Pharmacology of the single isomer, esuberaprost (beraprost-314*d*) on pulmonary vascular tone, IP receptors and human smooth muscle proliferation in pulmonary hypertension

Running title: Pharmacological profile of esuberaprost (beraprost-314*d*)

Lei Shen, PhD[1], Jigisha A. Patel, PhD[1], Xavier Norel, PhD[2], Shahin Moledina, MD[3], Brendan J. Whittle, PhD, DSc[4], Kirby von Kessler[5], Prakash Sista, PhD[5], and Lucie H. Clapp, PhD[1]

[1]Institute of Cardiovascular Science, University College London, London, UK

[2] INSERM U1148, CHU X. Bichat, 75877 Paris Cedex 18, France

[3] Paediatric Cardiology, Great Ormond Street Hospital, London

[4]William Harvey Research Institute, Barts and the London School of Medicine, Queen Mary University of London, London, UK

[5]Lung Biotechnology, PBC, Silver Spring, MD, USA

Corresponding Author:

Professor Lucie Clapp, Institute of Cardiovascular Science, University College London, 5 University Street, London WC1E 6JF, UK

Phone: +44 (0)20 7679 6180

e.mail: l.clapp@ucl.ac.uk

Declaration of Interest Statement:

K. Von Kessler and P. Sista are employees of Lung Biotechnology, PBC (USA). L.H.C., X.N and B.J.W have acted as consultants for United Therapeutics, the parent company of Lung Biotechnology, PBC. Authors have no other financial interests to declare.

Category: Cardiovascular Pharmacology

Abstract

Background and Purpose: Beraprost is a prostacyclin analogue and IP receptor agonist which is approved to treat pulmonary arterial hypertension (PAH) in Asia. The beraprost-314*d* isomer (esuberaprost) is one of four stereoisomers contained within the racemic mixture of beraprost. The pharmacological profile of esuberaprost is now evaluated to determine how stereoisomer separation affects its potency and mode of action in functional assays.

Experimental Approach: Vascular tone was assessed using wire myography in rat and human distal pulmonary arteries (PAs) pre-contracted with U46619 (100nM). HEK-293 cells stably expressing the human IP receptor (HEK-293-IP) and pulmonary arterial smooth muscle cells (PASMCs) derived from PAH patients were used to assess cyclic AMP (cAMP) generation and cell proliferation, respectively.

Key Results: Esuberaprost relaxed rat PAs with a 5-fold greater potency compared with beraprost, and effects were strongly inhibited by RO3244794 (IP receptor antagonist) or L-NAME (NO synthase inhibitor). Esuberaprost caused EP_3 receptor-dependent vasoconstriction at high concentrations ≥1000nM, but contractions were 50% lower compared to beraprost. In HEK-293-IP cells, esuberaprost was 26-fold more potent (EC_{50} 0.4 nM) at increasing cAMP than beraprost. In human PASMCs, esuberaprost was 40-fold more potent than beraprost at inhibiting cell proliferation (EC_{50} 3nM *versus* 120nM), contrasting the 5-fold potency difference for cAMP elevation. Antiproliferative effects of esuberaprost appeared more dependent on NO than on the IP receptor. In PAs from patients with pulmonary hypertension, esuberaprost, caused some relaxation whereas beraprost instead produced a weak contraction.

Conclusions and Implications: Stereoisomer separation of beraprost has a significant effect on the pharmacology of the individual isomer, esuberaprost, identified in *vitro* as a highly potent prostanoid IP receptor agonist.

Key Words: vascular tone; smooth muscle cell proliferation; cyclic AMP; beraprost optical isomers; prostanoid IP and EP_3 receptors; pulmonary hypertension

1. Introduction

The pulmonary pharmacology of the prostacyclins is an area of considerable interest because of the widespread use of prostacyclin (epoprostenol) and its stable mimetics in the treatment of lung and other cardiovascular diseases, particularly pulmonary arterial hypertension (PAH) [1]. Prostacyclin analogues, iloprost (inhaled, *i.v.*) and treprostinil (*s.c.*, *i.v.*, inhaled, oral) are extensively used in the treatment of PAH, a highly proliferative, vascular remodelling disease eventually leading to right heart failure and death [2]. Moreover, an oral formulation of the stable prostacyclin analogue, beraprost has been approved for use in primary pulmonary hypertension since 1999 in Asian countries such as Japan, Korea and Indonesia [2].

Beraprost is a racemic mixture consisting of equal amounts of four stereoisomers: beraprost-314*d*, -314*l*, -315*d*, and -315*l*. Studies *in vitro* identified esuberaprost (beraprost-314*d*) as the most pharmacologically active isomer, with the relative binding affinity of the four isomers varying around 100 fold at a prostacyclin displaceable site in human platelets [3].

Prostacyclin and its mimetics are potent activators of the prostanoid IP receptor, a G_s-coupled receptor linked to cyclic AMP (cAMP) elevation, vasorelaxation, inhibition of platelet aggregation as well as to inhibition of smooth muscle cell proliferation [4-7]. However, prostacyclin and certain analogues can activate multiple prostanoid receptors at concentrations achieved at clinical doses, including vasodilator EP_2 and DP_1 receptors (treprostinil) as well as contractile prostanoid EP_1 (iloprost) and EP_3 (prostacyclin) receptors

[1,7-9]. EP_1 and EP_3 receptors, because they increase calcium and/or are negatively coupled to cAMP production through the inhibitory G-protein, G_i, they may counteract the actions of prostacyclin and its analogues acting on IP, EP_2 and DP_1 receptors in functional assays [1]. Beraprost has a low affinity (K_i >3 µM) for the above-mentioned EP_1, EP_2 and DP_1 prostanoid receptors, with the exception of EP_3 receptors, where the K_i for binding to the human receptor is 680 nM [10]. This contrasts with selexipag, a novel non-prostanoid and IP receptor agonist pro-drug, recently approved for the treatment of PAH [11], where the IP receptor is considered the only significant biological target for its active metabolite, MRE-269 [7,10,12].

Previous work has shown significant dependency on endothelial-derived nitric oxide (NO) for beraprost- and treprostinil-induced relaxation of rat pulmonary arteries *in vitro* [13,14]. In contrast, relaxation to iloprost and the selexipag metabolite, MRE-269 is relatively insensitive to removal of the endothelium in rat and human pulmonary artery, suggesting a differential mechanism of action of these two prostacyclin mimetics compared to beraprost and treprostinil [13,14].

To characterise more fully the pharmacological profile of the single isomer, esuberaprost, we assessed this in terms of functional responses mediated through prostanoid IP and EP_3 receptors. The dependence on NO in mediating functional effects in distal rat pulmonary arteries and in human cultured pulmonary arterial smooth muscle cells (PASMCs) derived

from PAH patients was subsequently evaluated. Here it is demonstrated that esuberaprost behaved as a highly potent agonist at stimulating cAMP, causing vasorelaxation in rat and inhibiting human vascular cell proliferation, and in some assays, was more potent compared with the raecemic mixture, beraprost than could be predicted from a "single active isomer" hypothesis. This may result in part from the pharmacological effects of other isomer(s) contained in the beraprost mixture thwarting its activity.

2. Materials and Methods

2.1 Materials

Beraprost sodium and esuberaprost (potassium salt) were provided by Lung Biotechnology (Silver Spring, MD, USA) and treprostinil by United Therapeutics Corp. (Silver Spring, MD, USA). Iloprost (50:50 R/S isomer; CAY 18215) and RO1138452 (CAY 10441) were purchased from Cambridge Bioscience Ltd (Cambridge, UK). U46619 was purchased from Enzo life Sciences (Exeter, UK) and L798, 106 (EP_3 antagonist) was purchased from Tocris (Bristol, UK). L-NAME were purchased from Sigma Aldrich (Poole, Dorset, UK). R-3-(4-fluoro-phenyl)-2-[5-(4-fluoro-phenyl)-benzofuran-2-ylmethoxycarbonylamino]-propion-ic acid (RO3244794) was a gift from Roche (Palo Alto, CA, USA). Stock solutions were made up in either distilled water (phenylephrine, acetylcholine, beraprost drugs, L-NAME) or DMSO (treprostinil, RO3244794, RO1138452, L798,106).

2.2 Experimental Methodology

2.2.1 Group sizes

For all experiments, the number of observations (group size) is provided in the figure legends, with a minimum of 5 independent observations performed in animals, patient samples (cell isolates and arteries) or in HEK-293 cells stably expressing the IP receptor. For cAMP, PCR and cell proliferation assays, measurements were performed in duplicate, triplicate or quintuplicate, respectively and an average taken in each cell isolate to calculate the final mean data.

2.2.2 Randomisation

Healthy adult female Sprague Dawley rats were in bred at UCL, London UK and were randomly assigned to the experimenter based on reaching the appropriate size (ranging 250-300 g). The pharmacological protocol was pre-determined before mounting the pulmonary arteries in each myograph. For cell culture experiments, plates were randomly assigned to drug treatment prior to any measurements. Pharmacological comparisons of esuberaprost and beraprost, were made in the same cell isolates at the same passage number (human PASMCs or HEK-293 cells) and where possible, in blood vessels taken from the same animals or patients.

2.2.3 Blinding

Experimental blinding was not used for this study as there was one core experimenter

responsible for each of the protocols described, where individuals also performed the subsequent analysis. In order to limit experimental bias, analysis was not routinely performed until experimental data set was complete.

2.2.4 Normalisation

Relaxation responses in distal pulmonary arteries were expressed as a percentage of the contractile response induced by the thromboxane mimetic, U46619, measured just before the addition of the lowest concentration of the vasorelaxant agonist. These were expressed as mean percentages of the constriction ± standard error of the mean (S.E.M) consistent with previously published data [6,15,16]. This allowed for comparison of agonist responses independent of size of vessel or contraction.

