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HL3501, a Novel Selective A3 Adenosine Receptor Antagonist, Lowers Intraocular Pressure (IOP) in Animal Glaucoma Models

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Purpose: The A3 adenosine receptor (A3AR) is a known therapeutic target for glaucoma treatment. In this study, we developed HL3501 and examined its selectivity profile and in vitro and in vivo effects.

Methods: For the rabbit model, intraocular pressure (IOP) was increased by laser photocoagulation of the trabecular meshwork (TM). The rabbits were then topically treated with HL3501, latanoprost, timolol, or vehicle for 3 weeks. For the mouse model, HL3501, latanoprost, or vehicle was administered following induced IOP elevation by dexamethasone (Dex). The IOP of all rabbits and mice was measured. Electroretinography was performed on both eyes of dark-adapted anesthetized mice on days 0 and 21. The mice's eyes were enucleated at the end of the treatment for immunofluorescence staining.

Results: HL3501 was highly specific to the A3AR and inhibitory of A3AR function. In the rabbit glaucoma model, HL3501 and latanoprost significantly decreased the IOP. In the Dex-treated mouse model, HL3501 and latanoprost significantly decreased the IOP and increased the b-wave amplitude as compared with the vehicle treatment. HL3501 and latanoprost also inhibited fibronectin and α-smooth muscle actin expression induced by Dex treatment.

Conclusions: HL3501 had effects similar to those of latanoprost in reducing ocular hypertension in animal models. HL3501 could be used as a novel approach to treat glaucoma.

Translational Relevance: HL3501 is a novel preclinical compound targeting the A3 adenosine receptor, which may also be a new treatment option to fill the unmet needs of many glaucoma patients.

Introduction

Elevated intraocular pressure (IOP) is a major risk factor for the development of glaucoma progression. Elevated IOP progressively induces optic neuropathy, which is characterized by the loss of retinal ganglion cells (RGCs) that can result in blindness.[1–3] The IOP is generated in the anterior eye via the aqueous humor (AH) circulation system. AH is produced by the ciliary body epithelium and drained by two main outflow pathways: the trabecular (conventional) and uveoscleral (unconventional) pathways.[4] The IOP is maintained in equilibrium when the rate of aqueous production is equal to the rate of aqueous outflow. The obstruction of the conventional and unconventional outflow pathways is associated with an elevated IOP, which is the only modifiable risk factor for glaucoma.[5] Controlling the IOP has been shown to protect against damage to the optic nerve in glaucoma.

Thus, reducing the IOP is the primary mechanism of anti-glaucoma medications (beta-blockers, alpha-2 agonists, epinephrine derivatives, carbonic anhydrase inhibitors, prostaglandin analogs, and rho kinase inhibitors), which act by decreasing AH production, by increasing trabecular outflow facility or uveoscleral outflow, and/or by reducing episcleral venous pressure.[6,7]

Several effective glaucoma therapies are available, but some patients remain unresponsive to treatment.[8] Thus, novel classes of IOP-lowering agents need to be developed for non-responders. Adenosine receptors (ARs) are expressed in the ciliary epithelium, and the activation or inactivation of ARs regulates AH production and outflow to maintain IOP equilibrium.[9] The A3 adenosine receptor (A3AR) has an important role in IOP regulation, and antagonists of A3AR can be potential treatments for similar pathologic conditions such as glaucoma. A3AR is known as a therapeutic target for glaucoma treatment because its antagonists prevent chloride (Cl^-) release and reduce AH production in the non-pigmented epithelial cells in the ciliary body.[10,11] The selective activation of A3AR can increase AH production, and consequently IOP, by a mechanism that involves the activation of Cl^- channels in the non-pigmented ciliary epithelial (NPE) cell. In previous studies, A3AR knockout mice showed lower IOP phenotype, and A3AR antagonists have reduced the IOP in mice.[12,13]

The A3AR antagonists known to date have shown IOP-lowering potential in studies of in vitro cells or mice, but only a few studies have used a rabbit model or focused on the co-administration of A3AR antagonists and other classes of anti-glaucoma medicines. HL3501, which was developed by us, is a novel small molecule selectively targeting A3AR. In this study, we examined the selectivity profile of HL3501 using in vitro binding and functional assays, in addition to the in vivo effects of HL3501 using both mouse and rabbit models.

