

Bimatoprost, Prostanamide Activity, and Conventional Drainage

Zhou Wan,¹ David F. Woodward,² Clive L. Cornell,³ Hans G. Fliri,³ José L. Martos,³ Simon N. Pettit,³ Jenny W. Wang,⁴ Alexander B. Kharlamb,⁴ Larry A. Wheeler,⁴ Michael E. Garst,⁵ Kari J. Landsverk,⁵ Craig S. Struble,⁵ and W. Daniel Stamer^{1,6}

PURPOSE. Despite structural similarity with prostaglandin F_{2α}, the ocular hypotensive agent bimatoprost (Lumigan; Allergan, Inc., Irvine, CA) shows unique pharmacology in vitro and functional activity in vivo. Unfortunately, the precise mechanisms that underlie bimatoprost's distinctive impact on aqueous humor dynamics are unclear. The purpose of the present study was to investigate the effects of bimatoprost and a novel prostanamide-selective antagonist AGN 211334 on human conventional drainage.

METHODS. Two model systems were used to test the consequences of bimatoprost and/or AGN 211334 treatment on conventional drainage. Human anterior segments in organ culture were perfused at a constant flow rate of 2.5 μL/min while pressure was recorded continuously. After stable baseline facilities were established, segments were treated with drug(s), and pressure was monitored for an additional 3 days. In parallel, the drugs' effects on hydraulic conductivity of human trabecular meshwork (TM) cell monolayers were evaluated. Pharmacological properties of AGN 211334 were characterized in isolated feline iris preparations in organ culture and heterologously expressed G-protein-coupled receptors were examined in vitro.

RESULTS. Bimatoprost increased outflow facility by an average of 40% ± 10% within 48 hours of treatment (*n* = 10, *P* < 0.001). Preincubation or coinubation with AGN 211334 significantly blunted bimatoprost's effects by 95% or 43%, respectively. Similar results were obtained in cell culture experiments in which bimatoprost increased hydraulic conductivity of TM cell monolayers by 78% ± 25%. Pretreatment with AGN 211334 completely blocked bimatoprost's effects, while coinubation decreased its effects on average by 74%. In both

models, AGN 211334 alone significantly decreased fluid flux across trabecular tissues and cells.

CONCLUSIONS. The findings indicate that bimatoprost interacts with a prostanamide receptor in the trabecular meshwork to increase outflow facility. (*Invest Ophthalmol Vis Sci.* 2007;48:4107-4115) DOI:10.1167/iov.07-0080

Glaucoma is a leading cause of adult blindness, affecting nearly 70 million people worldwide.^{1,2} The most common form, primary open-angle glaucoma, is characterized by decreased outflow through the conventional drainage pathway that results in ocular hypertension.³⁻⁵ Elevated intraocular pressure (IOP) over time appears to contribute to blindness by increasing mechanical stress on the optic nerve head, resulting in irreversible damage to retinal ganglion cell axons.^{6,7}

Because of their efficacy at lowering IOP, prostaglandin (PG) compounds have been widely used in clinical practice to treat ocular hypertension. The first PG mimetic used in the successful management of IOP was latanoprost, a synthetic PGF_{2α} analogue. Latanoprost is relatively inactive until its isopropyl ester is hydrolyzed to create a biologically active free acid that then functions as an FP receptor agonist.⁸ Because of the efficacy of latanoprost, two additional mimetics, travoprost and unoprostone, have been developed for the treatment of ocular hypertension. The hypotensive activity of these three F_{2α} analogues seems to be accomplished by long-term remodeling of the extracellular matrix in the ciliary body.^{9,10} Thus, the IOP lowering by PGs appears to be predominantly due to enhanced uveoscleral (unconventional) outflow.¹¹

Recently, a related PG compound, the prostanamide bimatoprost, was introduced, and has been shown to be an effective ocular hypotensive agent in patient studies.¹²⁻¹⁴ Bimatoprost is synthetic molecule derived from anandamide that has structural and pharmacological similarity to PGF_{2α} ethanolamide. Although structurally similar, evidence shows that bimatoprost possesses unique pharmacologic and pharmacokinetic properties, distinct from known FP receptor agonists. For example, 1000-fold higher concentrations of bimatoprost than PGF_{2α} are necessary to induce [Ca²⁺]_i mobilization in cells that express endogenous FP receptors or cells that heterologously express human FP receptors.^{14,15} Moreover, clinical pharmacologic studies with bimatoprost reveal that, unlike latanoprost, bimatoprost is not significantly metabolized, because of the absence of free acid hydrolysis product in systemic circulation after topical ocular administration to human volunteers.^{16,17} The hydrolysis of bimatoprost to a free acid occurs at a very slow rate (<1% per hour) when exposed to several ocular and nonocular tissues in three studies^{14,16,18} and at a higher rate in two other studies.^{19,20} Last, bimatoprost fails to activate more than 100 known drug targets, including a variety of receptors that may be involved in regulating IOP.¹⁴ Unfortunately, because a prostanamide receptor has not been cloned, the existence of prostanamides is currently based on pharmacologic criteria.

From the Departments of ¹Ophthalmology and Vision Science and ⁶Pharmacology, University of Arizona, Tucson, Arizona; the Departments of ²Biological Sciences and ⁴Chemical Sciences, Allergan, Inc., Irvine, California; the ³Department of Chemistry, Selcia, Ltd., Ongar, United Kingdom; and ⁵Covance, Inc., Madison, Wisconsin.

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Corresponding author: W. Daniel Stamer, Department of Ophthalmology and Vision Science, The University of Arizona, 655 North Alvernon Way, Suite 108, Tucson, AZ 85711; dstamer@eyes.arizona.edu.

TABLE 1. Characteristics of Human Donor Eyes

Donor	ID	Age (y)	Gender	Death to Enuc. (h)	Death to Perf. (h)	Histo Score	CCT Slope ($\mu\text{m}/\text{h}$)
1	164	71	F	7.0	25	2.3	-1.5
	163					3.0	-1.6
	160					2.6	-2.2
2	159	91	M	16.0	40	2.3	-0.9
	158					3.0	-3.8
3	157	67	M	2.7	41	2.8	-4.2
	156					2.3	-0.6
4	155	76	M	4.0	34	2.5	-2.6
	150					3.1	-4.6
5	149	67	M	3.5	36	3.2	-2.8
	148					2.9	0.1
6	147	50	F	8.0	35	2.9	-1.2
	146					1.5	0.5
7	145	65	F	6.0	32	2.0	0.5
8	144	67	M	4.0	39	2.3	-0.7
	140					2.6	0.3
9	139	63	F	5.0	33	3.1	-0.3
	138					3.0	-0.8
10	137	49	M	5.0	36	2.0	-1.6
11	135	76	F	8.0	30	2.9	-0.4
Mean \pm SEM		67.5 \pm 3.5	5F, 6M	6.3 \pm 1.1	34.6 \pm 1.6	2.7 \pm 0.1	-1.4 \pm 0.3

ID, identification; Enuc., enucleation; Perf., perfusion; Histo, histology.