To control for variable growth rates in different PAH patient cell lines, agonist responses were normalised to % change in cell proliferation relative to the growth response induced by serum alone (100%) as previously described [5,7]. Likewise, cAMP levels were normalized to the peak response induced by either beraprost or esuberaprost in each PAH cell isolate. In all previous studies from the Clapp laboratory, phosphodiesterase (PDE) activity has not been inhibited prior to extraction so as to assess receptor activation under "quasi" physiological/therapeutic conditions [5,7,17]. Such an approach will inherently give rise to a greater variation in cAMP levels, particularly in PAH cells, where PDE activity is high [1].

2.2.5 Ethical statement

All animal care and experimental procedures were approved by the institution's Animal Welfare and Ethical Review Body of UCL and is in compliance with the guidelines of Animal Research: Reporting of In Vivo Experiments (ARRIVE). Human lung tissue, from which pulmonary arteries were isolated and smooth muscle cell lines derived, were obtained after patient or relative consent with Ethics Committee approval from Great Ormond Street Hospital (ICH and GOSH REC 05/Q0508/45) and the Assistance Public - Hôpitaux de Paris (Institutional Review Board IRB00006477, agreement No. 11-045) as previously described [7].

2.3 Animals

The standard animal housing and care was in the central biological service unit at UCL; all rats were housed in climate-controlled conditions with a 12-h light/dark cycle and had free access to normal pelleted rat chow and drinking water. On the day of experiment, healthy female Sprague–Dawley rats (20 weeks, 250-300g) were sacrificed by cervical dislocation followed by removal of the lung. The procedure is in compliance with the guidelines of Animal Research: Reporting of In Vivo Experiments (ARRIVE) as described previously [6]. The lung tissue was then placed in physiological salt solution (PSS) containing in mM the following: 112 NaCl, 5, KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 0.5 KH_2PO_3, 0.5 NaH_2PO_3, and 10 glucose (gassed with 95% O_2/5% CO_2 to pH 7.4). Third order (or lower for human) pulmonary arterial branches were cleaned of connective tissue and cut into segments (2 mm in

length) for mounting in a wire myograph.

2.4 HEK-293-IP cell lines and culture

A HEK-293 cell line stably expressing the human IP receptor gene (HEK-293-IP) has been generated as previously described [18]. Cells were maintained in minimal essential medium (MEM) containing Earle's salts and L-glutamine (Life Technologies, Paisley, UK) supplemented with 9% foetal bovine serum (FBS; Gibco, Life Technologies, Paisley, UK), 1% penicillin-streptomycin (1000 units/ml stock; Life Technologies, Paisley, UK) and Zeocin (400 μg/ml; Life Technologies, Paisley, UK) to maintain stable IP receptor expression. Zeocin was not present in the culture media for actual experiments.

2.5 Source of lung tissue from patients with pulmonary hypertension (PH)

Lung samples were obtained from patients diagnosed with group 1 (PAH; n=10) and group 3 PH (PH associated with lung diseases; n=1) who had undergone surgery for lung transplantation. Patients had a mean age of 13.3 ± 3.3 yrs and a mean pulmonary artery pressure of 64.6 ±5.2 mm Hg (range 38-94 mm Hg) prior to transplantation.

2.6 Isolation and culture of PASMCs from patients with PAH

Primary cell lines of distal PASMCs (n=7) from PAH patients were derived by enzymatic dissociation as previously described [5,7]. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in human smooth muscle basal medium-2 (SMBM; Lonza, Slough,

UK) supplemented with 9% FBS (Life Technologies, Paisley, UK) and 1% penicillin/streptomycin (Life Technologies, UK). After reaching confluence, cells were washed with PBS (Life Technologies, Paisley, UK) and treated with 0.05% trypsin-EDTA (Life Technologies, Paisley, UK) for further passage. Cells isolated and cultured in this way displayed classic “hill and valley” morphology, and a high percentage of cells expressed known smooth muscle cell markers [7].

2.7 Experimental Protocols

2.7.1 Small vessel myography

PAs were mounted on wires in an isometric myograph (500A JP Trading, Denmark). Vessels were continuously aerated (95% O_2, 5% CO_2) at 37 ^{O}C in PSS and pre-tensioned to an equivalent of ~22 mmHg (3K Pascal). The normalised luminal diameter of each segment was obtained as described previously [6,19], averaging 339 ± 20 µm (n=39) and 273 ± 33 µm (n=22) when pressurised in rat and human arteries, respectively. An equilibration period of at least 1 hr was allowed during which time tissues were contracted with a single application of the thromboxane A_2 mimetic, U46619 (50 nM) followed by 2-3 washes. Pulmonary arteries were then contracted with 100 nM U46619 and the presence of functional endothelium was assessed by examining responses to the endothelium-dependent vasorelaxant acetylcholine (10 µM), which gave on average a 60% relaxation of the contractions induced by U46619 in rat PAs but only 6% in human PAs obtained from patients with PH.

Contractions were allowed to plateau, before cumulative concentration–response curves (1–30,000 nM) were constructed for the two prostacyclin analogues, beraprost and esuberaprost in the absence and presence of an IP receptor inhibitor, EP_3 receptor inhibitor or the nitric oxide synthase (NOS) inhibitor, Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), which were added at least 60 mins before the addition of either U46619 or phenylephrine.

2.7.2 Real-time quantitative PCR (RT-qPCR)

RNA was extracted from cultured HEK-293-IP and cDNA synthesised. The primer set of human PGTIR (NM_000960; IP receptor), PTGER1 (NM_000955; EP_1 receptor) PTGER2 (NM_000956; EP_2 receptor), PTGER3 (NM_000957; EP_3 receptor), PTGER4 (NM_000958; EP_4 receptor), PGTDR (NM_000953; DP_1 receptor), and the reference gene, β-actin (NM_001101) were purchased from Qiagen (Manchester, UK). Real-time quantitative PCR was performed using an automated thermal cycler (ABI Prism 7900HT; Applied Biosystems, Basingstoke, UK). RT-qPCR was used to determine the relative expression of different prostanoid receptors using a modified method [20]. PCR cycle was 50 ^{o}C for 2 mins, 95 ^{o}C for 15 mins, followed by 40 cycles at 94 ^{o}C for 15 seconds, 56 ^{o}C for 30 seconds and 76^{o}C for 30 seconds The relative amount of cDNA was calculated using the ‘2–[delta] CT threshold cycle’ method [21].

2.7.3 Cyclic AMP measurement

Human PASMCs or HEK 293-IP cells were grown to 70-80% confluence in either MEM

(HEK-293-IP) or DMEM/F12 (Gibco, Loughborough, UK, PASMCs) supplemented with 9% FBS and penicillin (100 U/ml)/streptomycin (100 µg/ml; Life Technologies, Paisley, UK). Cells were stimulated for 15 mins after treatment with various agonists acting on the IP receptor and cAMP measured using a competitive enzyme immunoassay kit (ADI-900-163, Enzo Life Sciences, Exeter, UK) as previously described [7]. The protein concentration in the cell lysate was determined using a Bradford based protein assay (BCA; Novagen, EmD Chemicals, San Diego, CA, USA).

2.7.4 Cell proliferation assays

Human PASMCs were grown in human smooth muscle basal medium-2 (SMBM; Lonza, Slough, UK) containing 9% FBS for 24 hr and the media then switched to one containing no FBS for 48 hr to growth-arrest cells. Cells were then incubated in SMBM containing 9% FBS with and without the test agent (s) for 4 days. Cell proliferation was assessed using an MTS (Promega, Southampton, UK) assay kit as previously described [7]. Cells between passages 2 and 9 were used for experiments.

2.8 Data & Statistical analysis

Data are presented as mean ± standard error of mean (S.E.M.) of n observations. Agonist log-concentration curves were constructed and fitted using the non-linear fitting routine in GraphPad Prism 7 (San Diego, CA, USA). To obtain best fit of data, a single or double sigmoidal function was used where the slope was not usually constrained unless the fitting

routine required this to converge. The concentration of agonist causing 50% of the maximal response (E_{Max}) was expressed as the negative logarithm of the EC_{50} value (pEC_{50}) and extrapolated from the fits of individual data sets, along with E_{Max}. Statistical analysis was performed in GraphPad Prism 7 using a non-paired Student t-test or ANOVA with post-hoc correction as indicated in the figure or table legends. P values < 0.05 were considered statistically significant.

3. Results

3.1 Comparison of esuberaprost with beraprost in rat pulmonary arteries

Distal pulmonary arteries were contracted with 100 nM of the thromboxane A_2 mimetic, U46619, which unlike phenylephrine, is one of the few agents to strongly contract these small pulmonary arteries [16]. Both beraprost and esuberaprost produced concentration-dependent relaxation of pre-contracted rat pulmonary arteries over the concentration range tested (Figure 1A). Contractions to U46619 were not significantly different between the two groups, being 3.2±0.4 and 3.4±0.5 mN (n=35) in all experiments for esuberaprost and beraprost, respectively. There were two components to the relaxation curve induced by these two agents (Figure 1B), with esuberaprost being significantly more potent (5.4-fold) and efficacious at activating the first component compared to beraprost (EC_{50} 33 versus 181 nM). However, both appeared equipotent at activating the second component of the relaxation curve (EC_{50} ~12-13 μM), although E_{Max} was greater for esuberaprost (88% versus 78%).

