The ultralong-acting intraocular pressure reduction produced by JV-GL1 is mediated entirely by prostanoid EP2 receptors, according to gene deletion

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Abstract

BACKGROUND AND PURPOSE: A single dose of JV-GL1 lowers intraocular pressure (IOP) for a week according to previous studies on non-human primates. This highly protracted effect did not correlate with its ocular bio-disposition, where the drug was undetectable inside the eye after only one day post-dosing. Our current studies were intended to determine the role of EP2 receptors in mediating the long-term ocular hypotensive activity of JV-GL1 and utilized mice deficient in EP2 receptors. EXPERIMENTAL APPROACH: The protracted intraocular pressure reduction produced by JV-GL1 was investigated in C57BL/6J and EP2 receptor knock-out mice (B6.129-Ptger2tm1Brey/J, EP2KO). Both ocular normotensive and steroid induced ocular hypertensive (SI-OHT) mice were studied. Intraocular pressure was measured tonometrically under general anesthesia. Aqueous humor outflow facility was measured ex vivo using the iPerfusion system in normotensive C57BL/6J mouse eyes perfused with 100 nM de-esterified JV-GL1 and in SI-OHT C57BL/6J mouse eyes that had received topical JV-GL1 (0.01%) 3 days prior. KEY RESULTS: In SI-OHT, JV-GL1 did not lower IOP in EP2 KO mice. However, in WT mice with SI-OHT, JV-GL1 lowered IOP for 4-6 days. JV-GL1 did not alter outflow facility in WT mice at 3 days after topical administration. CONCLUSIONS AND IMPLICATIONS: The long-term effect of JV-GL1 on IOP in the SI-OHT model of glaucoma is EP2 receptor dependent. Such protracted activity of a single dose of a small molecule (JV-GL1) is unprecedented. Future studies on JV-GL1 may eventually lead to "once-weekly" small molecules, with reduced drug prices and better disease control.

Introduction

Primary open-angle glaucoma is the second leading cause of blindness (Resnikoff *et al.* , 2004). First-line glaucoma therapy typically consists of eye drops that lower intraocular pressure (IOP) and containing prostaglandin analogues such as bimatoprost, latanoprost, and travoprost (Sambhara and Aref, 2014). These drugs primarily target the uveoscleral tissues (Schachtschabel, Lindsey and Weinreb, 2000; Winkler and Fautsch, 2014), lowering resistance to aqueous humour outflow, reducing IOP, and thereby protecting retinal ganglion cells from pressure induced damage. Once daily dosing is required to effectively manage IOP, however 25% of patients are non-responders (Scherer, 2002; Sakurai *et al.* , 2014), and poor patient compliance is commonplace (Okeke *et al.* , 2009; Boland *et al.* , 2014). Poor compliance correlates with more severe visual field loss (Sleath *et al.* , 2012). It follows that a relaxed dosing regimen, for example once or twice weekly with a long acting drug, may improve compliance and treatment outcomes accordingly.

JV-GL1 (PGN 9856-isopropyl ester), was recently reported as a highly selective and potent EP_2 receptor agonist (Colemanet *al.* , 2018), which profoundly lowers IOP in Cynomolgus monkeys for an unprecedented

duration of 5-10 days after a single dose (Woodward, *et al.* , 2019a). The well-researched EP_2 agonist, butaprost (Nilsson *et al.* , 2006), and the more contemporary EP_2 agonists taprenepag (Prasanna *et al.* , 2011) and omnidenepag isopropyl (Fuwa *et al.* , 2018) have comparatively short 24 hour durations of action.

Understanding the mechanism behind the uniquely long-acting ocular hypotensive effect of JV-GL1 is of interest in the development of superior ocular hypotensive agents. Given the lack of correlation between the ultra-long duration of action of JV-GL1 and its relatively short bioavailability in the eye (Woodward et al., 2019a), an EP_2 receptor mediated mechanism seemed unlikely. In the present investigations, we examined the IOP-lowering effect of JV-GL1 in ocular normotensive and hypertensive mice. Ocular hypertension was induced in mice using a recently developed method of locally injected dexamethasone eluting nanoparticles (Agrahari, 2017; Wang *et al.* , 2018). The role of the EP_2 receptor in mediating the IOP effects of JV-GL1 was then investigated using EP_2receptor deleted mice (Kennedy *et al.* , 1999).

Methods

Animals

All procedures on living mice were carried out in compliance with the Imperial College Statement for Use of Animals in Research, under a UK Home Office project license. All animal care and experimental protocols adhered to (a) the US National Research Council's Guide for the Care and Use of laboratory animals, (b) the ARVO guidelines and (c) the 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).

Prostaglandin E receptor 2 (EP_2) knock-out mice (B6.129-$Ptger2^{tm1Brey}$ /J) were purchased from The Jackson Laboratory (Bar Harbour, Maine, USA), supplied via Charles River Ltd. UK. These mice were originally created in a C57BL/6J background via homologous recombination and exhibit increased sensitivity to dietary salt induced hypertension and produce small litter sizes due to a defect in embryo implantation (Kennedy *et al.* , 1999). We therefore adopted the following breeding strategy.$Ptger2^{+/-}$ breeding pairs were purchased from the Jackson Laboratories. Male $Ptger2^{-/-}$offspring were then crossed with female$Ptger2^{+/-}$ offspring to produce$Ptger2^{-/-}$ individuals. Separately,$Ptger^{+/+}$ offspring were crossed with$Ptger2^{+/-}$ offspring to produce$Ptger2^{+/+}$ individuals.$Ptger2^{-/-}$ and$Ptger2^{+/+}$ individuals were thus separated by 1 generation. For experiments that used only WT mice, C57BL/6J mice were purchased from The Jackson Laboratories and used without breeding. Mice were housed in individually ventilated cages with a 12-hour light/dark cycle, maintained at 21°C, with food and water ad libitum. Following arrival from the commercial supplier, mice were allowed to acclimatise for a week before any regulatory procedures were performed.

Genotyping followed the recommended protocol provided by the Jackson Laboratory for B6.129-$Ptger2^{tm1Brey}$ /J mice. Genotyping was carried out on DNA extracted from ear tissue sampled at weaning, following the manufacturer's instructions (Express Extract, Kapa Biosystems, Cambridge, MA, USA). KAPA2G Robust HotStart ReadyMix (Kapa Biosystems) was used for PCR reactions. Knock-out sense primer (ATTAAGGGCCAGCTCATTCC), wild-type sense primer (TGCTCATGCTCTTCGCTATG) and common antisense primer (CGTACTCCCCGTAGTTGAGC), with annealing temperatures of 60°C and 28 cycles, yielded predicted products of 300 bp for knock-outs and 165 bp for wild-types (Supplemental Figure 1D). PCR products were resolved by gel electrophoresis (1% agarose) in the presence of a DNA gel stain (SYBR Safe, Invitrogen, Carlsbad,CA, USA). Bands were visualized on an imaging station (Biospectrum 500, UVP, Upland, CA, USA).