The mechanism by which prostamides differ from $\text{PGF}_{2\alpha}$ agonists in their efficacy toward IOP regulation is still unknown. A recent study showed that bimatoprost treatment dampens the increase in IOP caused by water drinking in a group of patients with glaucoma, suggesting an effect on the pressure-sensitive, conventional drainage pathway.¹² In recent clinical studies, bimatoprost successfully lowered IOP in patients who were refractory to latanoprost therapy, suggesting differences in the mechanism of action of prostamide and $\text{PGF}_{2\alpha}$ -receptor agonists.^{13,21} In addition to changes observed in the extracellular matrix of the ciliary body, bimatoprost-treated monkeys displayed morphologic changes in their conventional drainage pathway after 1 year of treatment.²² Taken together, these data suggest that bimatoprost acts on the conventional drainage tract.

To test specifically the effects of bimatoprost on conventional drainage, we used the anterior segment perfusion model, that preserves the architecture of the trabecular meshwork (TM) and allows the testing of conventional outflow function separately from unconventional function. To examine the role of prostamide receptors in control of conventional drainage, we tested the ability of a second-generation prostamide-selective antagonist, AGN 211334, to block bimatoprost's effects. AGN 211334 is the latest compound in the series and is more than 10 times more potent than the prototypical prostamide antagonist AGN 204396.²³ The presence of prostamide receptor activity in human TM cells was tested by recording changes in hydraulic conductivity of primary cultures of TM cell monolayers on bimatoprost/AGN 211334 treatments. Results show that bimatoprost interacted with prostamide receptors on TM cells to increase outflow facility in situ and hydraulic conductivity in vitro.

METHODS

Materials

Bimatoprost was synthesized by Allergan Inc. (Irvine, CA). AGN 211334 was designed and synthesized by Selcia Ltd. (Ongar, UK). Stock solutions were prepared by dissolving drugs in ethanol giving a stock concentration of 10^{-3} M for bimatoprost and 3×10^{-3} M for AGN

211334 that were kept at -20°C until use. Stock solutions of isoproterenol (10^{-2} M; Calbiochem, La Jolla, CA) were made fresh in perfusion medium. Final solutions were prepared fresh by diluting stock solutions in perfusion medium immediately before chamber exchanges.

Anterior Segment Perfusion Model

Fresh human eyes were obtained postmortem from the National Disease Research Interchange (Philadelphia, PA) and the Donor Network of Arizona (Phoenix, AZ). Characteristics of the eyes are shown in Table 1. The eyes were free of any known ocular disease, and were stored in moistened chambers at 4°C until dissected. Preparation and perfusion of anterior segments were performed exactly as previously described by our laboratory, slightly modifying original descriptions of perfusion methods.²⁴⁻²⁷ After dissection and mounting into culture chambers, anterior segments were perfused at a constant flow rate of $2.5 \mu\text{L}/\text{min}$ with Dulbecco's modified Eagle's medium (DMEM), to which antibiotics (penicillin 100 U/mL, streptomycin 100 $\mu\text{g}/\text{mL}$; Sigma-Aldrich, St. Louis, MO), bovine serum albumin (BSA) 25 mg/dL, and 1% fetal bovine serum (FBS) were added. The anterior segments were cultured at 37°C in humidified air containing 5% CO_2 . Intrachamber pressures were continuously recorded with dedicated pressure transducers that interfaced with a digital data recorder and computer.

When stable baseline facilities were reached (typically after 2-4 days of perfusion), medium in anterior segments was exchanged under approximately 10 mm Hg (13.6 cm H_2O) of constant pressure with medium containing 1 μM bimatoprost. We chose this concentration based on ciliary body and iris tissue concentrations of bimatoprost observed in topically treated nonhuman primates²⁸ and from preliminary studies in which we performed sequential dose-response exposures (10 nM-1 μM) and obtained consistent results only at 1 μM (not shown). Contralateral segments were exchanged with 30 μM AGN 211334 plus 1 μM bimatoprost (protocol 1) or 30 μM AGN 211334 alone and then were constantly perfused with drug solution. Anterior segments that initially received AGN 211334 were exchanged with medium containing 30 μM AGN 211334 plus 1 μM bimatoprost after 24 hours of pretreatment (protocol 2) and then were constantly perfused with drug solution. Forty-eight hours after initial drug treatments, medium from anterior segment pairs was exchanged with fresh me-

dium without drug(s), and the segments were perfused for an additional 3 to 4 days.

Central Corneal Thickness

A pachymeter (SP-100 Handy Pachymeter; Tomey Corp., Nagoya, Japan) was used to obtain central corneal thickness (CCT) measurements of whole globes on arrival at our laboratory and of anterior segments during perfusion.²⁷ After initial measurements on whole globes, CCT was measured on anterior segments 2 hours after the start of perfusion, and every 24 to 48 hours afterward.²⁷ Data points were the average of three readings taken sequentially. If one of the readings was significantly different from the other two (>100 μm), two more readings were made, and both the highest and the lowest were discarded. The slope was calculated from the CCT measurements obtained after the start of perfusion to the day of the first of the drug treatment(s).

Morphologic Analysis

At the end of perfusion, medium in anterior chambers was exchanged with 3% paraformaldehyde (PFA) in phosphate-buffered saline (pH 7.4) under 10 mm Hg pressure. After perfusion at 2.5 $\mu\text{L}/\text{min}$ for 1 hour with PFA, anterior segments were removed from culture chambers and several wedges (~2 mm wide) containing outflow tissues were cut from each of four quadrants using a no. 15 scalpel blade and were stored in 2% PFA. Representative wedges from each quadrant were embedded in Spurr's plastic according to standard methods and stained with toluidine blue.²⁶ Sagittally oriented 0.5 μm sections were viewed by light microscopy (BH-2; Olympus, Tokyo, Japan) with an upright microscope at magnifications of 200 \times and 400 \times . All sections were evaluated in a masked fashion by two observers according to a grading scheme that is described elsewhere²⁷: 0, no cells in the trabecular meshwork (TM) or only a few swollen cells, with inner wall disruption (breaks, other damage) present; 1, only a few cells in the TM, typically in the juxtacanalicular tissue (JCT), but existing cells show little or no swelling, with the inner wall intact; 2, JCT well populated with cells, corneoscleral and uveal meshworks contain few or no cells, and intact inner wall; 3, JCT and most of corneoscleral meshwork filled with cells, normal-appearing cells (no swelling), and intact inner wall; and 4, essentially normal-looking trabecular meshwork, the cells present everywhere in the JCT and corneoscleral meshwork (uveal mesh not considered), and intact inner wall.