The IP receptor antagonist, RO3244794 (1 μM) abolished the first phase of relaxation to both beraprost (Figure 2A) and esuberaprost (Figure 2B) over the concentration range of 1-3000 nM. In contrast, RO3244794 did not inhibit the second component of relaxation to either agent, with the EC_{50} for beraprost and esuberaprost, being 17.2 μM and 10.6 μM, respectively in the presence of the IP receptor antagonist.

The role of NO in mediating relaxation to the two drugs was assessed by examining the effects of esuberaprost and beraprost in the absence or presence of L-NAME (100 μM), a cell-permeable NO synthase (NOS) inhibitor which has been used previously in similar vascular preparations *in vitro* at this concentration [14]. In rat pulmonary arteries pre-contracted with U46619 (100 nM), L-NAME inhibited the relaxation to beraprost over the entire concentration range tested (Figure 3A). The first phase of the relaxation was substantially reduced (≥80%); there was also a reduction of the second phase of relaxation, albeit to a lesser extent (≤50%). Likewise, for esuberaprost, L-NAME inhibited relaxation over the whole concentration range tested, though overall, the residual relaxation left in the presence of L-NAME was greater compared with beraprost ($P<0.001$, 2 Way ANOVA).

Phenylephrine (PE) has previously been shown to synergise with and enhance the contractile action of agonists capable of activating EP_3 receptors [22,23]. Thus, contractile responses to beraprost and esuberaprost were observed in rat pulmonary arteries primed with 100 nM PE (Figure 4A), with mean concentration-response curves shown (Figure 4B). The estimated

EC_{50} values for contraction induced by both drugs were similarly high, being 4.6 µM and 4.7 µM (n=10) for beraprost and esuberaprost, respectively, although contractions (normalised to unit length) were significantly greater for beraprost, being 2-fold greater at 10 µM. The involvement of the EP_3 receptor was studied using L-798,106, a potent and highly selective prostanoid EP_3 receptor antagonist with a 3000-fold selectivity over other prostanoid receptors [24]. Contractions to both beraprost and esuberaprost at these high concentrations in small pulmonary arteries were substantially reduced by the EP_3 receptor antagonist, L-798,106 (1 µM), which inhibited the contractions by 57% and 70%, respectively.

3.2 Cyclic AMP elevation in HEK-IP and in human PASMCs

In previous work, HEK-293 cells stably expressing the human IP receptor have been used to assess the potency and efficacy of IP receptor agonists on cAMP generation [5,9,18]. In the present study, RT-qPCR was used to confirm stable expression of IP receptors in HEK-293 cells and also to estimate its relative expression compared to other prostanoid receptors. Data shown in Figure 5A confirm that the IP receptor is expressed at levels >280 fold compared with other prostanoid receptors in these HEK-293-IP cells. The magnitude of cAMP elevation in cells stimulated for 15 mins in the absence and presence of either beraprost or esuberaprost over a wide concentration range (0.001 to 1000 nM) was then compared (Figure 5B). Both agents increased cAMP levels in a concentration-dependant manner which peaked at 10 nM for esuberaprost and 100 nM for beraprost. Esuberaprost was 26-fold ($P<0.001$; n=10-12) more potent than beraprost at cAMP generation in HEK-293-IP cells, with EC_{50} values being

0.4 nM versus 10.4 nM, respectively. Both esuberaprost and beraprost gave similar E_{Max} values (Table 1).

In other studies where the activity of two other prostacyclin analogues were similarly evaluated, esuberaprost was >100 fold ($P<0.001$; One-Way ANOVA) more potent (EC_{50} 45 nM; n=8) than treprostinil and significantly ($P<0.05$; One-Way ANOVA) more potent than iloprost (EC_{50} 2.7 nM; n=6; Table 1).

Cyclic AMP generation induced by beraprost and esuberaprost in human PASMCs derived from PAH patients was assessed over the concentration range from 0.1 nM to 10 µM. A 15 mins treatment period was used as this was the time-point used in HEK-293-IP cells, but also the time- point when cAMP induced by beraprost was previously found to be maximal in normal human PASMCs [17]. Data has been normalised to total cell protein and to the maximal cAMP response obtained at either 1 µM or 10µM of either agent (Figure 5C). Both esuberaprost and beraprost elevated cAMP in a concentration-dependent manner, with the EC_{50} being 4.6-fold lower for esuberaprost compared to beraprost (183 nM and 845 nM respectively); however, E_{Max} was comparable.

3.3 Antiproliferative effects in human PASMCs from PAH patients

Antiproliferative effects of the two drugs were assessed in human PASMCs derived from PAH patients, with all data collected and compared using the same patient cell isolates (n=5). Cells

were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations (0.001-10,000nM) of beraprost or esuberaprost (Figure 6A-C). The results indicate that the lowest dose of esuberaprost to cause significant inhibition was 1 nM compared to 10 nM with beraprost. In addition, the inhibition of proliferation at the highest concentration tested (10 μM) was greater with esuberaprost, being 61% as opposed to 43% (Figure 6B). Extrapolated EC_{50} values showed that esuberaprost is 36-fold more potent than beraprost in preventing cell proliferation, having an EC_{50} of 3 nM compared to 120 nM for beraprost.

To determine the role of the IP receptor, responses to esuberaprost were assessed in the absence and presence of the IP receptor-antagonist RO1138452 at 1 μM [25]. In previous studies, 1 μM RO1138482 has been shown to fully inhibit the antiproliferative effects of the selective IP receptor agonist, MRE-269 [7] and is the concentration required to maximally inhibit the IP receptor-dependent relaxation to prostacyclin analogues in rat and human arteries [6,15,26]. This antagonist shifted the concentration-response curve of esuberaprost to the right, changing the EC_{50} from 3.6 nM to 187 nM, although E_{Max} remained unchanged (Figure 6D).

The antiproliferative effects of esuberaprost were significantly ($P<0.01$) reduced over a wide concentration range (from 0.1 to 10,000 nM) in the presence of the NOS inhibitor, L-NAME (100 μM), which reduced E_{Max} from 49.7 ± 7.8% to 12.2 ± 4.8% (n=5).

3.4 Vasorelaxant effects in distal human pulmonary arteries from PH patients

Distal human pulmonary arteries obtained from PH patients were pre-contracted with U46619 (100 nM), which induced contractions of 1.2±0.1 mN and 1.1±0.3 mN (n=11) in the esuberaprost and beraprost groups, respectively (Figure 7A). Esuberaprost produced relaxation of arteries, the magnitude of which was substantially smaller than that observed in rat PAs, with relaxation only reaching 19% at 10 µM (Figure 7B). Overall, beraprost failed to cause relaxation at any concentration tested (1-30,000 nM) and instead caused a weak contraction (Figure 7A), with responses being significantly different from esuberaprost ($P<0.05$).

4. Discussion

In the current study, we evaluated the *in vitro* functional effects of esuberaprost and compared its pharmacological activity to beraprost, which is a racemic mixture comprised of equal amounts of 4 stereoisomers. Consistent with the notion of a predominant pharmacologically active isomer, esuberaprost behaved as a more potent agonist than beraprost in all functional assays.

In some assays however, esuberaprost had more than the anticipated increase in potency compared to beraprost. Moreover, in cAMP assays in HEK-293-IP cells, esuberaprost was found to be the most potent agonist on IP receptors than any of the currently approved prostacyclin analogues. It was also 36 fold more potent than beraprost as an anti-proliferative

agent in distal human PASMCs from PAH patients. Both esuberaprost and beraprost relaxed distal rat pulmonary arteries in an IP receptor- and NO-dependent manner, whereas the antiproliferative effects of esuberaprost in PAH cells were less dependent on the IP receptor but were more strongly dependent on the NO pathway. In PAs from PH patients, esuberaprost, caused relaxation whereas beraprost did not.

4.1 Activity of esuberaprost and beraprost

The observation that esuberaprost had high IP receptor activity in HEK-293-IP cells, some 26-fold higher than beraprost, was unexpected. One explanation is that the single isomers interact with additional cellular targets but with differential potencies and/or efficacy, and in the absence of the remaining 3 isomers, the true potency of esuberaprost is unmasked. Indeed, evidence from the current study suggests that esuberaprost activates EP_3 receptors to a lesser extent than beraprost in rat distal pulmonary vessels. Moreover, beraprost caused contraction in PAs from PH patients, suggesting isomer(s) contained in the beraprost mixture, have undesirable pharmacological effects which thwart its activity.

Of note, the binding affinity of esuberaprost in platelets closely approximated that for prostacyclin [3]. Thus the EC_{50} value (0.4 nM) for esuberaprost-induced cAMP generation in HEK-293-IP cells reported in the present study, fits well with the K_i (determined from the K_d) for saturable prostacyclin binding of 2 nM in human platelets [27]. Moreover, the EC_{50} values for cAMP generation calculated in the present work for the prostacyclin analogues, iloprost

(2.7 nM) and treprostinil (45 nM) in HEK-293-IP cells are close to the K_i of these drugs (4 and 32 nM, respectively) documented in binding studies in HEK-293 cells expressing the human IP receptor [9]. Binding studies comparing the K_i of iloprost and prostacyclin at the human IP receptor [1] and prostacyclin with esuberaprost [3], predict that esuberaprost should be slightly more potent than iloprost, as indeed was actually observed in HEK-293-IP cells. With respect to beraprost, a K_i value of either 19 or 38 nM was reported against the rat or human IP receptor, respectively expressed in CHO cells [10]. Thus the rank potency order for analogue-induced IP receptor activation is esuberaprost>iloprost>beraprost>treprostinil, similar to the rank order of analogue-dependent relaxation of proximal human pulmonary arteries for the latter three agents [26].