Methods

Radioligand Binding Assay and A3AR Functional Assay

Both binding and functional assays were performed by Eurofins Cerep (Celle-Lévescault, France). All human AR (A1R, A2aR, A3R) binding experiments were performed according to the protocol described in Table 1. [^{3}H]CCPA (1 nM), [^{3}H]CGS21680 (6 nM), and [^{125}I]AB MECA (0.15 nM) were incubated with hA1-Chinese hamster ovary (CHO), hA2-human embryonic kidney (HEK), and hA3-HEK cell membranes, respectively in the presence or absence of HL3501 for 1 or 2 hours in 100 µL of buffer containing 50-mM Tris-HCl, 10-mM $MgCl_2$, and 1-mM EDTA (pH 8.0). The reactions were terminated by filtration through GF/B filters and radioactivity was determined using a liquid scintillation analyzer. Non-specific binding was defined by using 10 µM N^6-cyclopentyladenosine (CPA), 10 µM *N*-ethylcarboxamidoadenosine (NECA), and 1 µM N^6-(3-iodobenzyl)-5'-*N*-methylcarbaxamidoadenosine (IB-MECA) on A1, A2a, and A3 binding assays, respectively. Compound binding was calculated as percent inhibition of the binding of a radioactively labeled ligand specific for each target. Fluorescent imaging plate reader (FLIPR) assays were conducted to profile which compound acts as an agonist or antagonist on the A3 receptor. The A3AR agonist (NECA) and antagonist (VUF 5574; Sigma-Aldrich, St. Louis, MO) were used as references. The FLIPR assay was performed using ChemiScreen A3 Adenosine Receptor stable cell line (HTS052C; Eurofins Cerep). On the day the assay was performed, the cells were loaded with GPCR Profiler Assay Buffer (Eurofins Discovery Service). The A3 agonist assay was conducted on a FLIPR^{TETRA} instrument where

Table 1. Binding Experiment Protocols

Receptors	Source	Ligand	Concentration	K_d	Non-Specific	Incubation
A_1 (*h*) (agonist radioligand)	Human recombinant (CHO cells)	[^{3}H]CCPA	1 nM	0.7 nM	CPA (10 µM)	60-min RT
A_{2A} (*h*) (agonist radioligand)	Human recombinant (HEK293 cells)	[^{3}H]CGS 21680	6 nM	27 nM	NECA (10 µM)	120-min RT
A_3 (*h*) (agonist radioligand)	Human recombinant (HEK293 cells)	[^{125}I]AB-MECA	0.15 nM	0.22 nM	IB-MECA (1 µM)	120-min RT

RT, room temperature.

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the HL3501, vehicle control, and reference agonist (NECA) were added to the assay plate after the fluorescence/luminescence baseline was established. The agonist assay was performed for a total of 180 seconds and was used to assess the ability of each compound to activate each G-protein-coupled receptor (GPCR) assayed. The antagonist assay was performed using EC_{80} potency values determined during the agonist assay, and all pre-incubated test compound wells were challenged with the EC_{80} concentration of the reference agonist (NECA) after the establishment of a fluorescence/luminescence baseline. The antagonist assay was conducted using the same assay plate that was used for the agonist assay. The EC_{50} values and IC_{50} values were determined via nonlinear regression analysis of the concentration–response curves using the Prism 8.0 software (GraphPad Software, La Jolla, CA).

Materials

HL3501, an A3AR antagonist, was synthesized by Handok Pharmaceuticals (Seoul, Republic of Korea). Xalatan (latanoprost, 0.005%) and timolol (0.5% timolol XE) were purchased from Pfizer (New York, NY) and Merck (Kenilworth, NJ), respectively.