The final reported grade for each anterior segment was calculated by averaging the scores of all four quadrants in an anterior segment from two observers (Table 1).

Cell Culture

Three previously characterized strains of human trabecular meshwork cells (HTM61, -86, and -89) were used in the present study.^{29,30} HTM cells were cultured in Dulbecco's modified Eagle's medium (low-glucose DMEM; Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (Gemini, Woodland, CA) and 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.29 mg/mL glutamine (Invitrogen), and grown in humidified air containing 5% CO₂ at 37°C. Cells (1 \times 10⁵) were seeded onto polycarbonate membrane filters (tissue culture treated, 12-mm diameter, 0.4- μm pore size; Corning Inc., Corning, NY) at confluence and the monolayers allowed to mature 9 to 12 days for before the experiments.

Cell Perfusion

Filters with cells were placed in an Ussing-type chamber filled with HEPES-buffered DMEM (25 mM HEPES; pH 7.4). The chamber, tubing, and reservoir were gently filled with DMEM+HEPES, and the cells were allowed to acclimate for 30 minutes at 37°C with no pressure gradient. The experiment was begun by raising the reservoir to 13.6 cm above the midline of the filter containing cells (giving a 10-mm Hg pressure differential across the cells) and allowed to perfuse for 30 minutes at 37°C, giving initial baseline measurement. Afterward, the

chamber was exchanged with fresh medium containing 1 μM isoproterenol, 1 μM bimatoprost, and/or 30 μM AGN 211334. The cell monolayers were again exposed to a pressure head of 10 mm Hg for 30 minutes, and hydraulic conductivity was recorded.^{31,32} The experiments were concluded by removing the filters from the chamber, rinsing cells twice in phosphate-buffered saline and fixing cells with 4% paraformaldehyde in PBS. For inclusion of the data, initial hydraulic conductivity measurements (both before and after mock exchange) must have been stable, (i.e., within 5% of each other) and in the span of 1.5 to 6 $\mu\text{L}/\text{min}/\text{mm Hg}/\text{cm}^2$ such that drug-induced changes relative to the baseline would remain in the range of detection for the force displacement transducer.

Feline Iris Contraction Model

Feline iris sphincter tissues prepared as described previously were mounted vertically under 50 to 100 mg tension in a jacketed 10-mL organ bath.³³ Smooth muscle tension of the isolated iris sphincter was measured isometrically with force displacement transducers (FT-03; Grass Telefactor, West Warwick, RI) and recorded on a polygraph (model 7; Grass Telefactor). The organ baths contained Krebs' solution maintained at 37°C by a heat exchanger and circulating pump. The Krebs' solution (118.0 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.9 mM CaCl₂, 1.18 mM MgSO₄, 25.0 mM NaHCO₃, 11.7 mM glucose, and 0.001 mM indomethacin) was gassed with 95% O₂ and 5% CO₂ to give a pH of 7.4. Tissues were allowed 60 minutes to stabilize before each experiment. The feline iris experiments were designed so that a direct, four-way comparison for antagonist versus prostamide, vehicle versus prostamide, antagonist versus corresponding PG, and vehicle versus corresponding PG was provided in tissue preparations obtained from a single animal. One cumulative dose-response curve to agonist was obtained in each tissue. Vehicle (ethanol) and antagonist (AGN 211334) were given 30 minutes before the agonist dose-response curves were constructed. The response to PGF_{2 α} 10⁻⁷ M was determined at the beginning and end of each dose-response curve, with appropriate washout, and responses were calculated as the percentage of this reference contraction.

Ca²⁺ Signaling Studies on Human Recombinant Prostanoid Receptors

The use of chimeric G protein cDNAs (prostanoid DP, EP₁, EP₂, EP₄, FP, IP, and TP) stably expressed in HEK-293 EBNA cells allowed responses to G_s- and G_i-coupled prostanoid receptors to be measured as a Ca²⁺ signal, as previously described.³³ Ca²⁺ signaling studies were performed with an FLIPR (fluorometric imaging plate reader). Cells were seeded at a density of 5 \times 10⁴ cells/well in a poly-D-lysine-coated (BioCoat), black-walled, clear-bottomed, 96-well plates (BD Biosciences, Franklin Lakes, NJ) and allowed to attach overnight in an incubator at 37°C. The cells were then washed twice with HBSS-HEPES buffer (Hanks' balanced salt solution without bicarbonate and phenol red, 20 mM HEPES; pH 7.4) with a plate washer (Denley Cellwash; LabSystems, Franklin, MA). After 45 to 60 minutes of dye loading in the dark using the Ca²⁺-sensitive dye Fluo-4AM, at a final concentration of 2 \times 10⁻⁶ M, the plates were washed four times with HBSS-HEPES buffer to remove excess dye and leaving 100 μL of buffer in each well. The plates were then placed in the FLIPR instrument and allowed to equilibrate at 37°C. Compound solutions were added in a 50- μL volume to each well to give the desired final concentration. Cells were excited through an argon laser at 488 nm, and emission was measured through a 510- to 570-nm band width emission filter (FLIPR; Molecular Devices, Sunnyvale, CA). The peak increase in fluorescence intensity was recorded for each well.

The experimental design for the FLIPR studies was as follows. On each plate, four wells each served as negative (HBSS-HEPES buffer) and positive controls (standard agonist: for DP, BW 245C; for EP₁-EP₄, PGE₂; for FP, PGF_{2 α} ; for IP, carbaprostacyclin; and for TP, U-46619). The peak fluorescence change in each well containing drug was expressed relative to the control. To obtain concentration-response