There are several observations to note from the cAMP generation experiments in PASMCs isolated from patients with PAH. Compared with HEK-293-IP cells, the mean difference in the EC_{50} values between esuberaprost and beraprost was much smaller (4.6-fold). The actual EC_{50} for cAMP generation was significantly higher, being 183 nM and 845 nM for esuberaprost and beraprost compared to 0.4 nM *versus* 10.4 nM in HEK-293-IP cells, respectively. The higher EC_{50} values could reflect an overall higher PDE activity in PAH compared to HEK-293 cells, coupled with a lower expression of IP receptors, which is consistently reported in lung tissue and PASMCs from PAH patients [5,7,28,29]. Beraprost was also able to elevate cAMP in PAH cells, whereas previously this agent was reported to only increase cAMP in human PASMCs from PAH patients in the presence of a broad

spectrum PDE inhibitor [28]. The reason for this discrepancy is not immediately apparent, though an intriguing observation is that both the cAMP elevating and antiproliferative effects of beraprost were greater in mouse PASMCs expressing a BMPRII mutation found in PAH patients compared to wildtype cells [30]. However, our smooth muscle cell lines were not derived from any patients with heritable PAH.

In the vascular cell proliferation studies, not only was esuberaprost significantly more effective over what is likely to be plasma concentration (1-10nM) achieved by the clinical dose range of this drug, but it was 40-fold more potent than beraprost, having an EC_{50} of 3 nM compared to 120 nM. In a previous publication, beraprost inhibited cell proliferation in normal human PASMCs with an EC_{50} of 40 nM [17]. This reduced potency seen in PAH-diseased cells in the current work with beraprost may again point to a lower IP receptor expression and/or high PDE activity in PASMCs isolated from PAH patients [7,28]. On the other hand, the EC_{50} for esuberaprost is in a similar range to that previously determined for treprostinil in human PASMCs derived from control [17] and PAH [7] patients (EC_{50} 4 nM and 11 nM, respectively). Thus in the absence of the other 3 sterioisomers, the antiproliferative activity of esuberaprost may be in a similar potency range to that of treprostinil.

4.2 Role of IP receptor

There were two components to the relaxation induced by beraprost and esuberaprost in small

rat pulmonary arteries, with the first phase being abolished by the IP receptor antagonist, RO3244794 while the second phase was insensitive to this agent. Previous experience suggests that 1 μM RO3244794 is sufficient to effectively block IP receptor function even at high agonist concentrations (1-10 μM), so the residual relaxation is likely to reflect an additional target activated, independent of the IP receptor. Indeed under similar experimental conditions, the same IP receptor antagonist abolished the treprostinil-induced relaxation, while only marginally inhibiting responses to iloprost in distal rat pulmonary arteries [6].

The extent to which the IP receptor contributes to the inhibitory effects of beraprost on cell proliferation in human PASMCs has not previously been investigated. Cyclic AMP-dependent pathways have been reported to underlie beraprost inhibition of cell growth in these cells, induced either by hypoxia or transforming growth factor β (TGFβ) or indeed inhibition of cardiac fibroblast proliferation driven by the TGFβ-Smad pathway [30-32]. Our results now show overall weak effects of IP receptor inhibition on the antiproliferative responses to esuberaprost in human cells, which is similar to what was found with treprostinil and iloprost in PASMCs from PAH patients [5,7]. With respect to treprostinil, a predominant role of EP_2 receptors in mediating the antiproliferative effects of this agent was reported in human PASMCs from PAH patients [7]. However, IP receptors do appear to be functional in these diseased patient cells, as MRE-269, the active metabolite of selexipag, inhibited cell proliferation in a fully IP receptor-dependent manner, being completely inhibited by the IP receptor antagonist, RO1138452 [7].

4.3 Nitric oxide is an important mediator of the functional effects of esuberaprost

Previous work has shown a high degree of dependency on the endothelium for beraprost-induced relaxation in both large and small rat pulmonary arteries, with a greater role in smaller arteries reported [13,14]. In our studies, endothelial function (as assessed by acetylcholine) was minimal in human PAs from PH patients, which could explain in part the relatively poor ability of the beraprost isomer, esuberaprost to promote vasorelaxation. In other studies, beraprost induces near maximal relaxation (≥80%) in control human proximal [26] and tertiary intralobar [14] PAs, and can do so even in the absence of the endothelium [14]. It should be noted that the contractile agents used here (U46619) and in these previously published studies (norepinephrine and prostaglandin $F_{2\alpha}$), were different, which can influence prostanoid analogue responses on pulmonary tone [16]. Nonetheless, downregulation and desensitisation of the IP receptor is likely to contribute to reduced efficacy of prostacyclin analogues on pulmonary vascular tone in PH, although other pathways are also likely to be involved [1].

Based on the inhibition of vasorelaxation by L-NAME observed in the present study, suggests that esuberaprost relaxes rat small pulmonary arteries in part via release of NO, as indeed previously reported for beraprost in the same species and tissue [14]. In other studies, however, MRE-269- and iloprost-induced relaxation of rat pulmonary arteries was reported to be insensitive both to the removal of the endothelium as well as to the NOS inhibitor, L-NAME even when used at a concentration of 100 μM [13,14]. Given that this high

concentration of L-NAME was used in the current studies to ensure near maximal NOS inhibition, the data strongly suggest a differential mechanism of action of esuberaprost and beraprost compared to iloprost and MRE-269 in promoting vasorelaxation in rat pulmonary artery.

L-NAME inhibited both components of relaxation induced by esuberaprost and beraprost, findings which suggest an IP receptor dependent and also an IP- independent involvement of endothelial NOS (eNOS) contributing to relaxation. Peroxisome proliferator-activated receptors (PPARs), recognised as cellular targets for prostacyclin and some analogues, can be activated either through direct binding and/or by the IP receptor [1]. IP receptor-dependent activation of PPARβ, resulting in the downstream activation of calcium–activated potassium channels, has been reported to contribute to pulmonary smooth muscle relaxation induced by iloprost, although the mechanism appeared not to involve NO [33]. Direct binding of beraprost to PPARβ has been shown in murine vascular smooth muscle cells [34], although its involvement in promoting vasorelaxation to this analogue is unknown.

The present results show that NO is likely to be a major component of the mechanisms underlying the antiproliferative effects of esuberaprost in PASMCs from PAH patients. The mechanism is likely to be mediated by both IP receptor-dependant and IP receptor-independent pathways, but based on a relatively modest reversal by the IP receptor antagonist, it is concluded that NOS activation is most likely to occur via non-IP receptor mechanisms.

This could involve transcriptional upregulation of either eNOS or iNOS, direct phosphorylation of NOS and/or activation and enhanced expression of PPARβ, all of which have been reported *in vitro* with beraprost [34,35]. With respect to the involvement of PPARs, PPARβ, appears to contribute to the antiproliferative effects of beraprost in murine aortic smooth muscle [34,35] and of treprostinil in fibroblasts [36], while in human PASMCs from PAH patients, PPARγ appears to play a major role in treprostinil effects on cell proliferation [5].

4.4 Role of contractile EP_3 receptors

Many studies have provided evidence that the contractile effects of prostacyclin analogues are mediated through EP_3 receptors [13,15,16,37,38]. In contrast, the active metabolite of selexipag, MRE-269, as a selective IP receptor agonist, appears not to activate EP_3 receptors [13,14]. It is important to understand the role of receptors involved in the contractile response, as these could limit the doses of prostacyclin analogues given therapeutically or potentially give rise to unwanted side effects. The magnitude of esuberaprost contractile responses, being ~50% lower than that of beraprost over a wide concentration-range in the rat, could be considered advantageous in a clinical setting and possibly contribute to the higher potency of esuberaprost compared to beraprost that we observed in the functional assays. Indeed, lower activity at the EP_3 receptor may explain why esuberaprost causes relaxation whereas beraprost produces small contractions in PAs from PH patients. While, EP_3 receptors are not predicted to be substantially activated by beraprost in the therapeutic dose-range in control arteries,

given that significant contractions were only observed at or above 1000 nM, this may not be the case in PH. Indeed, EP_3 receptor agonism is known to be enhanced in an animal model of PAH [38], and inhibition of EP_3 receptors attenuates PAH in rats [39]. This may arise from thromboxane (TP) and/or α_1 receptor-induced synergism with EP_3 receptors, as 'priming' with a contractile agonist at either of these two receptors is reported to markedly increase both the potency and size of contraction to EP_3 agonists [16,23]. Indeed, upregulation of EP_3 receptors was concluded to account for a reduction in beraprost-induced relaxation in monocrotaline-treated rats with PAH [38]. Furthermore, 'priming' of rat pulmonary arteries with the α_1 agonist, phenylephrine was required before any contraction to treprostinil could be observed, albeit $\geq$10μM of the drug [16].

4.5 Summary and Conclusion

Esuberaprost is a highly potent IP receptor agonist, possibly the most potent prostacyclin analogue reported *in vitro* thus far. Its substantially greater antiproliferative profile in human cells compared to beraprost cannot solely be explained by differences in IP receptor potency or cAMP generation, It could be that expression of this antiproliferative activity may have been thwarted by the pharmacological effects of the other 3 other isomers that contribute to the activity seen with beraprost itself. This may also reflect in part, the lower functional activity of esuberaprost at EP_3 receptors, which may assume a greater relevance in PH. Furthermore, the antiproliferative and vasorelaxation effects of esuberaprost in distal PASMCs and arteries show a high dependence on NO, but a variable role for the IP receptor.

In summary, isomer separation of beraprost to yield esuberaprost has significant effects on its pharmacology, which warrants further molecular and pharmacological investigation.