Laser-Induced Ocular Hypertension Rabbit Model

All New Zealand white (NZW) rabbit experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee of KNOTUS (approval no. 15-KE-192, Incheon, Republic of Korea). The NZW rabbits weighing between 2 and 2.5 kg were supplied by Koatech (Pyeongtaek, Republic of Korea). The rabbits were housed separately in stainless steel cages (500 mm wide × 800 mm long × 500 mm high) in an environmentally controlled room (temperature, 23°C ± 3°C; relative humidity, 55% ± 15%; 12-hour/12-hour light/dark cycle of 150–300 Lux; ventilation, 10–20 times per hour). Food and sterilized water were available ad libitum. To perform laser photocoagulation, the rabbits were anesthetized using Zoletil 50 (Virbac France, Carros, France) and xylazine (Rompun; Bayer AG, Berlin, Germany). After a gonioscopy lens was placed on the rabbit's right eye, a 75-µm spot size laser beam was irradiated onto the pigmented trabecular meshwork (TM) (0.1 second, 1.0 W) using a slit lamp equipped with a laser to induce internal photo ablation. We used a diode laser (LightLas532; LIGHTMED, San Clemente, CA) at 532 nm. After a 5-day recovery period, the rabbits were then administered 50 µL of the eye drops with HL3501 (0.02%, BID), latanoprost (0.005%, BID), timolol (0.5%, BID), or vehicle in the right eye, twice daily (at 9 AM and 5 PM) for 3 weeks ($n = 4$ rabbits per drug-treated group; $n = 2$ rabbits per vehicle-treated group). The IOP of all rabbits was measured using a TONOVET tonometer (iCare, Vantaa, Finland) immediately before drug treatment in the morning on days 0, 1, 3, 5, 7, 10, 14, 17, and 21. Because latanoprost and timolol are currently the preferred anti-glaucoma medications, these medicines were used as the comparative drugs to evaluate the potency of HL3501.

Dexamethasone-Induced Ocular Hypertension Mouse Model

Animals, Induction of High IOP, IOP Measurement, and Electroretinogram

Male C57BL/6 mice (11 weeks old) were purchased from Koatech. After acclimation for 1 week, 0.1% dexamethasone (Dex; 20 µL/eye) was topically administered in both eyes of mice three times a day for 2 weeks. After initiating the test compound treatment, dexamethasone administration was continuously maintained. After the first 2 weeks of induced IOP elevation via Dex treatment, HL3501 alone (0.04%, BID), latanoprost alone (0.005%, QD), or a combination of both was topically administered for 21 days, with each dose administered at 5-minute intervals. Each medication group was comprised of 8 to 10 mice. The individual body weight of each mouse was measured once a week during the test period. During the treatment period, the IOP was measured on days 0, 1, 3, 5, 7, 10, 14, 17, and 21 with a TONOLAB rebound tonometer (iCare). Electroretinography (ERG) was performed on both eyes of dark-adapted anesthetized mice using the MICRON Ganzfeld ERG system (Phoenix-Micron, Bend, OR). The pupils were fully dilated with proparacaine (0.5%). A drop of methylcellulose was applied on the cornea, and the electrodes were placed on the skin, tail, and cornea. The response was measured by stimulating the retina with a single white light (0.3 log cd·s/m^2 of flash intensity), and the b-wave amplitude was analyzed using the LabScribe ERG software (iWorx, Dover, NH).

Hematoxylin and Eosin Staining

After in vivo testing, the mice were euthanized and the eyes were enucleated for histology analysis. The eyes were fixed in 10% formalin for 24 hours, embedded in paraffin, and sectioned. For the observation of RGCs, hematoxylin solution (0.1% hematoxylin plus

10% ammonium) was added to the retinal sections for 3 to 5 minutes. The sections were then washed three times with distilled water, rinsed with 95% alcohol, and stained with 1% eosin Y-solution for 1 minute. The eosin Y was washed off with a series of ethanol solutions (85%, 90%, and 100%), carboxylene, and xylene for 3 minutes each, and the sections were coverslipped with a mounting medium and scanned using a digital slide scanner (NanoZoomer 2.0 RS; Hamamatsu Photonics, Hamamatsu, Japan).

Immunofluorescence Staining

Each section was incubated overnight at room temperature with primary antibodies (fibronectin, α-smooth muscle actin [α-SMA]). The next day, the sections were washed three times in 0.1-M phosphate buffered saline with Tween 20 (PBST) and incubated with secondary antibodies with fluorescence for 1 hour. Afterward, the sections were washed three times in 0.1-M PBST solution, were counterstained using DAPI Fluoromount-G Mounting Medium (Thermo Fisher Scientific, Waltham, MA), and were analyzed with a fluorescence microscope (TE2000-U; Nikon, Tokyo, Japan). The primary antibodies used were fibronectin antibodies (1:200, ab2413; Abcam, Cambridge, UK) and α-SMA antibodies (1:200, ab5694; Abcam). The secondary antibodies used were Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:500, A-21206; Thermo Fisher Scientific) and Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (1:500, A-31572; Thermo Fisher Scientific).