Preparation of dexamethasone-eluting nanoparticles

Initial attempts to replicate the method described by Agrahari *et al* . (Agrahari, 2017) to synthesise dexamethasone loaded nanoparticles using the penta-block co-polymer PGA-PCL-PEG-PCL-PGA (poly(glycolic acid)-poly (caprolactone)-poly (ethylene glycol)-poly (caprolactone)-poly (glycolic acid) were unsuccessful. In our hands we could not dissolve the co-polymer in dichloromethane, perhaps due to the crystallinity of high molecular weight poly-glycolic acid (Hacker and Mikos, 2011). Therefore, we chose to use a variant

of the penta-block co-polymer described by Tamboli *et al* . (Tamboli, Mishra and Mitra, 2013), which was (poly(*d,l*)lactide-poly (caprolactone)-poly(ethylene-glycol)-poly (caprolactone)-poly(*d,l*)lactide (PDLLA-PCL-PEG-PCL-PDLLA).

Nanoparticles were synthesised from the PDLLA-PCL-PEG-PCL-PDLLA co-polymer using the oil-in-water solvent evaporation technique (McCall and Sirianni, 2013). 75 mg of penta-block co-polymer (AK099, PolySciTech, Akina Inc. West Lafayette, IN, USA) and 5 mg of dexamethasone (D4209, Sigma-Aldrich St. Louis, MO, USA) were added to 1.2 ml of ethyl acetate and vortexed into solution. The solution was added dropwise to 2 ml of vortexing 0.1% aqueous D-alpha-tocopherol polyethylene glycol 1000 succinate (E-TPGS, 57668, Sigma-Aldrich St. Louis, MO, USA). Immediately after mixing, the pre-emulsion was probe ultrasonicated (Branson 450 digital Sonifier, Branson Ultrasonic Corporation, CT, USA) in 10 x 30 second bursts at 20% power on ice. Between bursts, the emulsion was allowed to cool for 10 seconds. The emulsion was immediately added dropwise to 45 ml of 0.3% aqueous E-TPGS on a magnetic stirrer (300-400 rpm) for overnight solvent evaporation at room temperature. Nanoparticles were recovered by ultracentrifugation at 20,000 RPM for 1 hour at 4°C and washed 3 times with distilled water to remove E-TPGS and un-entrapped dexamethasone. Nanoparticles were lyophilized for 72 hours with trehalose, a lyoprotecterant (Zhou*et al.* , 2013), in a weight ratio of 0.5:1, trehalose:polymer (A19434.06, Alfa Aesar Haverhill, MA, USA), and stored at 4°C for further studies. Synthesis of unloaded nanoparticles was identical with the omission of dexamethasone. Average particle size was 167 nm [145, 189] (mean, [95% CI]) based on 150 measurements of individual particles in six scanning electron microscope images (JSM-5610, JEOL Ltd. Akishima, Tokyo, Japan) using the 'measure' function in ImageJ (Schneider, Rasband and Eliceiri, 2012).

We used mass spectrometry to measure the mass of dexamethasone contained within 5 mg of loaded nanoparticles. Nanoparticles were first dissolved in DMSO. An Agilent 6130 Quadrupole LC-MS coupled to an Agilent 1260 Infinity LC using a 150 X 4.6 mm Phenomenex Gemini NX-C18 column with a 110 Å pore size and 5 µm particle size was used to quantify dexamethasone concentration. Ultrapure water and acetonitrile, each containing 0.1% (v/v) formic acid (VWR) by volume, were used for the mobile phase at a flow rate of 1 ml/min. Samples were eluted with a gradient of 95% (v/v) water to 95% (v/v) acetonitrile over 10 minutes. The electrospray source was operated with a capillary voltage of 3.2 kV and a cone voltage of 25 V with nitrogen used as the nebulizer and desolvation gas at a total flow of 600 l/h. Detection was between 100-1000 Da in both positive and negative ionisation mode.

On the day of use, lyophilised nanoparticles were re-suspended in sterile PBS by bath sonication to a concentration of 0.25 mg/ml dexamethasone. Injections were performed under general anaesthesia induced by 5 minutes exposure to 4% isoflurane and 1 l/min oxygen in an anaesthetic chamber. Following loss of consciousness, confirmed by toe pinch, mice were transferred to a Bain co-axial circuit fitted with a mouse nose-cone to maintain anaesthesia. Mice received topical anaesthesia (Lidocaine hydrochloride 4%, Chauvin Pharma, Aubenas, France) immediately prior to injection of 20 µl nanoparticle suspension into the periocular tissue of each eye using a Hamilton syringe and a 30 gauge needle. Mice recovered in a 36°C chamber and were housed in groups of 5 for the remainder of the study.

Preparation of Ophthalmic Solutions

JV-GL1 isopropyl ester (and de-esterified JV-GL1) were supplied by JeniVision Inc. Irvine, CA, USA). Butaprost was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Both drugs were dissolved in 100% EtOH and made up to 0.01% in vehicle, composed of 0.2 µm sterile filtered PBS, 0.2% DMSO and 1% polysorbate 80.

Investigating the IOP effects of JV-GL1

The effects of JV-GL1 on IOP were investigated in four experiments. In all studies IOP was measured under general anaesthesia, bilaterally in each mouse between 09:00 AM and 12:00 noon using a commercial rebound tonometer (TonoLab; Icare, Helsinki, Finland). For each time point IOP was calculated as the mean of three consecutive tonometer readings, each containing 6 rebound events, as previously described (Overby *et al.* , 2014). We designed 4 experiments to test the effect of JV-GL1 on IOP.

The first experiment investigated the IOP effect of JV-GL1 in normotensive *Ptger2*$^{-/-}$ mice (11 males & 3 females, 10.7 [10-12] weeks old, mean [95% CI]) and*Ptger2*$^{+/+}$ mice (6 males & 10 females, 12.6 [12-13] weeks old). Baseline IOP was established prior to unilateral administration of either 0.01% JV-GL1 (*Ptger2*$^{-/-}$ n=7,*Ptger2*$^{+/+}$ n=9) or 0.01% butaprost (*Ptger2*$^{-/-}$ n=7,*Ptger2*$^{+/+}$ n=7), with contralateral eyes receiving vehicle. IOP was measured at 3 and 24 hours, with an additional measurement at 48 hours for the JV-GL1 treated group. Data were presented as the mean difference between paired treated and untreated eyes (ΔIOP).

The second experiment investigated the IOP effect of JV-GL1 in wildtype C57BL/6J mice (10 week old males, n=18) that had been rendered ocular hypertensive by subconjunctival injection of dexamethasone-eluting nanoparticles. Baseline IOP was established over two time points prior to bilateral nanoparticle injection. IOP was measured after two weeks to establish the level of ocular hypertension induced by the glucocorticoid, referred to as the pre-treatment time point. Mice were treated bilaterally with either 0.01% JV-GL1 (n=9) or 0.01% butaprost (n=9). IOP was measured in both eyes at 3 hours, followed by measurements at 2, 6 and 9 days. JV-GL1 treated eyes were additionally measured at 12 and 16 days, whereupon a second challenge with 0.01% JV-GL1 was administered to one eye, with contralateral receiving vehicle. After 19 days, IOP was measured for a final time and mice culled for outflow facility measurements. Data was presented as the mean change in IOP from the pre-treatment time point (δIOP).

The third experiment aimed to compare the IOP-lowering effect of JV-GL1 between normotensive versus hypertensive WT C57BL/6J littermates (n=8 vs 9; 10 week old males). Normotensive individuals received unloaded nanoparticles. Baseline IOP was established once prior to bilateral injection of either dexamethasone loaded or unloaded nanoparticles into the periocular tissues. IOP was measured after 1 week to establish the level of ocular hypertension. One eye was then treated with 0.01% JV-GL1 with contralateral eye receiving vehicle. IOP was measured in both eyes at 3 and 24 hours, followed by measurements at 4 and 6 days. Data were presented as the mean difference between paired treated and untreated eyes (ΔIOP).