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Author contributions

L.S**.** performed all quantitative PCR, cAMP assays and myography experiments, performed initial data analysis, and prepared the first draft of the paper. J.A.P. derived primary cell lines from PAH patients and performed the cell proliferation assays and associated data analysis. B.J.W., X.N. and S.M. had input in aspects of study methodology and B.J.W the editing of the draft manuscript. K.V.K, P.S. and L.H.C. contributed to study conception and design. L.H.C. further analysed and interpreted the data as well as writing and editing the manuscript. All authors critically reviewed the manuscript for intellectual content and approved the final version.

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	pEC_{50}	EC_{50} (nM)	E_{Max} (pmol cAMP/mg prot)
HEK-293-IP			
Esuberaprost	9.40 ± 0.21 (12)	0.40	715 ± 67 (12)$^{\#}$
Beraprost	7.98 ± 0.15 (10)***	10.35	701 ± 62 (10)$^{\#}$
Iloprost	8.56 ± 0.50 (6)*$^{\#}$	2.73	1273 ± 320 (6)
Treprostinil	7.35 ± 0.29 (8)***	44.94	1323 ± 225 (8)
Human PASMCs			
Esuberaprost	6.74 ± 0.09 (5)	183	799 ± 135 (5)
Beraprost	6.07± 0.07 (5)***	845	909 ± 223 (5)

Table 1. Comparison of the potency and efficacy of prostacyclin analogues on cAMP levels in HEK-293 cells stably expressing the IP receptor (HEK-293-IP) and in human pulmonary arterial smooth muscle cells (PASMCs) from pulmonary artery hypertensive (PAH) patients. The mean log EC_{50} and E_{Max} values have been obtained from sigmoidal (variable slope) fitting of the mean concentration-response data in each drug group. *=P<0.05, ***=P<0.001 when compared to esuberaprost and $^{\#}$=P<0.05 when compared to treprostinil (One way ANOVA with Newman-Keuls for multiple comparisons or t-test in PASMCs).

Figure legends:

Figure 1. Comparison of the vasorelaxant effects of beraprost and esuberaprost in rat distal pulmonary arteries. Rat distal pulmonary arteries were contracted with 100 nM U46619 and contractions allowed to plateau before incremental (1-30,000 nM) doses of beraprost and esuberaprost were added in a cumulative fashion (A). Concentration-relaxation curves for beraprost and esuberaprost where data have been expressed as % (mean ± SEM, n=12-15) of the contractile response induced by U46619, the magnitude of which was determined just before the addition of the lowest dose of either prostanoid and taken as 100% (B). Data were analysed for best fit with a double sigmoid function generated in Prism (GraphPad software). *=P<0.05, **=P<0.01 or ***=P<0.001, 2-way ANOVA with Bonferroni post-hoc correction.

Figure 2. IP receptor involvement in esuberaprost and beraprost relaxation in rat pulmonary artery. Concentration-relaxation curves for beraprost (A) and esuberaprost (B) in the absence or presence of 1 μM RO3244794 in rat pulmonary arteries pre-contracted with 100 nM U46619. Data are expressed as % (mean ± SEM, n=7) of the contractile response induced by U46619, the magnitude of which was determined just before the addition of the lowest dose of either prostanoid and taken as 100%. The curves were generated by fitting data with a double sigmoid function in Prism (GraphPad software). **=P<0.01 and ***= P<0.001 compared with RO3244794 group (Two-way ANOVA with Bonferroni post-hoc correction).

Figure 3. Relaxation to beraprost and esuberaprost involves endothelial-derived nitric oxide (NO) in rat pulmonary artery. Concentration-relaxation curves for beraprost (A) and esuberaprost (B) in the absence or presence of 100 μM L-NAME (a non-selective NO synthase inhibitor) in rat pulmonary arteries pre-contracted with 100 nM U46619. Arteries were pre-treated with L-NAME for 1hr before the addition of U46619. Data are expressed as % of the contractile response induced by U46619, measured just before the addition of the lowest prostanoid dose and plotted as mean ± S.E.M (n=5-6). The curves were generated by fitting data with a double sigmoid in Prism (GraphPad software). *P<0.05, **=P<0.01 and ***= P<0.001 when compared to L-NAME (2-WAY ANOVA with Bonferroni post-hoc test).

Figure 4. Contractile effects of beraprost and esuberaprost at high concentrations in rat pulmonary arteries. Tracings (**A**) showing the contractile responses to beraprost and esuberaprost in rat pulmonary arteries primed with 100 nM phenylephrine (PE). Mean concentration-dependent contractile effects to beraprost and esuberaprost (**B**) determined from 10 independent experiments, where vessel tension has been normalized to vessel lumen diameter (mN/μm). Data are shown as mean ± SEM and are fitted with a unity slope sigmoidal fitting routine in GraphPad Prism. Concentration-dependent responses to esuberaprost (**C**) and beraprost (**D**) in rat pulmonary arteries primed with 100 nM phenylephrine (PE) in the absent and presence of 1 μM of the EP_3 receptor antagonist, L798,106. Data are shown as mean ± SEM and are fitted with a unity slope sigmoidal fitting routine in GraphPad Prism. *=P<0.05, **=P<0.01 and ***=P<0.001 when compared to beraprost (**B**) or the response in the absence of L798,106 (**C** and **D**) (2-way ANOVA, with

post hoc correction; n=6-10 observations).

Figure 5. Cyclic AMP generation mediated by beraprost and esuberaprost in HEK-293 cells stably expressing the IP receptor and in human PASMCs derived from PAH patients. (**A**) Messenger RNA was extracted from HEK-293 cells and expression levels of the different prostanoid receptors (IP, EP_1, EP_2, EP_3, EP_4, and DP_1) determined. Data were normalised to the house keeping gene, β-actin, and relative gene expression determined using the 2^(-delta CT) method and plotted on a split scale. *** P<0.001 compared with, one-way ANOVA with Dunnett's post-hoc test (n=6). Cyclic AMP levels were assessed in HEK-293 cells stably expressing the IP receptor (B) and in human PASMCs (C) treated with the two prostanoids over a range of concentrations (0.001- 10,000 nM) for 15 mins. Data were normalised to total cell protein (**B**) or to the maximal cAMP response obtained at either 1 μM or 10μM of either prostanoid (**C**) and analysed using a variable slope sigmoidal-curve fitting function in GraphPad Prism. Extrapolated EC_{50} and E_{Max} values were obtained from the mean fit to the data. **=P<0.01 and ***=P<0.001 when compared to esuberaprost alone (two-way ANOVA with Bonferroni post-hoc correction, n=10-12).

Figure 6. Comparison of the antiproliferative effects of beraprost and esuberaprost in human PASMCs derived from PAH patients; role of the IP receptor and nitric oxide. Human PASMCs were grown in smooth muscle basal media containing 9% FBS. Cell proliferation was determined with an MTS assay in PAH cells grown for 4 days in the absence

and presence of increasing concentrations (0.001-10,000nM) of beraprost or esuberaprost (**A**, **B, C**). Cell proliferation was also determined in the presence of increasing concentrations (0.001-10,000nM) of esuberaprost, with or without 1μM RO1138452 (IP receptor antagonist) or the NO synthase inhibitor, L-NAME (100 μM). Both antagonists were added 30 mins prior to receptor agonists and remained throughout (**D**). Data were expressed as % change in cell proliferation relative to the growth response induced by FBS alone (100%). In **C** and **D**, data have been analysed using a variable slope sigmoidal-curve fitting routine in GraphPad Prism. *=P<0.05, **=P<0.01, ***=P<0.001 when compared to either FBS alone (One ANOVA, Bonferroni post-hoc) or when compared (**C** and **D)** to esuberaprost alone (two-way ANOVA with Bonferroni post-hoc correction). All data are shown as mean ± S.E.M and comparisons made in the same patient cell isolates (n=5).

Figure 7. Effect of esuberaprost and beraprost on pulmonary vascular tone in arteries from patients with PH. Human distal pulmonary arteries derived from patients with pulmonary hypertension (PH) were contracted with 100 nM U46619 and incremental doses (1-30,000 nM) of esuberaprost and beraprost added in a cumulative fashion to the myograph chamber (A). Mean concentration-response effects on vascular tone for beraprost and esuberaprost where comparisons were made in arteries taken from the same five patients (B). Data have been expressed as % (mean ± SEM) of the contractile response induced by U46619 alone and taken as 100%. *=P<0.05 or **=P<0.01, 2-way ANOVA with Bonferroni post-hoc correction when compared to beraprost.

