RESEARCH PAPER

The affinity, intrinsic activity and selectivity of a structurally novel EP₂ receptor agonist at human prostanoid receptors

Correspondence Dr David F Woodward, JeniVision Inc., 5151 California Ave, Irvine, CA 92617, USA. E-mail: david.woodward@imperial.ac.uk

Received 16 December 2017; Revised 14 September 2018; Accepted 26 September 2018

R A Coleman¹, A J Woodrooffe¹, K L Clark¹*, C B Toris²†, S Fan², J W Wang³ and D F Woodward³

¹Asterand Bioscience, Royston, UK, ²University of Nebraska Medical Center, Omaha, NE USA, and ³JeniVision Inc., Irvine, CA USA

*Current addresses: KL Clark, GlaxoSmithKline Medicines Research Centre, Stevenage, UK.
†Current addresses: CB Toris, Department of Ophthalmology and Visual Science, Case Western Reserve University, Cleveland, OH, USA.

BACKGROUND AND PURPOSE
Prostanoid EP₂ 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 EP₂ receptor agonist, PGN-9856, and its therapeutic potential.

EXPERIMENTAL APPROACH
The pharmacology of a series of non-prostanoid EP₂ 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 EP₂ receptors. In addition to high affinity binding, it was a potent and full EP₂ receptor agonist with a high level of selectivity at EP₁, EP₃, EP₄, DP, FP, IP and TP receptors. In cells overexpressing human recombinant EP₂ receptors, PGN-9856 displayed a potency (pEC₅₀ ≥ 8.5) and a maximal response (increase in cAMP) comparable to that of the endogenous agonist PGE₂. 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 EP₂ receptor agonist properties.

CONCLUSIONS AND IMPLICATIONS
PGN-9856 is a potent, selective and efficacious prostanoid EP₂ 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
Introduction

The actions of the five naturally occurring prostanoid metabolites of arachidonic acid, namely, PGD\(_2\), PGE\(_2\), PGF\(_{2\alpha}\), prosta-cyclin (PGI\(_2\)) and Tx\(_{A2}\), 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 pharmaco logically 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\(_1\)–EP\(_4\), 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 confluence, scraped from culture flasks and centrifuged (800g 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 000g 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\(_3\) or EP\(_4\) receptors were incubated in Millipore Multiscreen\textsubscript{HTS}-HV (0.45 μm) plates (EMD Millipore, Billerica, MA, USA) containing assay buffer, radiolabelled \([\text{H}]\text{PGF}_2\alpha\) 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 PGF\(_{2\alpha}\). Bound and free radiolabel was separated by vacuum manifold filtration using appropriate wash buffers, and bound radiolabel was
determined by scintillation counting. Constituents of each of the buffers are detailed in Table 1. The affinity or pKi of each compound for each receptor was calculated from the concentration causing 50% radioligand displacement (IC50) using the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

\[ K_i = \frac{IC_{50}}{1 + \left( \frac{[\text{H}]PGE_2}{K_0} \right)} \]

### Radioligand displacement binding at prostanoid EP1 receptors

Membranes (protein 250 μg per well) expressing EP1 receptors were incubated in deep-well, 96-well plates containing assay buffer (10 mM Tris–HCl; 150 mM NaCl, 3 μM Indomethacin, 0.3% BSA), radiolabelled [3H] PGE2, 4 nM and test compounds (0.1 to 10 000 nM) at pH 7.4. Incubations were performed at 37°C for 60 min until equilibrium was reached. Non-specific binding was determined in the presence of 10 μM PGE2. After 60 min incubation at 37°C, the assay was terminated by filtration through a combination of GF-C and GF-B filters. The resulting pellet was resuspended in 5 mM Tris (pH 7.4), and bound radiolabel was determined by scintillation counting. The affinity or pKi of each compound for each receptor was calculated from the concentration causing 50% radioligand displacement (IC50) 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 the human DP1 receptor (Woodward et al., 2007, Wang et al., 2016). For assays, membranes (protein 15 μg per well) expressing the human DP1 receptor were incubated in Millipore Multiscreen™-HTS-HV (0.45 μm) plates containing assay buffer (50 mM Tris, 5 mM MgCl2, 10 μg·mL−1 saponin and 10 mM indomethacin), [3H] PGD2, 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 PGD2. 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 pKi of each compound was calculated from the concentration causing 50% radioligand displacement (IC50) using the Cheng–Prusoff equation as previously described.

### Table 1

Experimental conditions for radioligand displacement binding at recombinant EP2–4 prostanoid receptors

| Receptor | EP2 | EP3 | EP4 |
|----------|-----|-----|-----|
| Protein per well (μg) | 8 | 5 | 8 |
| Final [3H-PGE2] (nM) | 10 mM MES pH 6.0; 10 mM MgCl2; 1 mM EDTA | 10 mM MES pH 6.0; 10 mM MgCl2; 1 mM EDTA, 100 μM GTP-γ-S | 10 mM MES pH 6.0; 10 mM MgCl2; 1 mM EDTA, 3 μM indomethacin |
| Buffer Assay | 10 mM MES pH 6.0; 10 mM MgCl2 | 10 mM MES pH 6.0; 10 mM MgCl2 | 10 mM MES pH 6.0; 10 mM MgCl2 |
| Temperature (°C) | 30 | 30 | 30 |
| Incubation time (min) | 60 | 60 | 60 |

MES, 2-(N-morpholino) ethanesulphonic acid.
transports and 2 kinases. This work was carried by Cerep SA (Celle-Lévescault, Poitiers, France) and is reported as supplementary data.