The fourth experiment investigated the IOP effect of JV-GL1 in*Ptger2*$^{-/-}$ mice (5 males & 3 females, 12.5 [11, 14] weeks old) and *Ptger2*$^{+/+}$ mice (5 males & 5 females, 13 [12, 14.1] weeks old) that had been rendered steroid induced ocular hypertensive. Baseline IOP was established once prior to bilateral injection of dexamethasone loaded nanoparticles into the periocular tissues. IOP was measured after one week to establish the level of ocular hypertension. One eye was then treated with 0.01% JV-GL1 with contralateral eye receiving vehicle. IOP was measured in both eyes at 3 and 24 hours, followed by measurements at 4, 6 and 11 days. Data were presented as the mean difference between paired treated and untreated eyes (ΔIOP).

In all IOP studies, the following statistical analyses were applied. Normality was checked by the Kolgomorov-Smirnov test, equity of variances between groups was determined by Levene's test, and statistical significance between groups over multiple time points was established via a two-factor ANOVA. Errors are presented as 95% confidence intervals.

Measurement of Conventional Outflow Facility

In all perfusion studies mice were culled humanely by cervical dislocation, eyes enucleated immediately after death and outflow facility measured simultaneously in paired eyes using the*iPerfusion* system, as previously described (Sherwood *et al.* , 2016). Briefly, eyes were glued to a support platform submerged in PBS regulated at 35°C. The ocular anterior chambers were cannulated with 33-gauge bevelled needles (NanoFil, NF33BV-2, World Precision Instruments) attached to micro-manipulators and equilibrated for 30 minutes at 9 mmHg. Perfusate comprised 0.2 μm filtered DBG (PBS including divalent cations and 5.5 mM glucose). Flow was measured at pressure steps from 5 to 17 mmHg in 7 steps. The steady state criterion per step was 1 minute of <0.1 nl/min/mmHg/min variation in ratio of flow rate to pressure. Pressure steps that failed to reach steady state were excluded from further analysis, paired eyes with 4 or more successful steps were analysed. A Savitzky–Golay filter (60secs, first order) was applied to the digital pressure and flow data before calculation to increase precision without distorting the signal tendencies.

Mean steady state flow Q and pressure P for each included pressure step were calculated over a 4-minute

window and fit by the relationship

$$Q = C_r \left(\frac{P}{P_r} \right)^{\beta} P$$

C_r represents outflow facility at a reference pressure P_r(8 mmHg) and β characterizes the non-linearity of the$Q$-P relationship. Average relative change in C_r was compared between contralateral treated and control eyes (mean ± 95% CI) using a weighted t -test of the log-transformed data as described previously (Sherwood *et al.* , 2016).

Acute effects on outflow facility were determined by perfusing DBG containing 100 nM de-esterified JV-GL1 directly into the anterior chamber of normotensive C57BL/6J mouse eyes (n=7) and compared to paired vehicle perfused contralateral eyes. All pairs tested met the stability criteria. Long-term effects of topical JV-GL1 treatment on outflow facility were determined in steroid induced ocular hypertensive C57BL/6J mice (n=9) following unilateral treatment with 0.01% JV-GL1, with contralateral eyes receiving vehicle, outflow facility was measured ex vivo 3 days later. All pairs tested met the stability criteria.

Results

The effect of JV-GL1 in normotensive mice

As JV-GL1 is an EP_2 agonist (Coleman et al., 2018; Woodward et al., 2019a), our first aim was to measure the IOP-lowering effect of JV-GL1 in normotensive mice that express EP_2(*Ptger2* $^{+/+}$) versus mice that lack EP_2 (*Ptger2* $^{-/-}$). Between*Ptger2* $^{+/+}$ and*Ptger2* $^{-/-}$, there was no significant difference in baseline IOP nor outflow facility (Supplemental Figure 1). Three hours after unilateral treatment with JV-GL1 (0.01%, 10 µl), IOP was reduced in *Ptger2* $^{+/+}$ mice (ΔIOP =-3.1 [-4.1, -2.1] mmHg; mean [95% CI]; P <0.0001; n=9 mice) with respect to the vehicle-treated contralateral eye (Fig. 1A). In*Ptger2* $^{-/-}$ mice, however, there was no significant IOP reduction following treatment with JV-GL1 after 3 hrs (ΔIOP = 0.8 [-0.4, 2.0] mmHg; P =1, n=7). By 24 hours, IOP in the treated eye of *Ptger2* $^{+/+}$ mice had returned to baseline and was not statistically different from IOP in the control eye (ΔIOP = 0.0 [-1.0, 1.0] mmHg, P =1, n=9). Thus, the IOP-lowering effect of JV-GL1 requires *Ptger2* and lasts for less than 24 hrs, at least in normotensive mice.

We also compared the effects of JV-GL1 against the archetypical EP_2 agonist butaprost (Fig. 1A). Like JV-GL1, butaprost lowered IOP in the *Ptger2* $^{+/+}$ mice at 3 hrs relative to the contralateral vehicle-treated eye ([?]IOP = -2.4 [-4.2, -0.6] mmHg; P <0.01; n=7), but butaprost did not affect IOP in the *Ptger2* $^{-/-}$ littermates ([?]IOP = 0.4 [-0.7, 1.5] mmHg; P =1; n=7). The IOP reduction in response to butaprost was not significantly different from that of JV-GL1 at 3 hrs (P =1), and neither drug had an observable effect on IOP at 24 hrs. Thus, the magnitude and duration of IOP-lowering in response to JV-GL1 appears to be similar to that of butaprost in ocular normotensive mice.

To investigate the physiological mechanism of IOP reduction, we then examined the effect of JV-GL1 on pressure-dependent outflow. We observed no significant effect of de-esterified JV-GL1 (100 nM) on outflow facility when perfused directly into enucleated eyes of C57BL/6J mice (relative to contralateral vehicle-perfused eyes), which express*Ptger2* (-6% [-22, 13], P =0.4, n=5 pairs; Fig. 1B). Thus, consistent with other EP_2 agonists that are thought to lower IOP primarily by increasing unconventional outflow (Nilsson *et al.* ,2006; Woodward, *et al.* , 2019b), JV-GL1 does not appear to have an acute effect on outflow facility.