Figure 1

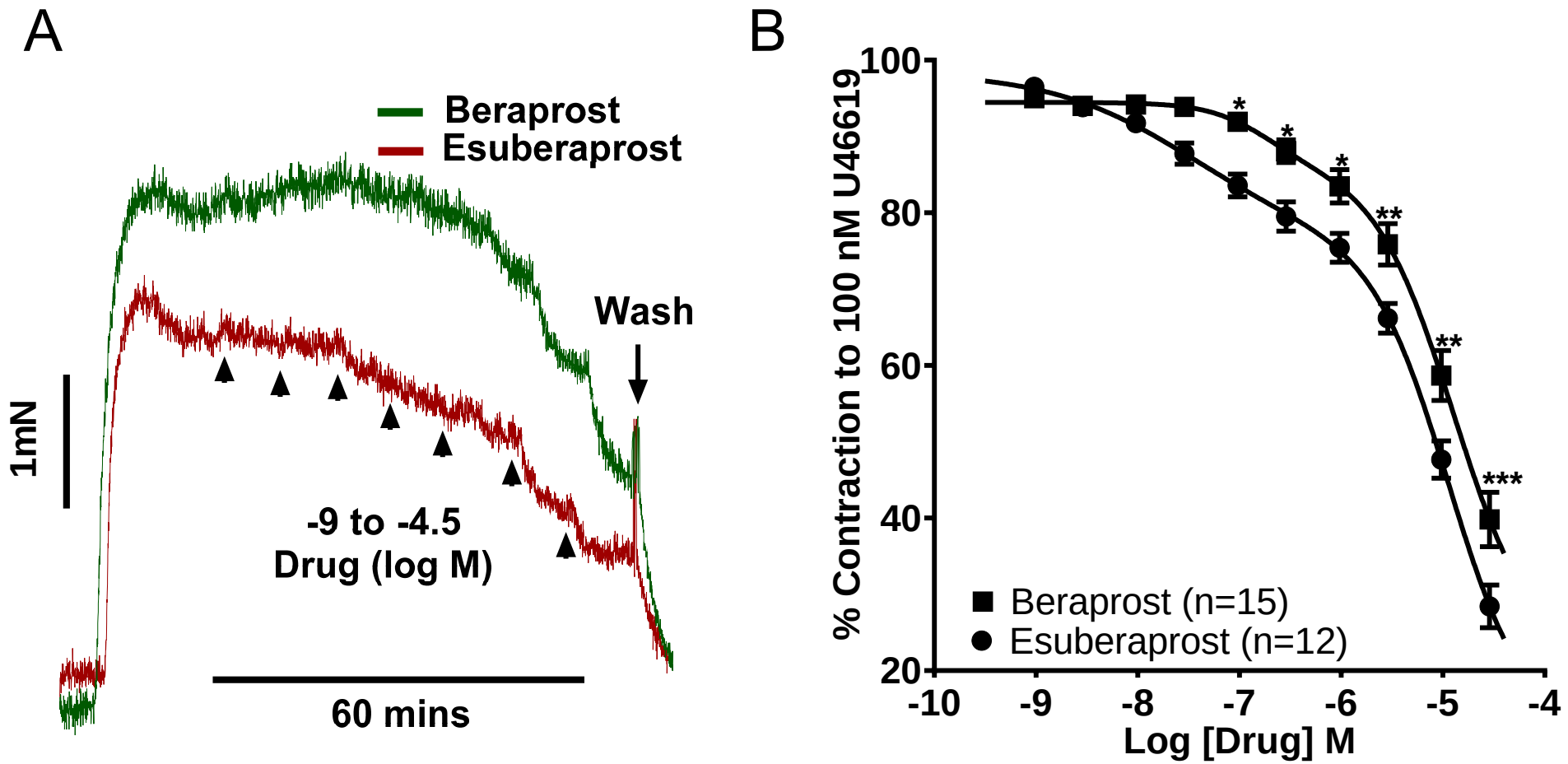
A
Beraprost
Esuberaprost
Wash
1mN
-9 to -4.5
Drug (log M)
60 mins
B
% Contraction to 100 nM U46619
100
80
60
40
20
-10
-9
-8
-7
-6
-5
-4
Log [Drug] M
Beraprost (n=15)
Esuberaprost (n=12)
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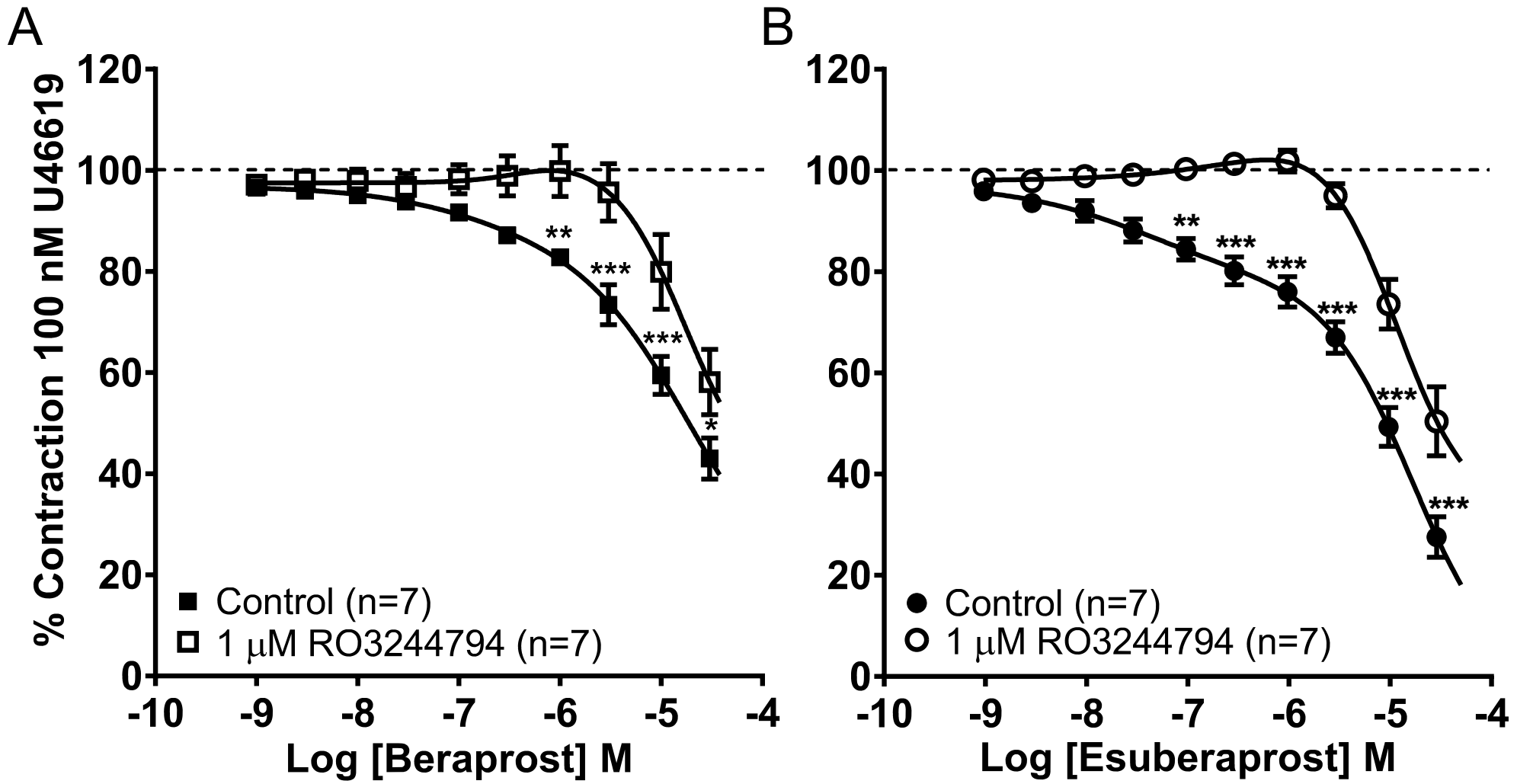
Figure 2


A
% Contraction 100 nM U46619
120
100
80
60
40
20
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-10
-9
-8
-7
-6
-5
-4
Log [Beraprost] M
Control (n=7)
1 μM RO3244794 (n=7)
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Log [Esuberaprost] M
Control (n=7)
1 μM RO3244794 (n=7)
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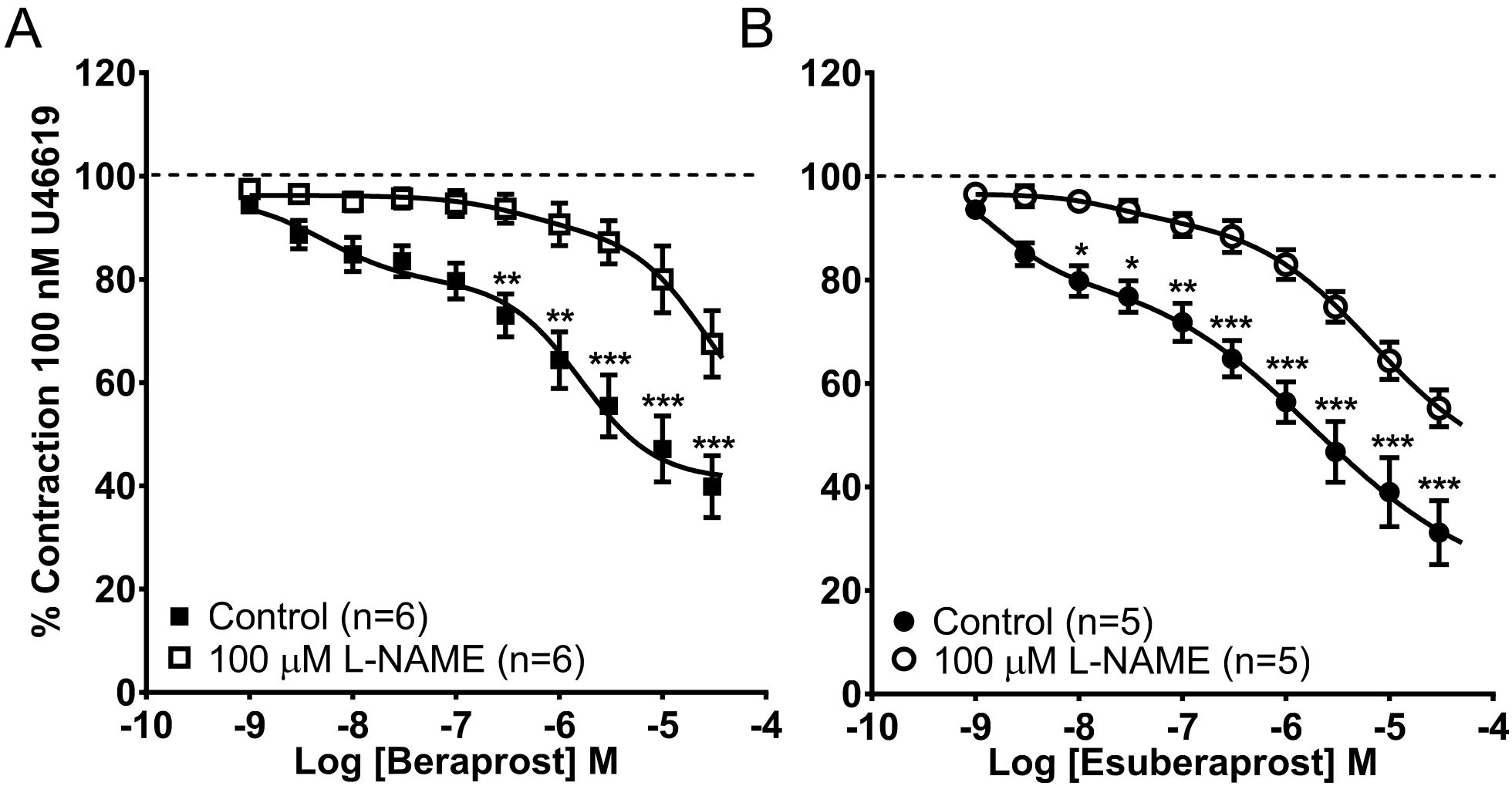
Figure 3