Assessment of agonist activity at recombinant EP2 and EP4 receptors (cAMP assay)
HEK cells stably expressing human EP2 or EP4 receptors (Wang et al., 2016) were routinely cultured at 37°C in a humidified 5% CO2 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 EP2 cell media, while 200 μg·mL⁻¹ hygromycin was included in the EP4 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 cyclooxygenase inhibitor indomethacin (3 μM). This was allowed to incubate for 1 h before the cells were stimulated with PGE2 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% polyethyleneimine. 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 [³²P]-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 PGE2, 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 x 10⁵ cell per mL, 100 μL per well) in complete medium. The plates were incubated for 24 h at 37°C with 5% CO2 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 x 10⁵ cells per well and pretreated for 1 h at 37°C/5% CO2 with PGE2 (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 RPMI 1640, 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 PGE2, 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% O₂/5% CO₂) Krebs solution at 37°C. The composition of the Krebs solution was as follows: NaCl (118.2 mM), KCl (4.69 mM), MgSO₄.7H₂O (1.18 mM), KH₂PO₄ (1.19 mM), glucose (11.1 mM), NaHCO₃ (25.0 mM), CaCl₂.6H₂O (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 EP₂ 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 (EC₅₀) 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 monocural photocoagulation 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 98561 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 pretreatment 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 9856.

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, PGD2, PGE2, iloprost, carbaprostacyclin, 17-phenyl-PGF2α, 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.

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
Ca2+ signalling (FLIPR assay)
The pEC50 values for PGN 9856, 9858, 9862 and 9863 agonist activity (intracellular Ca2+ release) at human recombinant DP1, EP1, EP2, EP3, EP4, FP, IP and TP receptors are shown in Table 2. All four compounds exhibited a high degree of selectivity for the EP2 receptor, the only measurable off-target activity was for PGN-9862 at the EP4 receptor. The rank order of potency at the EP2 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 pEC50) of compounds for intracellular Ca2+ release following stimulation of human recombinant DP1, EP1-4, FP, IP and TP receptors

| Compound   | DP1 | EP1 | EP2 | EP3 | EP4 | 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 1
Structures of compounds used in these experiments: PGN-9856, PGN-9858, PGN-9862 and PGN-9863.

Figure 2
Concentration-dependent displacement of the binding of radiolabelled PGE2 to human recombinant EP2 receptors by PGN-9856. Data are shown as mean ± SEM for n = 3–4 experiments.
was found to bind with high affinity to the human EP2 receptor expressed in a recombinant cell line (Figure 2). The results (Table 3) also show that the compound exhibits negligible affinity for EP1, EP3, DP and IP receptors and has 180-fold selectivity for EP2 over EP4 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 EP2 receptors

In HEK cells overexpressing the human recombinant EP2 receptor, PGE2 causes a concentration-dependent elevation of cAMP levels (Figure 3). This is a result of EP2 receptor stimulation as no response to PGE2 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 PGE2 (Figure 3). The pEC50 values obtained were 9.9 ± 0.1 and 9.7 ± 0.1 for PGN-9856 and PGE2 respectively.

### Agonist potency at recombinant EP4 receptors (cAMP)

In HEK cells overexpressing human recombinant EP4 receptors, PGE2 causes a concentration-dependent elevation of cAMP levels. This would be a result of EP4 receptor stimulation since no response to PGE2 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 EP4 receptor with a pEC50 of 6.1 ± 0.1, compared to 10.5 ± 0.1 for PGE2 (data not shown).

### Inhibition of TNF-α release from human peripheral blood monocytes

In human peripheral blood monocytes stimulated with LPS, the standard selective EP2 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. PGE2 was also tested in this system and behaved as a full agonist with a pEC50 = 7.5 ± 0.1. The pEC50 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 EP2 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. PGE2 was a full agonist (pEC50 = 7.7, n = 5). The pEC50 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

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**Table 3**

Affinity (pKi) of PGN-9856 for human recombinant EP1-4 and DP receptors and native IP receptors

| Compound | EP2 (n = 3) | EP1 (n = 2) | EP3 (n = 2–3) | EP4 (n = 3) | DP (n = 2) | IP (n = 2) |
|----------|-------------|-------------|--------------|-------------|------------|-----------|
| PGN-9856 | 8.5 ± 0.1   | <5          | <5           | 5.9 ± 0.1   | <5         | <5        |
| PGE2     | 8.2 ± 0.1   | 7.3         | 8.9 ± 0.1    | 8.8 ± 0.1   | PGD2 = 8.6 | Iloprost = 7.8 |

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

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**Figure 3**

Agonist potency (cAMP response) of PGN-9856, PGN-9858, PGN-9862, PGN-9863 and PGE2 at human recombinant EP2 receptors. Data are expressed relative to the response to 3 x 10⁻⁸M PGE2 (set at 100%). Values shown are means ± SEM from 3 individual experiments.
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 pEC50 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 EP2 receptor agonist, butaprost.