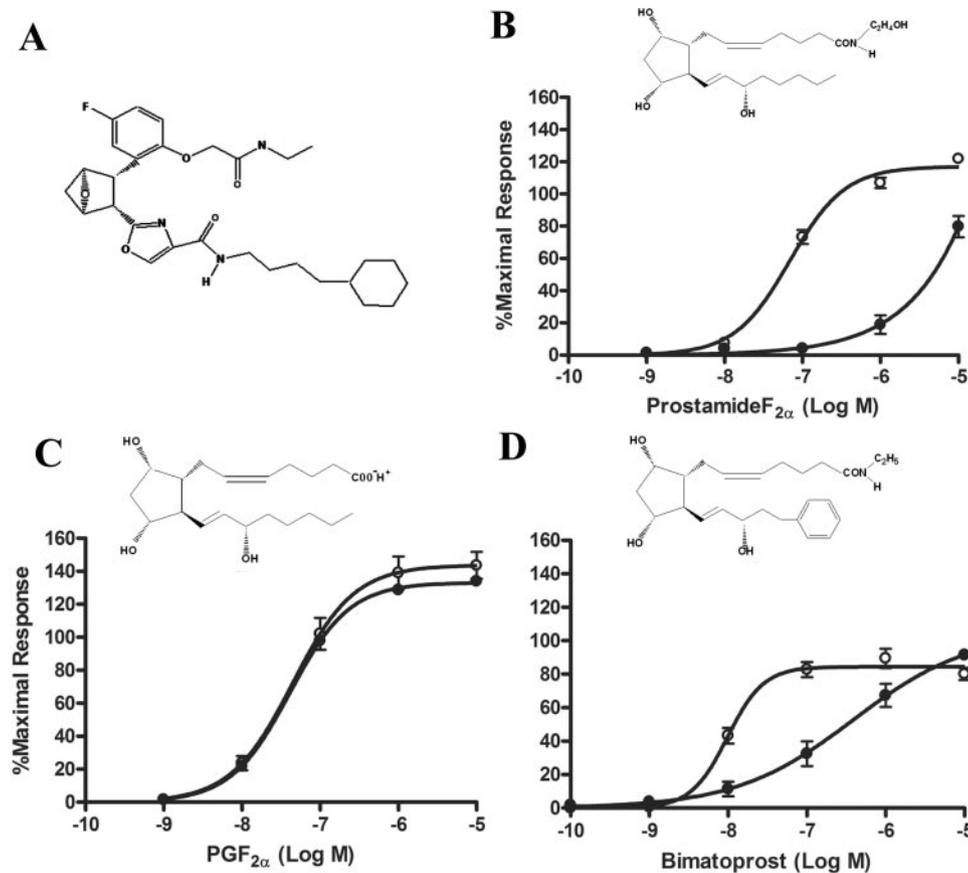


FIGURE 1. Specificity of the prostamide antagonist, AGN 211334. (A) Chemical structure of AGN 211334. Effects of the presence of AGN 211334 (30 μ M) on contraction of the feline iris produced by (B) prostamide F_{2 α} (C) PGF_{2 α} , and (D) bimatoprost are shown. Concentration-response curves are displayed for each agonist, either in the presence (●) or absence (○) of antagonist. Values shown are the mean \pm SEM. AGN 211334 significantly changed the EC₅₀ (median effective concentration) of bimatoprost and prostamide, but not of PGF_{2 α} ($P < 0.01$).

curves, compounds were tested in duplicate in each plate over the desired concentration range. Each compound was tested on at least three separate plates using cells from different passages to give $n = 3$.

Statistical Analysis

Drug effects were expressed as the percentage increase or decrease in outflow facility (or hydraulic conductivity) after drug administration (Cd) compared with baseline (Co) and calculated as $(Cd - Co)/Co \times 100\%$. A paired two sample *t*-test was performed for statistical analysis. $P < 0.01$ were considered to be statistically significant. For anterior segment perfusions, the Co was the mean outflow facility that was stable for at least 24 hours before any treatment. Cd was the mean facility of the second 12 hours after each treatment day. Washout facility was the mean facility of the second 12 hours after washout. Values are expressed as the mean \pm SEM.

RESULTS

AGN 211334 Blockade in the Feline Iris

The structures of a prostamide antagonist AGN 211334, prostamide F_{2 α} , PGF_{2 α} , and bimatoprost are shown in Figure 1. To examine the specificity of AGN 211334 as an antagonist, effects of AGN 211334 (30 μ M) on contractions produced by prostamide F_{2 α} , PG-F_{2 α} , and bimatoprost are shown in Figures 1B, 1C, and 1D, respectively. AGN 211334 produced a clear rightward shift of the bimatoprost concentration-response curve (10^{-8} – 10^{-5} M, Fig. 1D) and a clear rightward shift of the prostamide F_{2 α} concentration-response curve (10^{-9} – 10^{-5} M, Fig. 1B) as well, but no significant shift of the PGF_{2 α} concentration-response curve (Fig. 1C) in feline iris preparations.

AGN 211334 Blockade at Human Recombinant Prostanoid Receptors

The effects of a 30- μ M concentration of AGN 211334 on Ca²⁺ signals associated with human recombinant prostanoid receptor activation are summarized in Table 2. Antagonism was not apparent at the DP, EP₁, EP₂, EP₃, EP₄, FP, or IP receptors, but AGN 211334 was an efficacious TP receptor antagonist ($K_b = 16$ nM). The data in Table 3 are compared with the K_b value obtained for AGN 211334 versus prostamide F_{2 α} in the isolated feline iris, as previously described.^{3,4}

Bimatoprost and AGN 211334 Effects on Outflow Facility

Perfused human anterior segments in organ culture were used to assess the effects of bimatoprost and AGN 211334 on con-

TABLE 2. Antagonist Activity of AGN 211334 at Prostamide Receptors in the Feline Iris and at Human Recombinant Prostanoid Receptors

Prostanoid Receptor	Antagonist K_b (nM) for AGN 211334
Prostamide	236
DP	Inactive
EP ₁	Inactive
EP ₂	Inactive
EP ₃	Inactive
EP ₄	37,048
FP	Inactive
IP	Inactive
TP	16

ventional outflow function. Twenty anterior segments from 11 donors were examined. Age of donors ranged from 49 to 91 (mean, 67.5 ± 3.5 years), average time from death to enucleation was 6.3 ± 1.1 hours, whereas mean time from death to perfusion was 34.6 ± 1.6 hours (Table 1).

To examine effects of bimatoprost on conventional drainage, two experimental paradigms were used: In the first, one anterior segment from a pair with stable outflow facilities was treated with bimatoprost, and the contralateral segment was exposed to bimatoprost ($1 \mu\text{M}$) plus AGN 211334 ($30 \mu\text{M}$). In the second protocol, one segment of the pair was treated with bimatoprost, and the other was first pretreated with AGN 211334 and then 24 hours later was treated with bimatoprost plus AGN 211334. Examples of traces from anterior segments that were subjected to the two protocols are shown in Figure 2. Both traces show that bimatoprost's effects were immediate and steady over the 2 days of exposure (black traces). On washout of bimatoprost with fresh perfusion medium, we observed two types of responses: Either facility stabilized at a level higher than original baseline (Fig. 2B, $n = 5$), or it continued to increase (Fig. 2A, $n = 5$), but at a more gradual rate. We compared the slope of facility increase during bimatoprost treatment (0.03 ± 0.01) with the slope of increase after washout (0.01 ± 0.01) and found them to be different ($P =$

0.02). In contrast, outflow facility in all anterior segments treated with bimatoprost and AGN 211334 concurrently increased at a rate lower than bimatoprost treatment alone (Fig. 2A). Outflow facility in all anterior segments pretreated with AGN 211334 before cotreatment with bimatoprost remained similar to initial baseline measurements (Fig. 2B).