A
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% Contraction 100 nM U46619
120
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-4
Log [Beraprost] M
Log [Esuberaprost] M
Control (n=6)
100 μM L-NAME (n=6)
Control (n=5)
100 μM L-NAME (n=5)
*
**
***

Figure 4

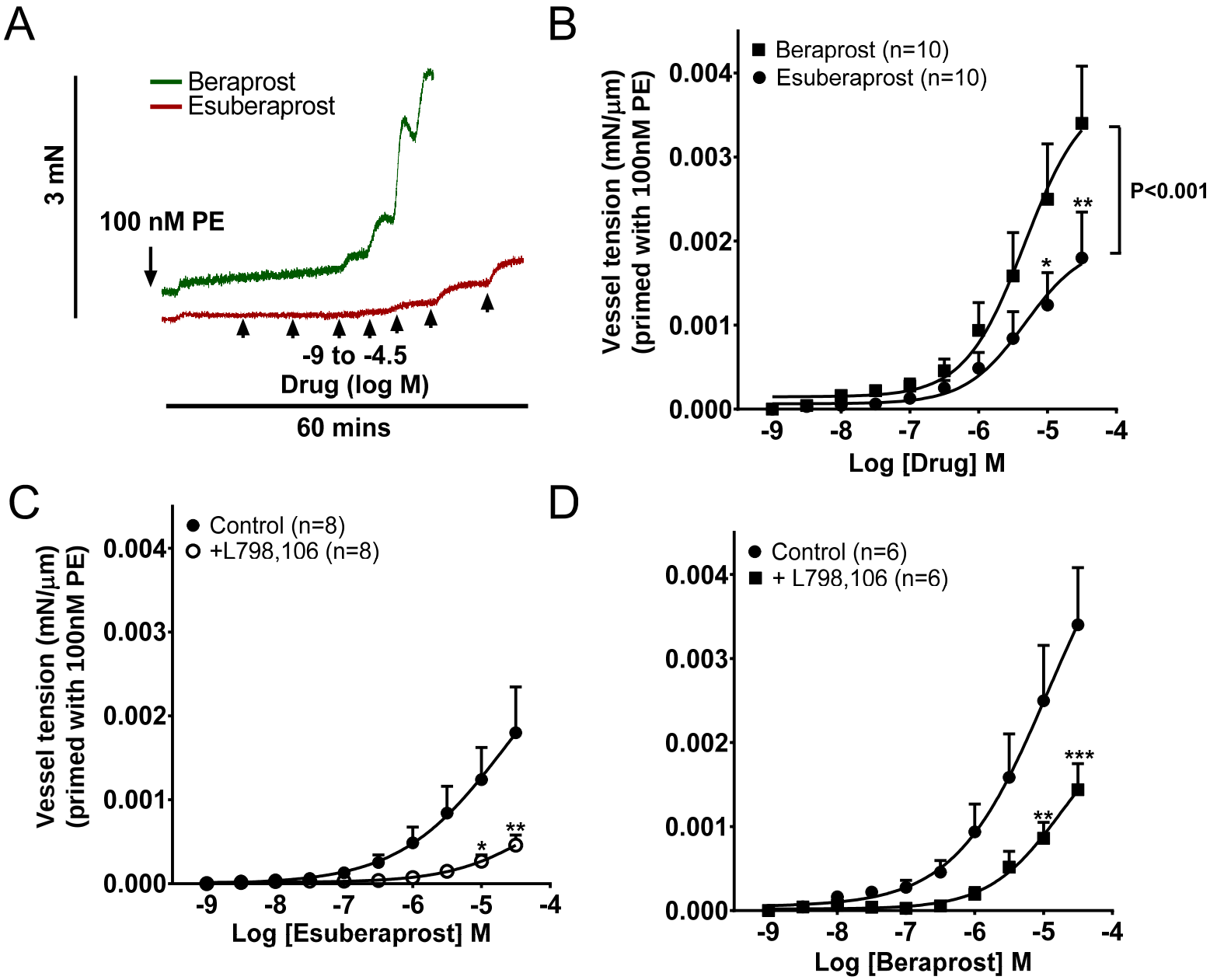

A
Beraprost
Esuberaprost
3 mN
100 nM PE
-9 to -4.5
Drug (log M)
60 mins
B
Beraprost (n=10)
Esuberaprost (n=10)
Vessel tension (mN/µm)
(primed with 100nM PE)
0.004
0.003
0.002
0.001
0.000
-9
-8
-7
-6
-5
-4
Log [Drug] M
P<0.001
*
**
C
Control (n=8)
+L798,106 (n=8)
Vessel tension (mN/µm)
(primed with 100nM PE)
Log [Esuberaprost] M
D
Control (n=6)
+ L798,106 (n=6)
Log [Beraprost] M
***

Figure 5

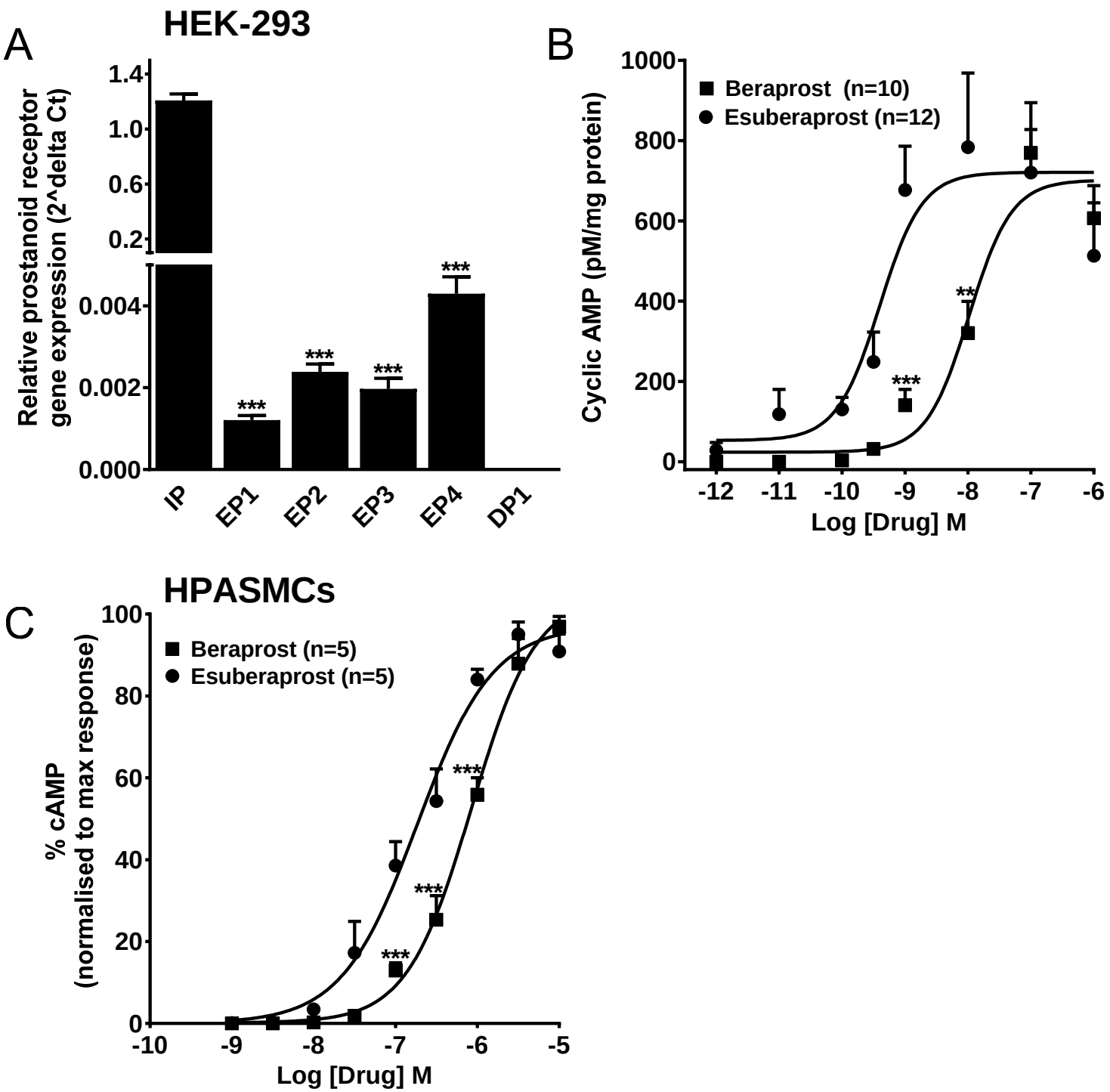

A
HEK-293
Relative prostanoid receptor gene expression (2^delta Ct)
1.4
1.0
0.6
0.2
0.004
0.002
0.000
***
***
***
***
IP
EP1
EP2
EP3
EP4
DP1
B
Cyclic AMP (pM/mg protein)
1000
800
600
400
200
0
Beraprost (n=10)
Esuberaprost (n=12)
**
***
-12
-11
-10
-9
-8
-7
-6
Log [Drug] M
C
HPASMCs
% cAMP (normalised to max response)
100
80
60
40
20
0
Beraprost (n=5)
Esuberaprost (n=5)
***
***
***
-10
-9
-8
-7
-6
-5
Log [Drug] M