**Relaxation of human myometrium**

Electrical field stimulation (EFS) caused frequency-dependent contractions of all myometrial tissues tested. At a submaximal 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 EP2 receptor agonist, butaprost, and a comparable maximum response (Figure 6A). The pEC50 for butaprost was 7.5 ± 0.2, and the % inhibitory response to SNP (100 μM) was 83 ± 5. The pEC50 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-isopropyl 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·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 PGE2 acts primarily via a family of four receptor subtypes, EP1, EP2, EP3 and EP4, 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.
(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’ EP2 receptor ligands. The compound described in this paper appears to meet this need.

The majority of receptor-selective prostanoid EP2 agonists had prostanoid-like structures. Until recently, the first selective agonists at EP2 receptors were AH-13205 (Nials et al., 1993) and butaprost (Gardiner, 1986). These were both structural analogues of PGE2. Neither compound was particularly potent, although both appeared to be selective for the EP2 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 EP2 receptor agonist (Abramovitz et al., 2000). 19-R (OH) PGE2 is unique in being a naturally occurring selective EP2 agonist (Woodward et al., 1993) but exhibits the chemical instability issues inherent in PGE2.ONO-AE1-259 has proven a useful compound in pharmacological characterization of EP2 receptor pharmacology in complex systems (Cao et al., 2002; Clarke et al., 2004; Jones and Chan, 2005) but is still another analogue of PGE2. 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 EP2 agonists only represent a small part of the potential therapeutic spectrum. There are a range of additional possible therapeutic opportunities.

Figure 6
(A) Inhibitory effects of PGN-9856 (n = 3) and the standard EP2 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^-7 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.
The potential therapeutic uses of selective EP<sub>2</sub> agonists also include treatment of bronchoconstriction, pre-term labour, systemic hypertension by stimulating natriuresis, neuroprotection and inflammation (Woodward <em>et al</em>, 2011). Prostanoid EP<sub>2</sub> receptors inhibit T-cell proliferation and regulate antigen-presenting cell function (Nataraj <em>et al</em>, 2001), inhibit TNF-α release from bone marrow-derived dendritic cells (Vassiliou <em>et al</em>, 2003), inhibit major histocompatibility complex class II expression in dendritic cells (Harizi <em>et al</em>, 2003), suppress IFN-α release by NK cells (Walker and Rotondo, 2004), inhibit Th1 and Th2 polarized antigen-specific T-cell responses (Okano <em>et al</em>, 2006) and augment the signalling and function associated with the anti-inflammatory cytokine IL-10 (Cheon <em>et al</em>, 2006). Thus, inhibition of cytokine release was a prominent feature of these present studies with PGN-9856. All the EP<sub>2</sub> 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<sub>2</sub> 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<sub>2α</sub> in the treatment of glaucoma, attention has turned to prostanoid EP<sub>2</sub> receptor agonists. Although effects of AH-13205 (Woodward <em>et al</em>, 1995) on monkey IOP are modest, butaprost (Nilsson <em>et al</em>, 2006) produces an effect greater than that of latanoprost (Stjernschantz <em>et al</em>, 1995) and bimatoprost (Woodward <em>et al</em>, 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<sub>2</sub> receptor agonists with an intended use in ophthalmology have been esterified, such as tarprenepag isopropyl (Prassana <em>et al</em>, 2011; Yanochko <em>et al</em>, 2014) and omidenepag isopropyl (Aihara <em>et al</em>, 2017). In order to determine the anti-glaucoma potential of PGN-9856, its effects were evaluated in the monkey model of glaucoma (Lee <em>et al</em>, 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 <em>et al</em>, 2011), which portends a lack of clinically useful anti-inflammatory activity for CP-533536 and PF-04217329.

It follows that, in order to fulfill the many potential therapeutic applications of an EP<sub>2</sub> 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<sub>2</sub> receptors, comparable to the natural ligand, PGE<sub>2</sub>, but also highly selective with no meaningful activity or affinity for other prostanoid receptors. It exhibited only very modest activity at EP<sub>2</sub> receptors in one functional assay employed, but this was more than 100-fold less than that for EP<sub>2</sub>. PGN-9856’s affinity for EP<sub>2</sub> receptors translated into agonist activity, producing potent, concentration-related elevation of levels of intracellular cAMP in a recombinant expression system. Moreover, its functional pEC<sub>50</sub> in this system, like that of PGE<sub>2</sub>, is approximately an order of magnitude lower than its affinity for the EP<sub>2</sub> 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<sub>2</sub> receptors, (Senior <em>et al</em>, 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<sub>2</sub> 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<sub>2</sub> receptor lead optimization programme, emerged as a potent, selective and specific EP<sub>2</sub> receptor ligand, which acts as a full agonist at human EP<sub>2</sub> 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 pneumatonometry. 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 pre-clinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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**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.