The effect of JV-GL1 in ocular hypertensive mice

We then examined the effect of JV-GL1 in C57BL/6J mice with ocular hypertension. We induced ocular hypertension by periocular injection of dexamethasone-eluting nanoparticles given bilaterally. This elevated IOP by 4.2 [3.5, 4.9] mmHg relative to baseline, and this elevation was sustained over 2 weeks following a single nanoparticle injection without a significant loss of body weight (n=18; Supplemental Figure 2). Ocular hypertensive mice were treated with a single topical drop of JV-GL1 (10 µl, 0.01%) given bilaterally. As with the normotensive mice, IOP was reduced after 3 hrs relative to the pre-treatment IOP (δIOP = -2.3 [-3.3,

-1.2] mmHg; P =0.01; n=9 mice, Fig. 2A). However, unlike the normotensives, the IOP reduction persisted for up to 6 days when δIOP = -2.8 [-3.7, -2.0] mmHg (P =0.001; n=9). Thus, a single treatment of JV-GL1 reduces IOP for several days in mice with steroid-induced ocular hypertension, in contrast to results from a different cohort of normotensive mice where the IOP-lowering effects of JV-GL1 lasted for less than 1 day (Fig. 1A)

After 9 days, the effects of JV-GL1 in hypertensives had diminished and IOP had returned to pre-treatment levels (δIOP = -0.5 [-1.5, 0.5] mmHg; P =1; n=9), which were still elevated with respect to baseline due to dexamethasone. We then administered a second dose of JV-GL1 (10 µl, 0.01%) to one randomly-chosen eye of each mouse, while the contralateral eye received vehicle (Fig. 2A *second red arrow*). After 3 days, IOP was again reduced in the JV-GL1-treated eye by -2.3 [-3.4, -1.2] mmHg (P= 0.04; n=9) relative to the contralateral eye, demonstrating at least partial repeatability. The mice were then euthanized 3 days after JV-GL1 treatment, and eyes were enucleated to measure outflow facility. In eyes treated with JV-GL1, outflow facility was not statistically different from that in contralateral vehicle-treated eyes (18% [-7, 50], P =0.2, n=9 pairs; Fig 2B). Thus, the IOP reduction observed in response to JV-GL1 in ocular hypertensive mice does not appear to coincide with an increase in outflow facility, similar to the absence of a facility response observed in normotensive mice.

To confirm that the long-lasting IOP reduction of JV-GL1 is not attributable to cohort differences between the normotensive and hypertensive groups, we compared the effects of JV-GL1 between wildtype littermates with or without ocular hypertension. Hypertension was induced by bilateral periocular injection of dexamethasone-eluting nanoparticles, while normotensives received a bilateral injection of unloaded nanoparticles. In the group receiving dexamethasone-eluting nanoparticles, IOP increased by 4.7 [3.4, 6.0] mmHg after 1 week, while IOP remained near baseline in the group receiving unloaded nanoparticles ([?]IOP = 0.0 [-1.6, 1.6] mmHg; Supplemental Figure 3). In the hypertensives, a single topical drop of JV-GL1 given unilaterally (10 µl, 0.01%) reduced IOP after 3 hrs by 3.1 [-4.4, -1.8] mmHg (P =0.01, n=8; Fig. 3). The IOP reduction in hypertensives persisted for up to 4 days ([?]IOP = -2.8 [-3.8, -1.8] mmHg,P =0.04), but had returned to pre-treatment levels by 6 days ([?]IOP = -1.4 [-2.9, 0.2] mmHg, P =0.5). In normotensive mice, however, the IOP reduction was short-lived and was observed only at 3 hrs ([?]IOP = -1.6 [-2.7, -0.5] mmHg, P =0.08; n=9). This confirms that a single dose of JV-GL1 is capable of reducing IOP for several days in mice with steroid-induced ocular hypertension, but the effect of JV-GL1 is relatively short-lived in normotensive C57BL/6J littermates.

To examine whether the long duration of JV-GL1 action was distinct from typical EP_2 agonism, we treated ocular hypertensive mice with a single bilateral dose of butaprost (10 µl, 0.01%). Butaprost reduced IOP after 3 hrs ([?]IOP = -2.5 [-4.2, -1.0] mmHg;P =0.07; n=9; Fig. 2A) but, as observed in normotensive mice, IOP returned to pre-treatment values by 2 days ([?]IOP = -0.8 [-1.5, 0.0] mmHg; P =0.6; n=9). This reveals that the long-lasting IOP reduction attributable to JV-GL1 under hypertensive conditions is distinct from that of typical EP_2 agonists.

The role of EP_2 in the long-lasting IOP reduction of JV-GL1 in ocular hypertensive mice

It is possible that the long-lasting IOP reduction in steroid-hypertensive mice is attributable to off-target effects of JV-GL1 apart from EP_2 (e.g., by inhibiting the steroid response). To explore this possibility, we compared the effects of JV-GL1 between *Ptger2*$^{-/-}$ and*Ptger2*$^{+/+}$ littermates, all with steroid-induced ocular hypertension. A single bilateral periocular injection of dexamethasone-eluting nanoparticles increased IOP by 4.9 [3.8, 5.9] mmHg over 1 week (n=18; Supplemental Figure 3), with no significant differences observed between *Ptger2*$^{-/-}$ and*Ptger2*$^{+/+}$ littermates.

In hypertensive *Ptger2* $^{+/+}$ mice, a single unilateral dose of JV-GL1 (10 µl, 0.01%) reduced IOP after 3 hrs by -4.2 [-5.6, -2.9] mmHg relative to the vehicle-treated contralateral eye (P <0.0001; n=10 mice, Fig. 4). IOP reduction persisted for up to 4 days, when ΔIOP = -2.6 [-3.3, -1.8] mmHg (P =0.02; n=10) but was undetectable by 6 days, when ΔIOP= -1.6 [-2.8, -0.4] mmHg (P =0.7; n=10). In contrast, JV-GL1 had no effect on *Ptger2*$^{-/-}$ mice at 3 hours, when ΔIOP= 0.67 [-0.3, 1.6] mmHg (P =1; n=8) nor any other time

point. Thus, the long-lasting effect of JV-GL1 on IOP reduction does not appear to involve off-target effects, but appears to require EP_2 receptor expression.

Discussion

The most important facet of JV-GL1 pharmacology is arguably its ultra-long duration of action (Woodward *et al* ., 2019a). The present studies have demonstrated that this prolonged effect on intraocular pressure is entirely mediated by a single pharmacological entity, the EP_2 receptor. Class selective long-acting β_2-agonists (LABAs) and muscarinic antagonists (LAMAs) have previously been discovered (Wold et al., 2019; Wendell et al., 2020) but their clinical dosing regimen is once daily, even when used in combination (Maqsood et al., 2019). For JV-GL1, a once weekly dosing regimen is feasible. Further research on JV-GL1 and the EP_2 receptor may lead to a wide range of small molecules dosed once-weekly that offer improved disease control and lower drug costs. At this juncture, the existing data suggests JV-GL1 may be an important new drug for women's health and inflammation (Coleman *et al* ., 2019). For glaucoma, the data indicate that JV-GL1 is a realistic proposition for a breakthrough drug in treating glaucoma (Coleman*et al* ., 2018; Woodward *et al.,* 2019a).

The long-acting effects of JV-GL1 on IOP in monkeys extend far beyond time points when detectable levels of the drug can be measured in anterior segment tissues of the eye (Woodward, *et al.* , 2019a). JV-GL1 was designed as an EP_2 receptor agonist and the long acting effects of a week or more were quite unexpected, given that the ocular hypotensive duration of action of the numerous EP_2 agonist family is essentially one day (Woodward,*et al.* , 2019b). This raised the possibility that the effects of JV-GL1 on IOP occurred as a result of two separate pharmacological activities; an initial EP_2 receptor mediated effect followed by a secondary long-acting phase that was independent of EP_2 receptors. In order to address this question, we studied the effects of JV-GL1 in gene deleted mice (Saeki *et al.* , 2009). Our studies demonstrate that the entire duration of ocular hypotensive action of JV-GL1 is EP_2 receptor mediated.