Figure 6

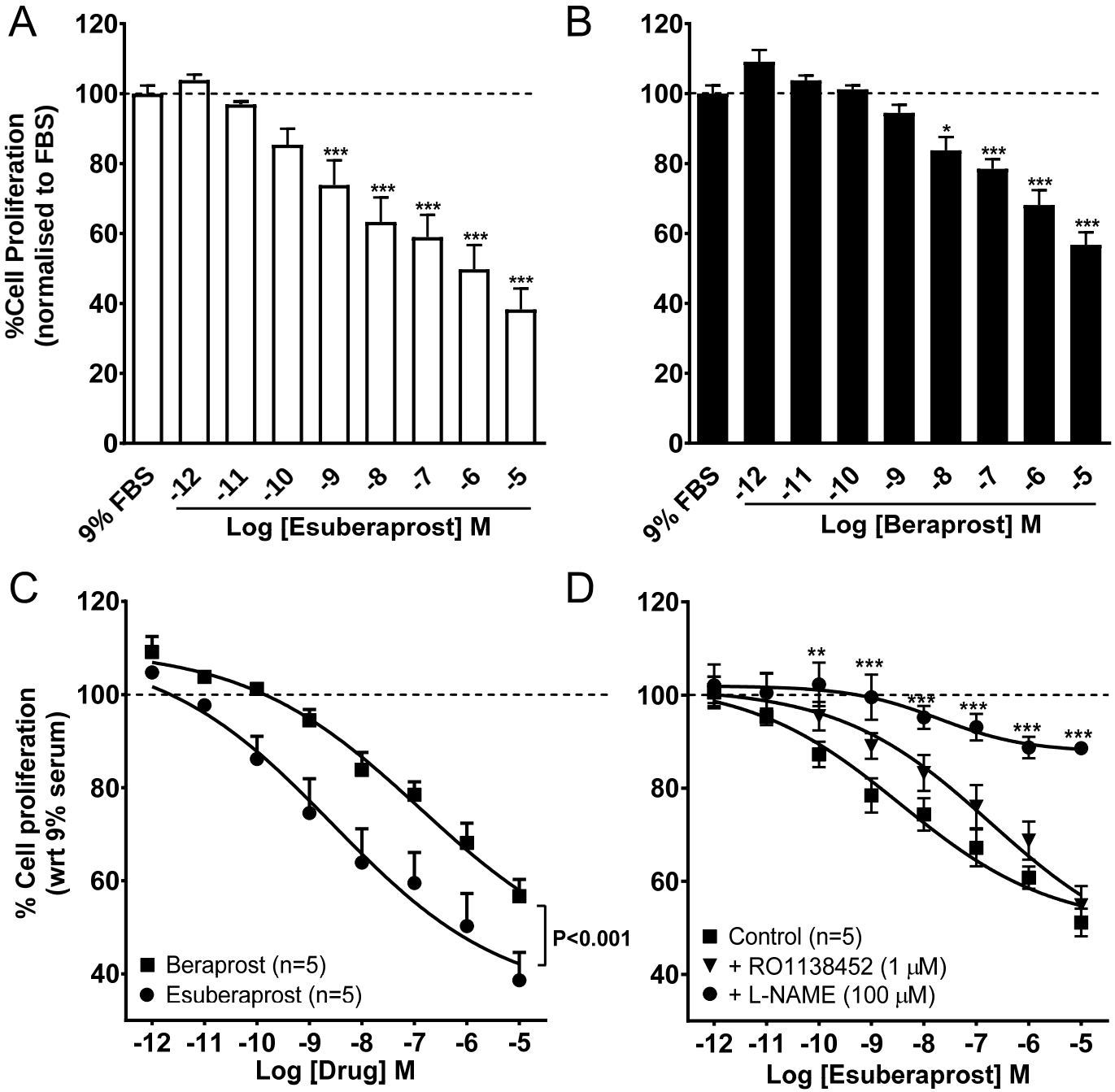
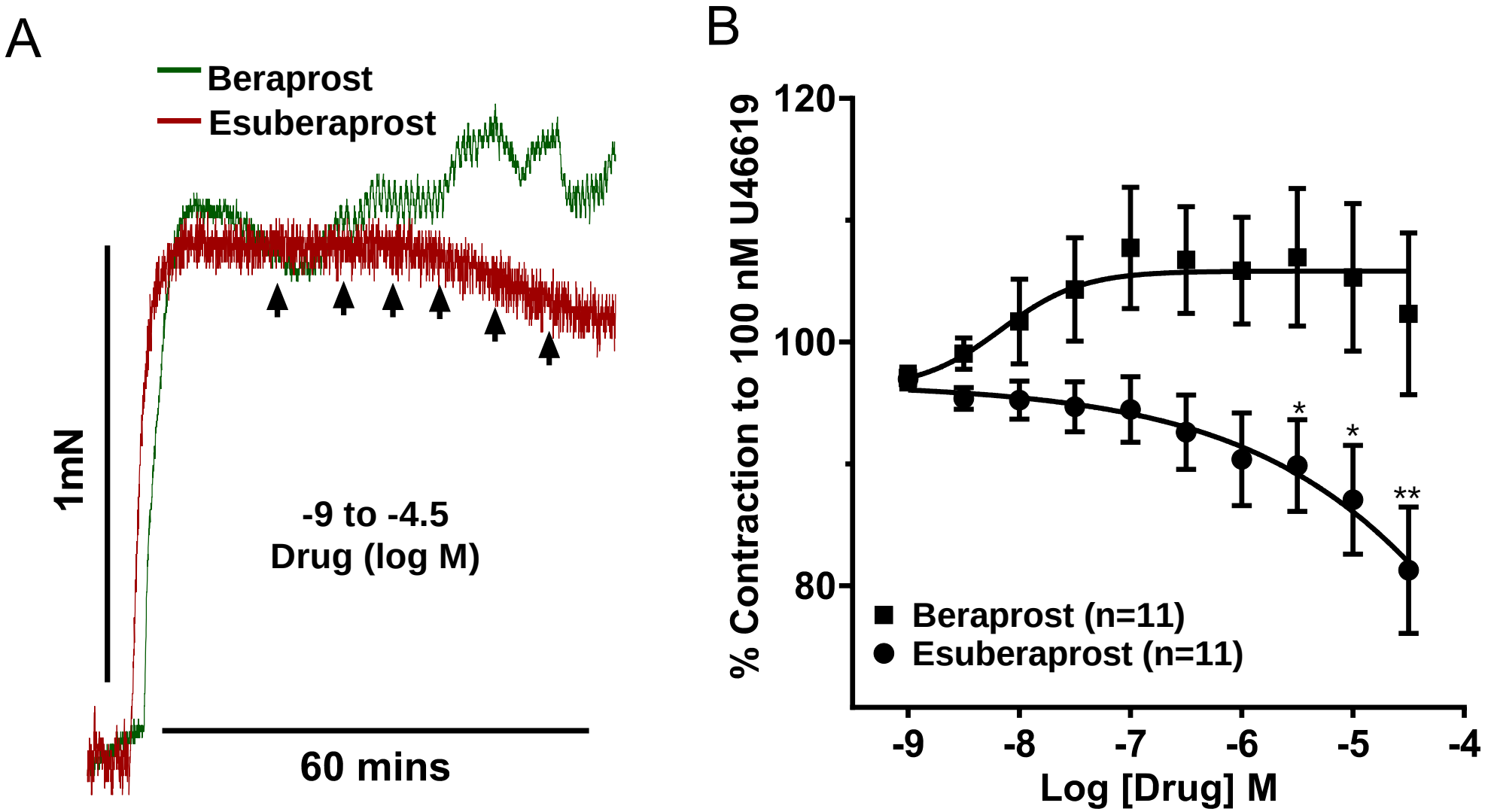

A
%Cell Proliferation (normalised to FBS)
120
100
80
60
40
20
0
***
9% FBS
-12
-11
-10
-9
-8
-7
-6
-5
Log [Esuberaprost] M
B
*
Log [Beraprost] M
C
% Cell proliferation (wrt 9% serum)
Beraprost (n=5)
Esuberaprost (n=5)
P<0.001
Log [Drug] M
D
**
Control (n=5)
+ RO1138452 (1 μM)
+ L-NAME (100 μM)
Log [Esuberaprost] M

Figure 7


A
Beraprost
Esuberaprost
1mN
-9 to -4.5
Drug (log M)
60 mins
B
% Contraction to 100 nM U46619
120
100
80
Beraprost (n=11)
Esuberaprost (n=11)
*
*
**
-9
-8
-7
-6
-5
-4
Log [Drug] M