A summary of outflow facility responses to drug treatments is shown in Figure 3. Ten anterior segments were exposed to bimatoprost with an average starting (baseline) facility of $0.18 \pm 0.04 \mu\text{L}/\text{min}/\text{mm Hg}$ (Table 2). When expressed as the percentage change from baseline, the average facility increase for the 10 segments was 14% after 24 hours ($P = 0.001$) and 40% after 48 hours ($P = 0.0002$). After washout, outflow facility continued to increase, but at a slower rate; reaching a maximum of 49% at 72 hours after initial exposure to bimatoprost ($P = 0.001$). The mean starting facility of four contralateral anterior segments cotreated with AGN 211334 plus bimatoprost was $0.24 \pm 0.09 \mu\text{L}/\text{min}/\text{mm Hg}$ (Table 2). The average increase in facility was 6% after 24 hours ($P = 0.02$) and 23% after 48 hours ($P = 0.03$). After washout, outflow facility on average was 17% higher than original baseline, but was not significantly different ($P = 0.3$). The average baseline outflow facility in six fellow segments pretreated with AGN 211334 alone was $0.15 \pm 0.02 \mu\text{L}/\text{min}/\text{mm Hg}$ (Table 3). On

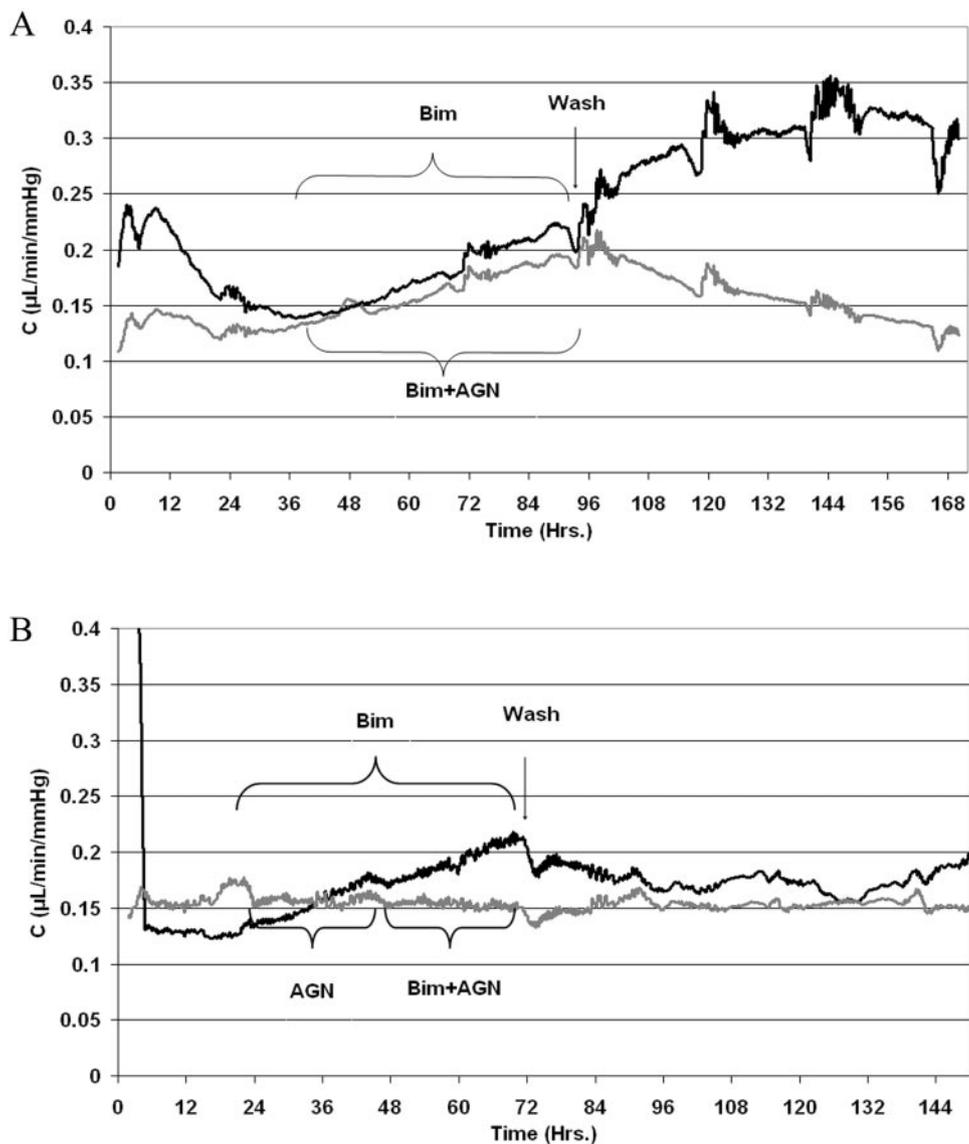


FIGURE 2. Facility traces of human anterior segment pairs treated with bimatoprost. After stable baseline facilities in both segment pairs were established, chambers were exchanged with medium containing $1 \mu\text{M}$ bimatoprost (BIM, black traces) or bimatoprost plus $30 \mu\text{M}$ of the prostanoid antagonist, AGN 211334 (AGN, gray traces). AGN 211334 was administered in one of two ways: contralateral segments were exposed to both compounds at the same time (A, segment pair 139/140), or control segments were first pretreated with AGN 211334 and then 24 hours later were treated with AGN plus bimatoprost (B, segment pair 149/150). Shown are traces representing data shown in Table 3.