A single topical application of 0.01% JV-GL1 significantly reduced IOP in both ocular normotensive and steroid-induced ocular hypertensive mice after 3 hours. However, only steroid treated mice exhibited a long-term pressure reduction, persisting for 4 to 6 days. JV-GL1 was incapable of reducing IOP in mice lacking the EP_2 receptor under both ocular normotensive and steroid ocular hypertensive conditions. Conventional interpretation of G-protein coupled receptor signalling would suggest that upon ligand binding to the EP_2receptor $G\alpha_s$ coupling activates adenylate cyclase which converts adenosine triphosphate to the second messenger, cyclic adenosine monophosphate (cAMP) (Regan *et al.* , 1994; Pierce*et al.* , 1995). The cAMP-dependent pathway promotes matrix metalloproteinase (MMP's) secretion (Shim, Kim and Ju, 2017). MMP's break down extracellular matrix material in the outflow tissues of the eye, reducing resistance and lowering IOP (Nilsson *et al.* , 2006).

In this study, topical JV-GL1 did not significantly affect outflow facility in the steroid ocular hypertensive mouse eye, nor in ocular normotensive mouse eyes perfused with de-esterified JV-GL1 over an acute time-scale. A study using cellular dielectric spectroscopy found JV-GL1 to have high activity in human ciliary muscle cells but little activity in human trabecular meshwork cells (3.9 nM vs >10 μM, EC_{50} values respectively) (Woodward, *et al.* , 2019a). This suggests a uveoscleral mechanism of action for JV-GL1, which correlates with the minimal effects on outflow facility observed in this study and with the mechanism of action proposed for the standard and most studied EP_2 agonist, butaprost (Nilsson*et al.* , 2006). Our perfusion results largely corroborate those obtained from perfusion studies in monkeys, where JV-GL1 did not significantly affect outflow facility. However, in monkeys JV-GL1 significantly increases uveoscleral outflow, as measured by fluorophotometry, and significantly reduces inflow by 20% over an acute timescale (Woodward, *et al.* , 2019a). A limitation of measuring outflow facility in enucleated mouse eyes is that inflow is essentially terminated once the eye is removed and the pressure independent nature of the uveoscleral pathway makes it challenging to measure using a pressure controlled perfusion system. Thus, any effect JV-GL1 may have on inflow or uveoscleral outflow cannot be measured. Unfortunately in monkey studies, inflow measurements were not continued over the time-course of IOP measurements, and the relationship between a reduction in inflow and IOP suppression remains unanswered.

In monkeys, JV-GL1 exhibited long-term IOP effects in both ocular normotensive and laser-induced ocular hypertensive animals, and demonstrated considerably greater pressure reduction (Woodward *et al.* , 2019a) compared to the observations made in this study with mice. These inconsistencies may simply be due to species differences. The relative size of the ciliary muscle is much smaller in mice than in primates (Ko and Tan, 2013) as mice do not accommodate for vision (Tamm and Lutjen-Drecoll, 1996). If JV-GL1 is thought to work primarily via the uveoscleral route, then the more developed ciliary muscle of the monkey, which is in frequent use and therefore containing greater amounts of extracellular matrix material, would be expected to respond better to remodelling effects of MMP's than the more vestigial ciliary muscle of the mouse.

Alternatively, it may be the different methods employed to induce ocular hypertension. Unlike laser-induced ocular hypertension, the steroid induced ocular hypertensive model relies on the persistent presence of dexamethasone in the eye, eluting from periocular depots of dexamethasone loaded nanoparticles. Steroid ocular hypertension is brought about by dexamethasone induced changes in extracellular matrix deposition and stiffness in the trabecular meshwork (Overby *et al.* , 2014; Raghunathan *et al.* , 2015). Increased extracellular matrix deposition is likely due to dexamethasone inhibiting MMP's, either through inhibition of transcription factors (Forster *et al.* , 2007) or by inducing tissue inhibitors of matrix metalloproteases (MMP's) (Xu *et al.* , 2001). Dexamethasone may have similar effects on the uveoscleral pathway. MMP's 2, 3 & 9 were found to be significantly reduced in ciliary body explants treated with dexamethasone, with MMP-3 eliminated after 72 hours of dexamethasone treatment (el-Shabrawi *et al.* , 2000). MMP-3 can break down a diverse range of extracellular matrix components (Matrisian, 1990). What is more, dexamethasone has been shown to inhibit the production of endogenous prostaglandins in human trabecular meshwork cells and scleral fibroblasts (Gerritsen *et al.* , 1986).

Prostaglandins have been shown to induce MMP production in the ciliary body (el-Shabrawi *et al.* , 2000). These MMP's are thought to be released into the aqueous and diffuse into the ciliary muscle where they break down extracellular matrix components between the longitudinal fibres of the ciliary muscle, allowing less restricted flow into the choroid (Ocklind, 1998). Consequently, we can speculate that JV-GL1 in the steroid ocular hypertensive mouse model would have to compete against the pro-fibrotic effects of constant dexamethasone delivery to the eye, limiting its ability to lower IOP to the same degree observed in the laser-induced ocular hypertensive monkey model, where no dexamethasone is present.

It is of interest that JV-GL1 has an effect on inflow in monkeys, as this has not been demonstrated with any other EP_2agonists to date (Woodward, *et al.* , 2019b). The most studied EP_2 agonist, butaprost, is efficacious at lowering IOP but has a short duration of action, with no effect on inflow and no effect on conventional outflow facility. The entirety of butaprost effects appear to be mediated by changes in uveoscleral outflow, specifically ciliary muscle resistance (Nilsson *et al.* , 2006). Therefore, JV-GL1 behaves somewhat differently when compared to other EP_2 agonists. Aqueous flow can be reduced by direct activators of adenylate cyclase such as forskolin (Caprioli *et al.* , 1984) and cholera toxin (Gregory *et al.* , 1981), via a cAMP-dependent process. In the latter case, reduction in IOP persisted for 6 days after a single intravitreal injection.

It is, of course, highly unlikely that JV-GL1 is directly and/or irreversibly activating adenylate cyclase. However, recent research has disrupted the conventional view of G-protein coupled receptor signalling, whereby active receptor conformations engage and activate only one of four classes of cytoplasmic heterotrimeric G-proteins ($G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12}$) and a more promiscuous model, whereby receptors can bind to more than one G-protein subtype, eliciting multiple G-protein dependent signalling events has emerged (Stallaert, et al., 2011; Hermans, 2003). Recent work investigating EP_2receptor activation with butaprost, demonstrated not only the conventional $G\alpha_s$ – cAMP pathway activation but also $G\alpha_{q/11}$ – calcium pathway activation upon ligand binding (Kandola *et al.* , 2014). Therefore, like forskolin and cholera toxin, which directly activate adenylate cyclase and thereby stimulate only the cAMP pathway, JV-GL1 may induce signal bias (Wold et al., 2019) and signal primarily via the $G\alpha_s$-cAMP pathway by virtue of a novel interaction with the EP_2 receptor. A study using cellular dielectric spectroscopy demonstrated that the EP_2 antagonist AH6809 could not completely block JV-GL1 at the EP_2 receptor, in human ciliary muscle cells

(Woodward, *et al.* , 2019a). This finding suggests that JV-GL1 may be binding to a possible allosteric site on the EP_2receptor where endogenous PGE_2 does not bind, or bind to the EP_2 receptor in a different way. Allosteric binding and a potential $G\alpha_s$-cAMP pathway bias may be responsible for JV-GL1 long duration effects. This would set it apart from unbiased EP_2 agonists such as butaprost and merit further research into its mechanism of action.

