharmacol 175: 987–993.

Gardiner PJ (1986). Characterization of prostanoid relaxant/inhibitory receptors (psi) using a highly selective agonist, TR4979. Br J Pharmacol 87: 45–56.

Green K, Cullen PM, Phillips CI (1984). Aqueous humor turnover and intraocular pressure during menstruation. Br J Ophthalmol 68: 736–740.

Harding SD, Sharman JL, Faccenda E, Southan C, Pawson AJ, Ireland S *et al*. (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucl Acids Res 46: D1091–D1106.

Harizi H, Grosset C, Gualde N (2003). Prostaglandin E_2 modulates dendritic cell function via EP_2 and EP_4 receptor subtypes. J Leukoc Biol 73: 756–763.

Jones RL, Chan KM (2005). Investigation of the agonist activity of prostacyclin analogues on prostanoid EP4 receptors using GW 627368 and taprostene: evidence for species differences. Prostaglandins Leukot Essent Fatty Acids 72: 289–299.

Kay LJ, Yeo WW, Peachell PT (2006). Prostaglandin E_2 activates EP_2 receptors to inhibit human lung mast cell degranulation. Br J Pharmacol 147: 707–713.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Lee P-Y, Podos SM, Howard-Williams JR, Severin CH, Rose AD, Siegel MJ (1985). Pharmacological testing in the laser-induced monkey glaucoma model. Curr Eye Res 4: 775–781.

Matias I, Chen J, De Petrocellis L, Bisogno T, Ligresti A, Fezza F *et al*. (2004). Prostaglandin ethanolamides (prostamides): *in vitro* pharmacology and metabolism. J Pharmacol Exp Ther 309: 745–757.

McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. Br J Pharmacol 172: 3189–3193.

Nataraj C, Thomas DW, Tilley SL, Nguyen MT, Mannon R, Koller BH *et al*. (2001). Receptor for prostaglandin E_2 that regulate cellular immune responses in the mouse. J Clin Invest 108: 1229–1235.

Nials AT, Vardey CJ, Denyer LH, Thomas M, Sparrow SJ, Shepherd GD *et al*. (1993). AH 13205, a selective prostanoid EP2-receptor agonist. Cardiovasc Drug Rev 11: 165–179.

Nilsson SFE, Drecoll E, Lütjen-Drecoll E, Toris CB, Krauss AH-P *et al*. (2006). The prostaglandin EP_2 receptor agonist butaprost increases uveoscleral outflow in the cynomolgus monkey. Invest Ophthalmol Vis Sci 47: 4042–4049.

Okano M, Sugata Y, Fujiwara T, Matsumoto R, Nishibori M, Shimizu K *et al*. (2006). E prostanoid 2 (EP_2)/EP_4-mediated suppression of antigen specific human T-cell responses by prostaglandin E_2. Immunology 118: 343–352.

Paralkar VM, Borovecki F, Ze HZ, Cameron KO Lefker B *et al*. (2003). An EP2 receptor-selective prostaglandin E_2 agonist induces bone healing. Proc Nat Acad Sci 100: 6736–6740.

Prassana G, Carreiro S, Anderson S, Gukasyan H, Sartnurak S *et al*. (2011). Effect of PF-04217329 a prodrug of a selective prostaglandin EP(2) agonist on intraocular pressure in preclinical models of glaucoma. Exp Eye Res 93: 256–264.

Schachar RA, Raber S, Courtney R, Zhang M (2011). A phase 2, randomized, dose-response trial of taprenepag isopropyl (PF-04217329) versus latanoprost 0.005% in open-angle glaucoma and ocular hypertension. Curr Eye Res 36: 809–817.

Senior J, Marshall K, Sangha R, Clayton JK (1993). *In vitro* characterization of prostanoid receptors on human myometrium at term pregnancy. Br J Pharmacol 108: 501–506.

Stjernschantz J, Selén G, Sjöquist B, Resul B (1995). Preclinical pharmacology of latanoprost, a phenyl-substituted PGF2 alpha analogue. Adv Prostaglandin Thromboxane Leukot Res 23: 513–518.

Toris CB, Gelfman C, Whitlock A, Sponsel WE, Rowe-Rendleman CL (2017). Making basic science studies in glaucoma more clinically relevant: the need for consensus. J Ocul Pharmacol Ther 33: 501–518.

Vassiliou E, Jing H, Ganea D (2003). Prostaglandin E_2 inhibits TNF production in murine bone marrow-derived dendritic cells. Cell Immunol 223: 120–132.

Walker W, Rotondo D (2004). Prostaglandin E_2 is a potent regulation of interleukin-12-and interleukin-18-induced natural killer cell interferon-γ synthesis. Immunology 111: 298–305.

Wang JW, Woodward DF, Martos JL, Cornell CL, Carling WR *et al*. (2016). Multi-targetting of selected prostanoid receptors yields agents with enhanced anti-inflammatory activity. FASEB J 30: 394–404.

Woodward DF, Bogardus AM, Donello J, Fairbairn CE, Gil DW*et al*. (1995). Molecular characterization and ocular pharmacology of the prostanoid EP_2 receptor. J Ocular Pharmacol 11: 447–454.

Woodward DF, Jones RL, Narumiya S (2011). Classification of prostanoid receptors, updating 15 years of progress. Pharmacol Rev 63: 471–538.

Woodward DF, Krauss AH-P, Wang JW, Protzman CE, Nieves AL *et al*. (2007). Identification of an antagonist that selectively blocks the activity of prostamides (prostaglandin-ethanolamides) in the feline iris. Br J Pharmacol 150: 342–352.

Woodward DF, Protzman CE, Williams LS, Krauss AH-P (1993). Identification of 19 (R)-OH prostaglandin E_2 as a selective EP_2-receptor agonist. PGs 46: 371–383.

Woodward DF, Wenthur SL, Rudebush TL, Shan F, Toris CB (2016). Prostanoid receptor effects on intraocular pressure, supported by ocular biodisposition experiments. J OcularPharmacol Ther 32: 606–622.

Woodward DF, Krauss AH-P, Chen J, Liang Y, Li C *et al*. (2003). Pharmacological characterization of a novel anti-glaucoma agent, Bimatoprost (AGN 192024). J Pharmacol Exp Ther 305: 772–785.

Yanochko GM, Affolter T, Eighmy JJ, Evans MG, Khoh-Reiter S *et al*. (2014). Investigation of ocular events associated with taprenepag isopropyl, a topical EP2 agonist in development for treatment of glaucoma. J Ocular. Pharmacol Ther 30: 429–439.

Zaslona Z, Okunishi K, Bourdonnay E, Domingo-Gonzalez R, Moore BB, Lukacs NW*et al*. (2014). Prostaglandin E2 suppresses allergic sensitization and lung inflammation by targeting the E prostanoid 2 receptor on T cells. J Allergy Clin Immunol 133: 379–387.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

https://doi.org/10.1111/bph.14525

Table S1 Summary results.
Table S2 Summary results.