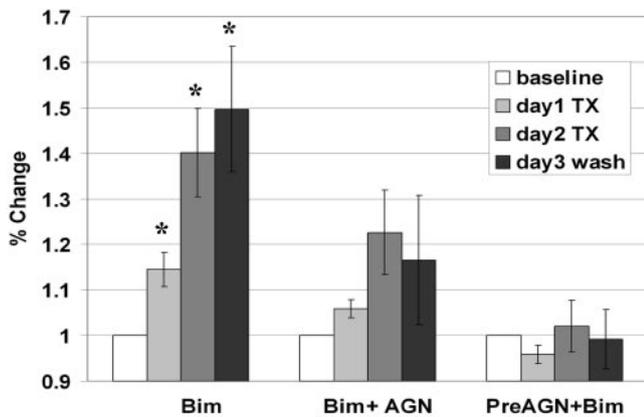


FIGURE 3. Summary of results showing effects of bimatoprost and/or AGN 211334 treatment on outflow facility in human anterior segments in organ culture. Data are expressed as the mean (\pm SEM) percentage of baseline outflow facility for comparisons between groups. Bimatoprost (BIM) treatment, significantly increased outflow facility after 24 hours (+14%, day 1; $P = 0.001$) and after 48 hours (+40%, day 2; $P = 0.0002$). After the chamber solution was exchanged with fresh medium (wash), average outflow facility measured 49% above original baseline ($P = 0.001$). For contralateral anterior segment controls, coadministration of bimatoprost with AGN 211334 (BIM+AGN) resulted in an outflow facility that was 6% above baseline by 24 hours ($P = 0.02$) and 23% by 48 hours ($P = 0.03$). After washout of the drugs, outflow facility was 17% above baseline ($P = 0.3$). For the anterior segments preincubated with AGN 211334 (PreAGN + Bim) facility decreased 4% with AGN 211334 alone ($P = 0.04$), increased by 2% after adding bimatoprost to AGN ($P = 0.3$), and was 1% less than baseline after washout ($P = 0.5$).

exposure of trabecular tissues to AGN 211334, outflow facility decreased by 4% after 24 hours ($P = 0.04$). Addition of bimatoprost to AGN-pretreated segments increased outflow facility by 2% above initial baseline ($P = 0.3$). After a chamber exchange

with fresh medium, average outflow facility in AGN-pretreated segments measured 1% lower than original baseline ($P = 0.5$). Shown in Figure 4 are representative images of histologic sections taken from an anterior segment pair that was treated with bimatoprost (Fig. 4A) or bimatoprost after pretreatment with AGN 211334 (Fig. 4B).

Bimatoprost and CCT

In addition to postperfusion evaluation of conventional outflow tissues by standard histology, CCT slope and outflow facility were two functional measurements in the present study that were used to assess the quality of anterior segment tissues during perfusion (Table 1).²⁷ The rate of CCT recovery was calculated as the rate of change of corneal thickness over the first 3 days of perfusion. On average, the CCT of the 20 anterior segments tested decreased by 1.4 $\mu\text{m}/\text{h}$ over the first 3 days of perfusion (Table 1).

After administrating drugs, we continued to measure CCT on all anterior segments. We observed that compared to initial measurements, CCT increased in 8 of 10 segments receiving bimatoprost alone. Shown in Figure 5, the average CCT in the bimatoprost alone group was $778 \pm 37 \mu\text{m}$ immediately before treatment and increased to $832 \pm 37.1 \mu\text{m}$ 2 days after treatment ($P = 0.015$). The CCT returned to an average of $784 \pm 56.8 \mu\text{m}$ 2 days after chamber exchange ($P = 0.4$). In contrast, anterior segments pretreated with AGN 211334 had no significant changes in CCT after drug treatment. The average CCT was $752 \pm 49 \mu\text{m}$ before treatment, $762 \pm 58 \mu\text{m}$ 2 days after treatment ($P = 0.3$), and $755 \pm 62 \mu\text{m}$ ($P = 0.5$) 2 days after washout.

Bimatoprost and AGN 211334 Effects on TM Monolayers

To determine whether bimatoprost’s molecular target in the conventional drainage tract resides on trabecular meshwork

TABLE 3. Summary of Facility Measurements, before and after Drug Treatments

Donor	ID	Drug Tx	Co ($\mu\text{L}/\text{min}/\text{mm Hg}$)	Cd ($\mu\text{L}/\text{min}/\text{mm Hg}$)			
				D1	D2	Wash	
	1	163	B	0.11	0.13	0.23	0.26
	2	160	B	0.09	0.10	0.14	0.18
	3	157	B	0.10	0.11	0.13	0.14
	4	156	B	0.13	0.13	0.14	0.15
	5	150	B	0.13	0.18	0.20	0.19
	6	148	B	0.51	0.54	0.54	0.53
	7	145	B	0.17	0.19	0.21	0.20
	9	140	B	0.16	0.17	0.22	0.28
	10	137	B	0.17	0.21	0.21	0.19
	11	135	B	0.19	0.21	0.28	0.28
		Mean \pm SE		0.18 ± 0.04	0.20 ± 0.04	0.23 ± 0.04	0.24 ± 0.04
	7	146	AB	0.13	0.14	0.19	0.20
	8	144	AB	0.19	0.21	0.24	0.23
	9	139	AB	0.16	0.16	0.18	0.16
	10	138	AB	0.50	0.51	0.51	0.44
		Mean \pm SE		0.24 ± 0.09	0.26 ± 0.09	0.28 ± 0.08	0.26 ± 0.06
	1	164	preA	0.12	0.12	0.13	0.13
	2	159	preA	0.13	0.11	0.10	0.09
	3	158	preA	0.09	0.09	0.10	0.10
	4	155	preA	0.16	0.15	0.15	0.15
	5	149	preA	0.15	0.15	0.16	0.15
	6	147	preA	0.23	0.22	0.27	0.26
		Mean \pm SE		0.15 ± 0.02	0.14 ± 0.02	0.15 ± 0.03	0.15 ± 0.03

ID, identification; Tx, treatment; Cd; outflow facility in the presence of drug; D1, day 1 treatment; D2, day 2 treatment; B, bimatoprost treat only; AB, AGN 211334 and bimatoprost co-treatment; preA, AGN 211334 pretreatment followed by bimatoprost treatment.