The long duration of action of JV-GL1 may result from allosteric binding, thereby implying that the binding of other anti-glaucoma EP_2 agonists (Woodward et al., 2019b) to their target receptor is orthosteric. The long duration of action on IOP and the inherent activity on human ciliary smooth muscle cells could be indicative of JV-GL1 as a positive allosteric modulator. One, and only one, possible published explanation (Jiang et al., 2010) directly relevant to the ultra-long action of JV-GL1 on IOP involves allosteric modulation of the EP_2 receptor. More pertinent to the extended ocular hypotensive activity is that allosteric potentiation of EP_2 receptor activity is favoured by an ester moiety compared to a carboxylate[22]. The unexpected activity of JV-GL1 on ciliary smooth muscle cells correlates with manifestation of activity in intact cells rather than cell-free assays for EP_2 allosteric potentiators[22]. JV-GL1 would be an ideal compound for elucidating the crystal structure of the EP_2 and thereby gain valuable insights into orthosteric and allosteric binding sites and drug design.

In summary, the long-acting effects of JV-GL1 on IOP are EP_2 receptor mediated and this provides an intriguing avenue of future investigation with respect to mechanisms that impart such extended biological activities. Beyond the significant potential benefits of JV-GL1 for improved glaucoma treatment, there are also implications for pre-term labour, asthma, and the anti-inflammatory effects of EP_2 agonists. Future research may reveal similar advantages for compounds designed to be ultra-long acting at other GPCRs.

Author contributions

Jacques A Bertrand, co-authored first draft of manuscript, performed all genetic/molecular biological studies, performed all intraocular pressure studies, and co-performed aqueous humor dynamics studies

David F Woodward, co-authored first draft of manuscript, conceived and directed the research, co-invented JV-GL1, co-authored final version.

Joseph M Sherwood, Invented and co-performed *iPerfusion* studies, devised graphical presentation, co-directed statistical analyses.

Jenny W Wang, co-invented JV-GL1 and provided samples of JV-GL1 and its free acid, co-authored second draft.

Robert A Coleman, provided expert advice on EP2 receptor pharmacology, co-authored second draft.

Darryl R Overby**, s** tudy director, co-directed data analysis and presentation, co-authored final version

Conflict of interest

DF Woodward and JW Wang are inventors of a patent asssigned to the company that they founded, JeniVision.

Declaration of transparency and scientific rigour

This declaration acknowledges that this paper adheres to the priciples for transparent reporting and scientific rigour of pre-clinical research recommnded by funding agencies, publishers, and other organisations engaged in supporting research.

Figures and Legends

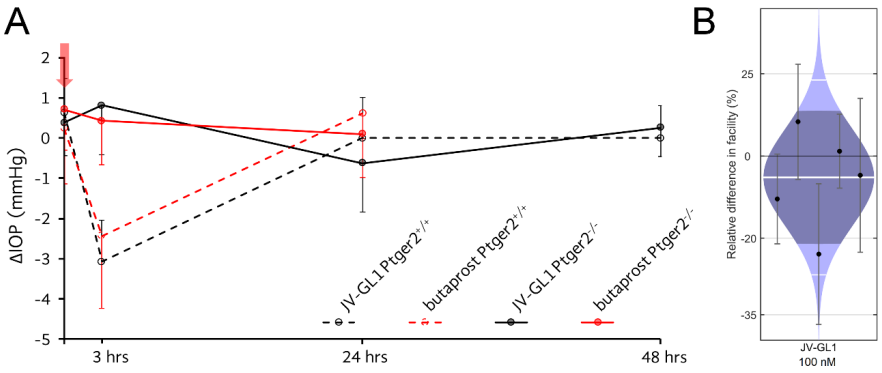

Figure 1. A) Effect of JV-GL1 and butaprost in normotensive Ptger2$^{+/+}$ and Ptger2$^{-/-}$ mice. Three hours after single topical applications of 0.01% JV-GL1 or 0.01% butaprost to Ptger2$^{+/+}$ mice, ΔIOP decreased to -3.1 [-4.1, -2.1] mmHg and -2.4 [-4.2, -0.6] mmHg respectively, returning to -0.002 [-1.0, 1.0] mmHg and 0.6 [-0.5, 1.5] mmHg by 24 hours. No effect was observed in Ptger2$^{-/-}$ mice after 3 hours (0.8 [-0.4, 2.0] mmHg vs 0.4 [-0.7, 1.5] mmHg, for JV-GL1 and butaprost respectively. Error bars are 95% confidence intervals. ΔIOP represents the difference in IOP between the treated eye relative to the contralateral untreated eye. **B)** Acute effect of 100 nM de-esterified JV-GL1 on outflow facility of enucleated C57BL/6J mouse eyes was not significantly different relative to contralateral vehicle perfused control eyes (-6% [-22, 13], P =0.4, n=5 pairs). Each data point shows the relative difference in reference facility C_r, between paired eyes comparing the treated with respect to the untreated control eye. The error bars represent the 95% confidence intervals on the relative difference in C_r. The light shaded region represents the best estimate of the sample distribution, with the geometric mean and two-sigma limits represented by the thick and thin horizontal white lines respectively. Dark central bands within the distribution represent the 95% CI on the estimated mean.

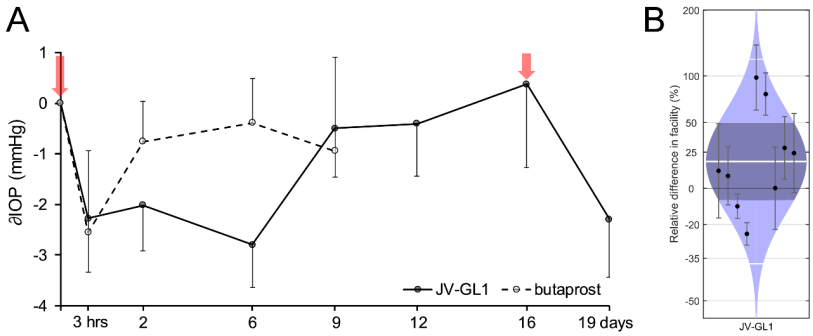

Figure 2. A) Effect of JV-GL1 and butaprost on IOP of SI-OHT mice relative to the pre-treatment time point (δIOP). Single, bilateral topical applications of 0.01% JV-GL1 to SI-OHT C57BL/6J mice (first red arrow) reduced IOP by -2.3 [-3.4, -1.2] mmHg (P =0.01; n=9) after 3 hours and maintained significant suppression for up to 6 days (-2.8 [-3.7, -2.0]; P =0.001; n=9). Comparatively, 0.01% butaprost reduced IOP after 3 hours by -2.5 [-4.2, -1.0] mmHg, returning to pre-treatment levels by the following 2-day time point (-0.8 [-1.5, 0.0] mmHg; P =0.6; n=9). A second unilateral topical application of 0.01% JV-GL1 (second red arrow) significantly reduced IOP of the treated eye by -2.3 [-3.4, -1.2] mmHg after 3 days, compared to contralateral vehicle treated eye (P =0.04, n=9). Error bars are 95% confidence intervals.**B)** 3 days after treatment with JV-GL1, outflow facility was not significantly altered (18% [-7%, 50%]; P=0.2, n=9 pairs) relative to vehicle treated eyes of SI-OHT C57BL/6J mice. Each data point shows the relative difference in reference facility, C_r, between paired eyes comparing the treated with respect to the untreated control eye. The error bars represent the 95% confidence intervals on the relative difference in C_r. The light shaded

region represents the best estimate of the sample distribution, with the geometric mean and two-sigma limits represented by the thick and thin horizontal white lines respectively. Dark central bands within the distribution represent the 95% CI on the estimated mean.

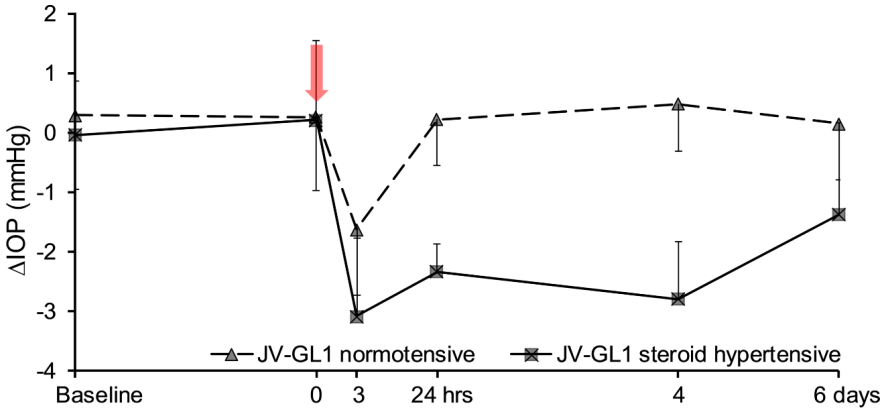

Figure 3. JV-GL1 decreases IOP in steroid treated ocular hypertensive mice after 3 hours, when ΔIOP = -3.1 [-4.4, -1.8] mmHg (P =0.01; n=8), with significant effects persisting for 4 days (ΔIOP = -2.8 [-3.8, -1.8] mmHg; P =0.04; n=8). In contrast, JV-GL1 decreases IOP in normotensive littermates at the 3 hour time point only (ΔIOP = -1.6 [-2.7, -0.5] mmHg; P =0.008; n=9). Red arrow indicates dosage time point and error bars are 95% confidence intervals.

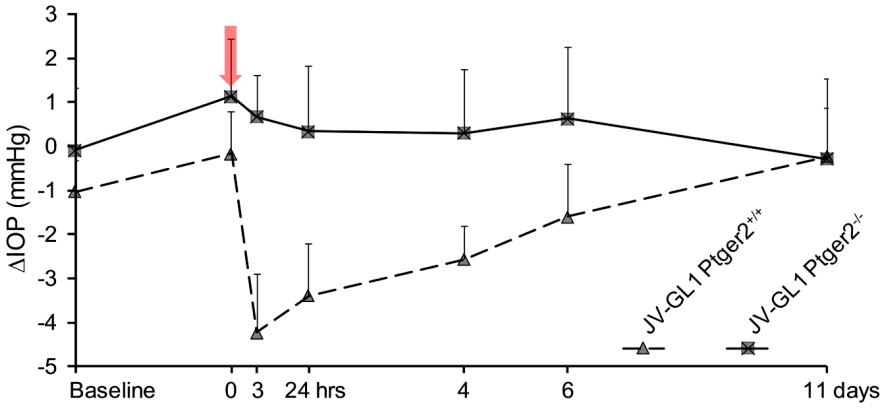

Figure 4. JV-GL1 decreases IOP in steroid induced ocular hypertensive *Ptger2*$^{+/+}$ mice after 3 hours, when ΔIOP = -4.2 [-5.6, -2.9] mmHg (P <0.0001; n=10), with significant effects persisting for 4 days (ΔIOP = -2.6 [-3.3, -1.8] mmHg (P =0.02; n=10). JV-GL1 does not significantly affect IOP in steroid induced ocular hypertensive*Ptger2*$^{-/-}$ mice. Red arrow indicates dosage time point and error bars are 95% confidence intervals.

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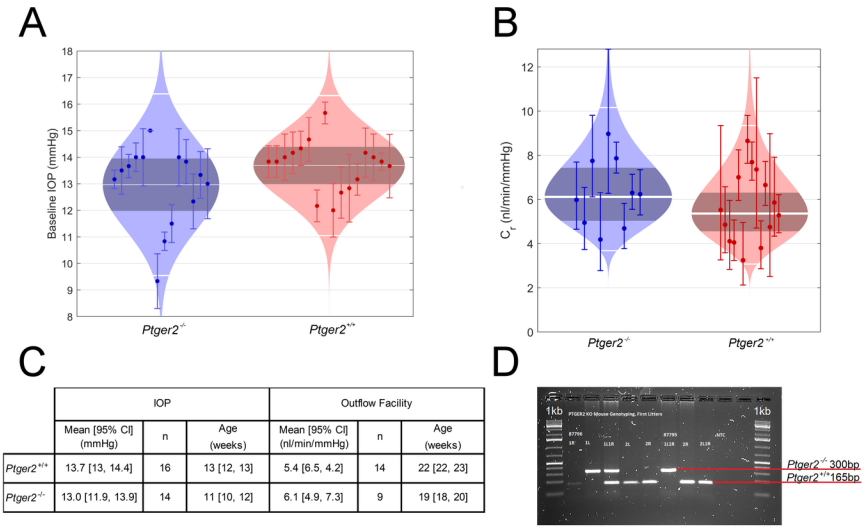
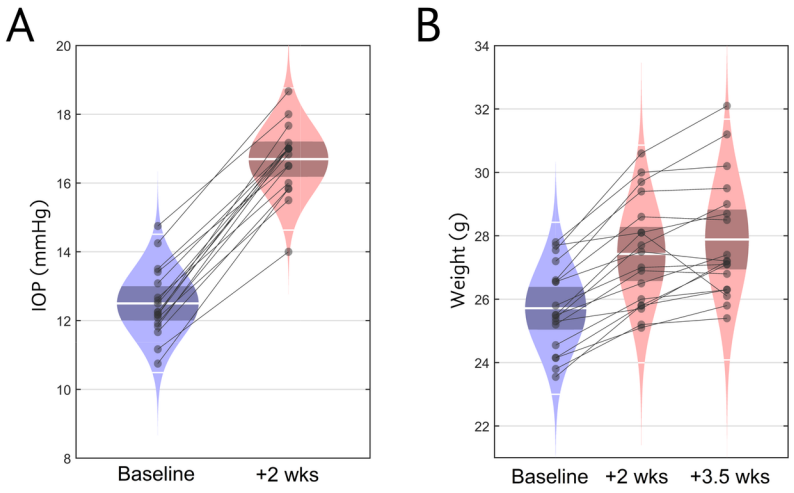
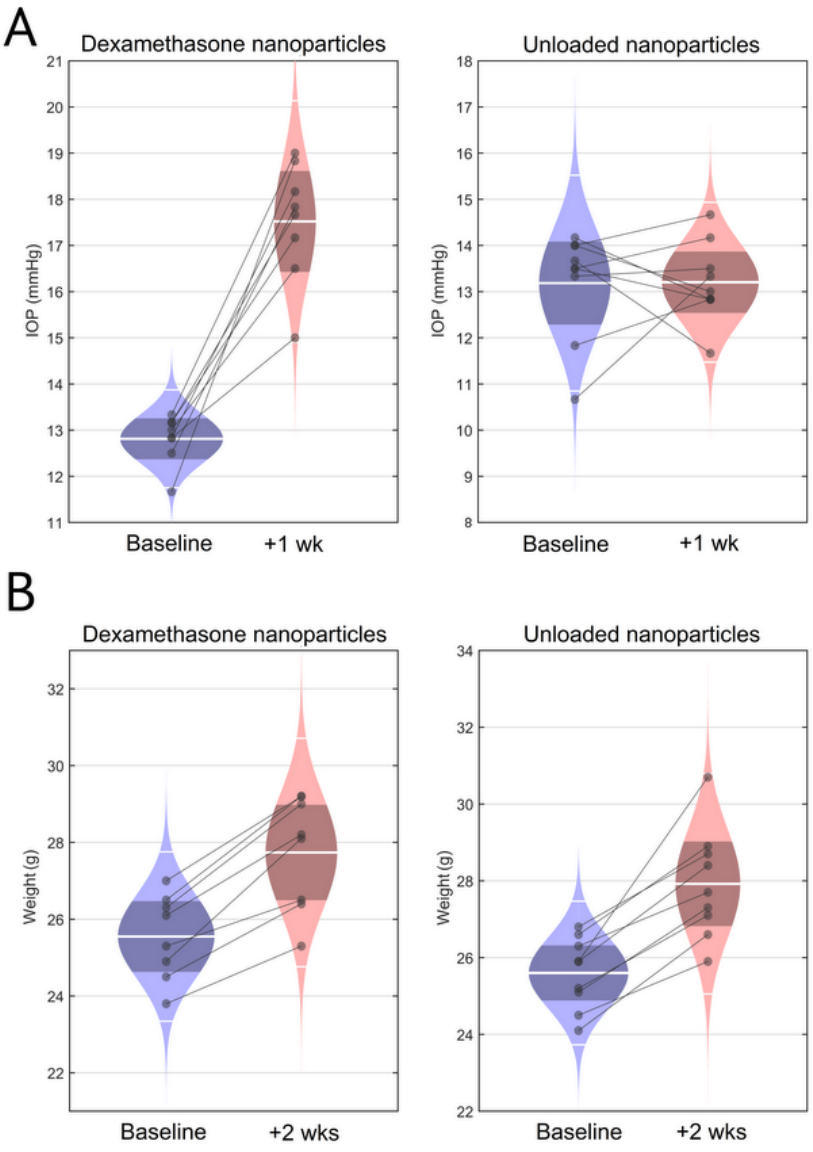