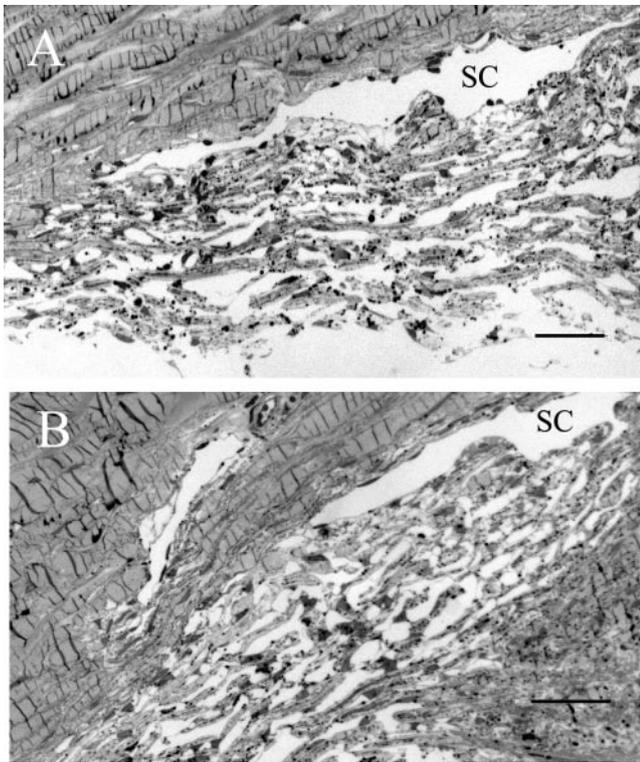


FIGURE 4. Histologic evaluation of conventional drainage tissues after drug treatment in organ culture. Shown are toluidine blue-stained, semithin sections from the anterior segment pair shown in Figure 2B. One anterior segment (A, segment 157) was treated with 1 μ M bimatoprost (BIM), whereas the contralateral segment (B, segment 158) was pretreated with AGN 211334 and then treated with 30 μ M AGN 211334 plus 1 μ M BIM. SC, Schlemm's canal. Bar, 100 μ m.

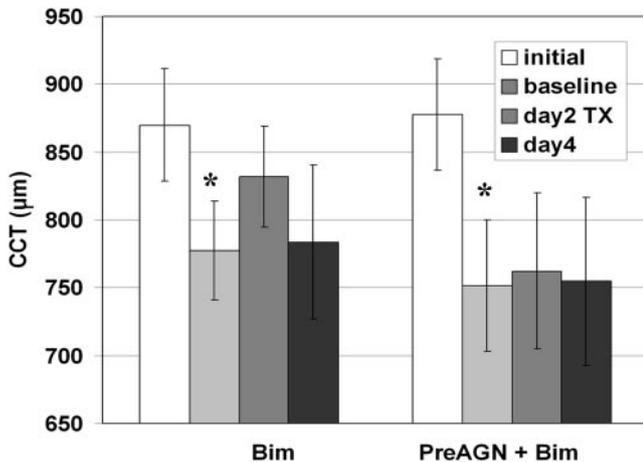


FIGURE 5. Summary of effects of bimatoprost treatment on CCT measurements of human anterior segments in organ culture. Initial average CCT at the start of perfusion was 870 ± 41 and 878 ± 41 μ m for bimatoprost (BIM)-treated and BIM plus AGN 211334-treated (pre-AGN+BIM) anterior segments, respectively. The average CCT decreased by 11% and 14% over the first 24 to 72 hours of perfusion for BIM- and pre-AGN+BIM-treated anterior segments, respectively. Forty-eight hours after treatment with BIM, average CCT of segments increased by 11% and 14% over the first 24 to 72 hours of perfusion for BIM- and pre-AGN+BIM-treated anterior segments, respectively. Forty-eight hours after treatment with BIM, average CCT of segments increased by 7% ($P = 0.015$). CCT began to return toward baseline measurements after washout of BIM. In pre-AGN segments however, BIM did not affect CCT measurements over time ($P = 0.3$).

cells, we examined the effects of bimatoprost on hydraulic conductivity (HC) across mature monolayers of human TM cells in culture. Figure 6A shows that bimatoprost (1 μ M) increased HC of TM monolayers by $78\% \pm 25\%$ and $75\% \pm 23\%$, respectively in two successive treatments ($P \leq 0.01$). As a positive control for this model, Figure 6B demonstrates that isoproterenol (1 μ M) significantly increased HC in two successive treatments as shown previously by others.³¹ However, Figures 6C and 6D show that when the drugs were used in combination, the effects of isoproterenol and bimatoprost were not additive or synergistic. To determine the specificity of AGN 211334 on bimatoprost's effects in this third model system, cell monolayers were either *preincubated* or *coincubated* with antagonist. Similar to results obtained in perfused human anterior segments, AGN 211334 (30 μ M) partially an-

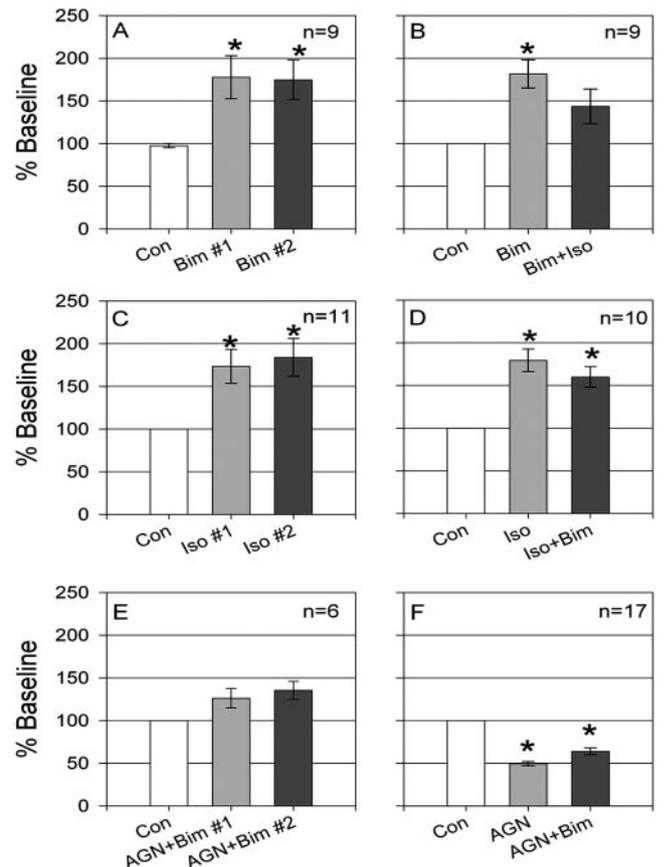


FIGURE 6. Effects of bimatoprost (BIM) and AGN 211334 effects on hydraulic conductivity of HTM monolayers. Fluid flow across cell monolayers in the apical to basal direction was driven by a pressure head of 10 mm Hg. Before drug treatments, baseline HC for each monolayer was measured (Con) and tested for stability after an exchange of the chamber solution with normal medium. Cells were then exposed to a single drug or drug combination by medium exchange. (A) Effect of two sequential treatments with BIM (1 μ M, BIM, $P = 0.01$ and $P = 0.008$ for BIM 1 and BIM 2, respectively). (B) Effects of BIM alone or in combination with isoproterenol (ISO; 1 μ M, BIM, $P = 0.001$ and $P = 0.06$ for BIM and Iso+BIM, respectively). (C) Results of two sequential ISO treatments ($P = 0.004$ and $P = 0.004$ for ISO 1 and ISO 2, respectively). (D) Effects of ISO and BIM treatments in reverse order of that shown in (B) ($P = 0.0002$ and $P = 0.0008$ for ISO and ISO+BIM, respectively). (E) Results when AGN 211334 (30 μ M, AGN) and BIM are used in combination ($P = 0.07$ and $P = 0.02$ for AGN+BIM 1 and AGN+BIM 2, respectively). (F) Effects of AGN alone or in combination with BIM ($P = 1 \times 10^{-12}$ and $P = 2 \times 10^{-8}$ for AGN and AGN+BIM, respectively). *Significant difference between the experimental and the control at $P < 0.01$.