	IOP			Outflow Facility		
	Mean [95% CI] (mmHg)	n	Age (weeks)	Mean [95% CI] (nl/min/mmHg)	n	Age (weeks)
Ptger2$^{+/+}$	13.7 [13, 14.4]	16	13 [12, 13]	5.4 [6.5, 4.2]	14	22 [22, 23]
Ptger2$^{-/-}$	13.0 [11.9, 13.9]	14	11 [10, 12]	6.1 [4.9, 7.3]	9	19 [18, 20]

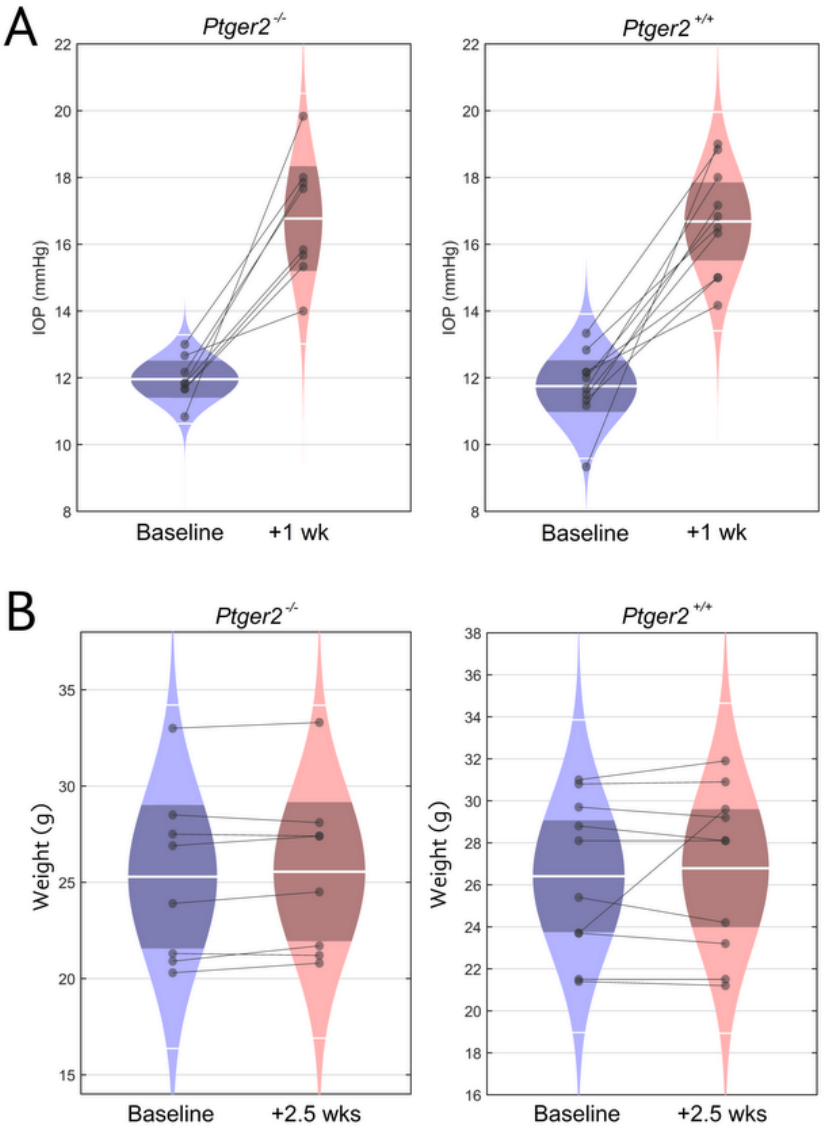
Supplemental Figure 1: IOP **(A)** and outflow facility**(B)** in *Ptger2* $^{+/+}$ mice and*Ptger2* $^{-/-}$ littermates. **(C)** Table of values. **(D)** Representative genotyping result from*Ptger2* $^{-/-}$ (300 base pairs) and littermate*Ptger2* $^{+/+}$mice (165 base pairs), ladder is 1 kb. The presentation of data in panels A and B is as described in Figures 1 and 2 of the main text.



Supplemental Figure 2: **(A)** IOP elevation in response to dexamethasone loaded nanoparticles. **(B)** Body weight of the mice was unaffected by the steroids, increasing from a mean of 25.7 [25, 26] grams to 27.9 [27, 29] grams over the duration of the study. The presentation of data in panels A and B is as described in Figures 1 and 2 of the main text, with grey lines connecting paired data at different time points.



Supplemental Figure 3: **(A)** IOP elevation in response to either dexamethasone loaded or unloaded nanoparticles in C57BL/6J mice. **(B)** Body weight of the mice was unaffected by the steroids. The presentation of data in panels A and B is as described in Figures 1 and 2 of the main text, with grey lines connecting paired data at different time points.



Supplemental Figure 4: **(A)** IOP elevation in response to dexamethasone loaded nanoparticles for *Ptger2*$^{-/-}$ and littermate *Ptger2*$^{+/+}$ mice. **(B)** Body weight of the mice was unaffected by the steroids. The presentation of data in panels A and B is as described in Figures 1 and 2 of the main text, with grey lines connecting paired data at different time points.