tagonized bimatoprost's effects when introduced to the cells together (Fig. 6E) and totally blocked bimatoprost's effects if the cells were pretreated (Fig. 6F). Of note, when the TM cells were exposed to AGN 211334 alone, fluid flow across the cells decreased by $51\% \pm 0.8\%$ ($P < 0.001$, Fig. 6F).

DISCUSSION

Our findings provide, for the first time, direct evidence that bimatoprost interacts with cells of the conventional drainage pathway to increase outflow facility. Such effects were attenuated significantly by pretreatment or coadministration with the prostamide-specific antagonist AGN 211334. Prostamide receptors appear to be located on TM cells, because AGN 211334 effectively blocked bimatoprost-induced increases in hydraulic conductivity of TM cell monolayers. Collectively, these results suggest that in addition to effects shown previously on uveoscleral outflow, bimatoprost working through prostamide receptors, increases conventional outflow.

To test our hypothesis that bimatoprost affects conventional drainage, fresh human anterior segments in organ culture were used, providing several advantages over other model systems. First, the architecture and cellular relationships in the conventional drainage tract are preserved; second, the anterior segment allows for long-term study of drug effects, enabling multiple manipulations (e.g., sequential drug treatments) to occur over the lifetime of the experiment³⁵; third, the conventional drainage pathway in humans differs from that in other species, including nonhuman primates, in terms of micro and gross anatomy³⁶⁻³⁹; last, and most relevant to the present study, nonhuman primates seem to differ from humans with respect to bimatoprost's effects on outflow facility. Thus, bimatoprost appears to increase conventional and unconventional outflow facility in humans, but only unconventional in monkeys.^{14,40}

The cellular target (receptor) for bimatoprost has been controversial. Some argue that bimatoprost is a prostaglandin $F_{2\alpha}$ prodrug, like latanoprost, which on application to the eye is hydrolyzed and behaves as an FP receptor agonist.^{19,20} In fact, when bimatoprost is hydrolyzed in the test tube, its free acid potently activates FP receptors.¹⁵ However, in some studies bimatoprost appears to be highly resistant to hydrolysis, and thus the appearance of the free acid of bimatoprost is rare in ocular tissues, particularly in regions such as the ciliary body thought primarily to mediate outflow effects.^{19,41} Other evidence suggests that $PGF_{2\alpha}$ and bimatoprost interact at different receptors. When tested in the same tissue preparation, $PGF_{2\alpha}$ and bimatoprost stimulate calcium transients in different cell populations^{34,42} and differentially stimulate connective tissue growth factor.⁴³ Last, bimatoprost shows no meaningful activity at prostaglandin FP receptors or other PG receptor subtypes ($K_d \geq 10^{-5}$ M).^{16,33}

In our hands, AGN 211334 effectively blocked bimatoprost- and prostamide $F_{2\alpha}$ - but not $PGF_{2\alpha}$ -mediated contractions. In addition, in both of our models for the conventional pathway, AGN 211334 antagonized prostamide's (bimatoprost's) effects on fluid flow through trabecular tissues and across trabecular monolayers. AGN 211334 alone decreased baseline outflow facility measurements in perfused anterior segment and initial hydraulic conductivity measurements for TM cell monolayers, suggesting that AGN 211334 interferes with endogenous signaling pathways or acts as an inverse agonist in these preparations. Because of potent and reproducible effects of AGN 211334 on hydraulic conductivity of TM cell monolayers, this model will serve as a useful tool to uncover the mechanism of AGN 211334 action in future studies.

Bimatoprost has been a safe and effective agent for lowering IOP in the management of ocular hypertension and open-angle

glaucoma. In the present study, we observed that bimatoprost treatment adversely affected CCT measurements, effects that were antagonized by AGN 211334. To our knowledge, corneal edema has not been reported during clinical trials with bimatoprost. A recent study indicated that other antiglaucoma drugs including latanoprost may affect the physiologic function of corneal endothelial cells through change of $[Ca^{2+}]_i$ mobility.⁴⁴ The effect of bimatoprost on the corneal endothelium is still unclear and requires further characterization.

In clinical studies bimatoprost appears to affect IOP earlier than other prostaglandin mimetics, and effects are long lasting. Bimatoprost demonstrated effective 24-hour IOP control after a single dose in both human and normal dogs, and almost 10 mm Hg was dropped 4 hours after a single dose in dogs with glaucomatous eyes.¹⁴ In the anterior segment perfusion model used in the present study, bimatoprost gradually increased outflow facility over the 2 days of exposure and continued to increase outflow facility in some segments after chamber exchange with fresh medium (probably because of difficulty in washing bimatoprost out of tissues). Consistent with this finding, careful examination of outflow tissues exposed to bimatoprost with the light microscope revealed no consistent morphologic changes (i.e., breaks in inner wall, data not shown). In contrast, bimatoprost's effects in the cell-perfusion model were observed immediately, during the first 30 minutes of exposure. Because bimatoprost's effects were not additive or synergistic with isoproterenol, we concluded that both drugs affect intracellular pathways that control cell contractility, as shown before.³¹ The reasons for the time differences between the models are unclear. However, in both cases, effects appeared sooner than would be anticipated if bimatoprost was influencing remodeling of extracellular matrix in juxtacanalicular tissues or in cell monolayers. Alternatively, bimatoprost may have two mechanisms of action: one that occurs immediately and another that occurs over time. For example, we cannot rule out that bimatoprost alters the extracellular matrix environment in the conventional drainage tract and/or the sclera, similar to effects of $PGF_{2\alpha}$ and its analogues.⁴⁵ Clearly, more work needs to be done to characterize bimatoprost's effects in the outflow tracts.

The unique pharmacology of bimatoprost and its effects on conventional drainage make it a leading compound for determining mechanisms that regulate resistance to outflow in the conventional drainage tract. Understanding these mechanisms will enable the design of more efficacious compounds with the ability to increase conventional outflow in those with ocular hypertension and glaucoma.

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