Western Blot Analysis

Three mice in each group were randomly chosen for the western blot. After the mice were euthanized, retinal tissues were collected. The retinal tissues were homogenized in RIPA buffer (150-mM NaCl; 1.0% IGEPAL CA-630; 0.5% sodium deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 50-mM Tris, pH 8.0; 1× protease inhibitors; and 1-mM phenylmethylsulfonyl fluoride) and centrifuged at 14,000*g* for 30 minutes at 4°C. Total protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Proteins (10 µg/lane) were loaded into a 10% SDS–polyacrylamide gel, subjected to electrophoresis, and transferred to a polyvinylidene difluoride or nitrocellulose membrane. The following primary antibodies were incubated overnight at room temperature: matrix metalloproteinase 2 (MMP-2), MMP-9, tissue inhibitor of metalloproteinase 1 (TIMP-1), and TIMP-2. The next day, the sections were washed three times in 0.1-M PBST and incubated with secondary antibodies for 1 hour. Protein bands were quantified by densitometry using enhanced chemiluminescence reagents (Amersham Bioscience, Amersham, UK) and ImageJ 1.48 (National Institutes of Health, Bethesda, MD). The primary antibodies used were MMP-2 (1:1000, ab235167; Abcam), MMP-9 (1:1000, ab228402; Abcam), TIMP-1 (1:1000, ab179580; Abcam), TIMP-2 (1:1000, ab180630; Abcam), and alpha-tubulin (1:2000, sc-5286; Santa Cruz Biotechnology, Dallas, TX). The secondary antibodies used for MMPs and TIMPs were anti-rabbit IgG and HRP-linked antibody (1:2000, 7074; Cell Signaling Technology, Danvers, MA), whereas the secondary antibodies used for alpha-tubulin were anti-mouse IgG and HRP-linked antibody (1:2000, 7076; Cell Signaling Technology). The expression values of MMP-2, MMP-9, TIMP-1, and TIMP-2 were normalized with α-tubulin. Each MMP expression value was then grouped according to the respective TIMP inhibitor expression values. The MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios were analyzed as the extracellular matrix (ECM) remodeling parameter. This study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Institutional Animal Care and Use Committee of Inje University Busan Paik Hospital (IACUC No. IJUBPH_2018-008-01).

Statistical Analyses

All data are presented as the mean ± SD. Comparisons among multiple groups were determined using one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistical analyses were performed using GraphPad Prism 6.0. $P < 0.05$ was considered statistically significant.

Results

HL3501 Is a Highly Selective A3AR Antagonist

We assessed the binding affinity of HL3501 on human A1, A2, and A3AR. Binding assays were performed using standard radioligands and membrane preparations from CHO cells (A1 and A3) or HEK293 cells (A2a) stably expressing hAR subtypes. HL3501 did not bind to A1 and A2a receptors, but it bound to the A3 receptor. The functional assay of HL3501 on the A3AR was assessed with a calcium flux assay in the Eurofins Discovery Service GPCR Profiler. NECA was the reference agonist for A3, and the E_{max} value was 1.25 µM. VUF 5574 was the reference antagonist for A3, and the I_{max} value was 0.0375 µM. HL3501 did not have an agonistic character on the A3AR until 10 µM, but the HL3501 had a potent antagonist effect on

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Table 2. HL3501 Is a Highly Selective A3AR Antagonist

	Binding Affinity Assay (K_i)			A3 Functional Assay Calcium Flux Assay (IC_{50})	
Compound	hA1	hA2a	hA3	Agonist Assay	Antagonist Assay
HL3501	>10 µM	>10 µM	20 nM	>10 µM	18 nM

All human AR experiments were performed using adherent CHO cells and HEK293 cells stably transfected with cDNA encoding the appropriate receptor. In the functional assay, NECA was the reference agonist for A3 (E_{max} = 1.25 µM), and VUF 5574 was the reference antagonist for A3 (I_{max} = 0.0375 µM).

the A3AR. The IC_{50} of HL3501 was 18 nM (Table 2). These results indicate that HL3501 is a highly selective human A3AR antagonist.

HL3501 Showed an IOP-Lowering Efficacy in Laser-Induced OHT Rabbit Models

NZW rabbits with laser photocoagulated TMs were used as reliable glaucoma animal models.[14–16] In the present study, we assessed whether HL3501 could reduce the IOP in a laser-induced glaucoma rabbit model. To evaluate the potency of HL3501, we used latanoprost and timolol as comparative drugs. As shown in Figure 1A, HL3501 (0.02%, BID), latanoprost (0.005%, BID), and timolol (0.5%, BID) consistently and effectively reached the lowest IOP level for 21 days in the laser-induced glaucoma rabbit model. Notably, on day 7, HL3501 significantly reduced IOP

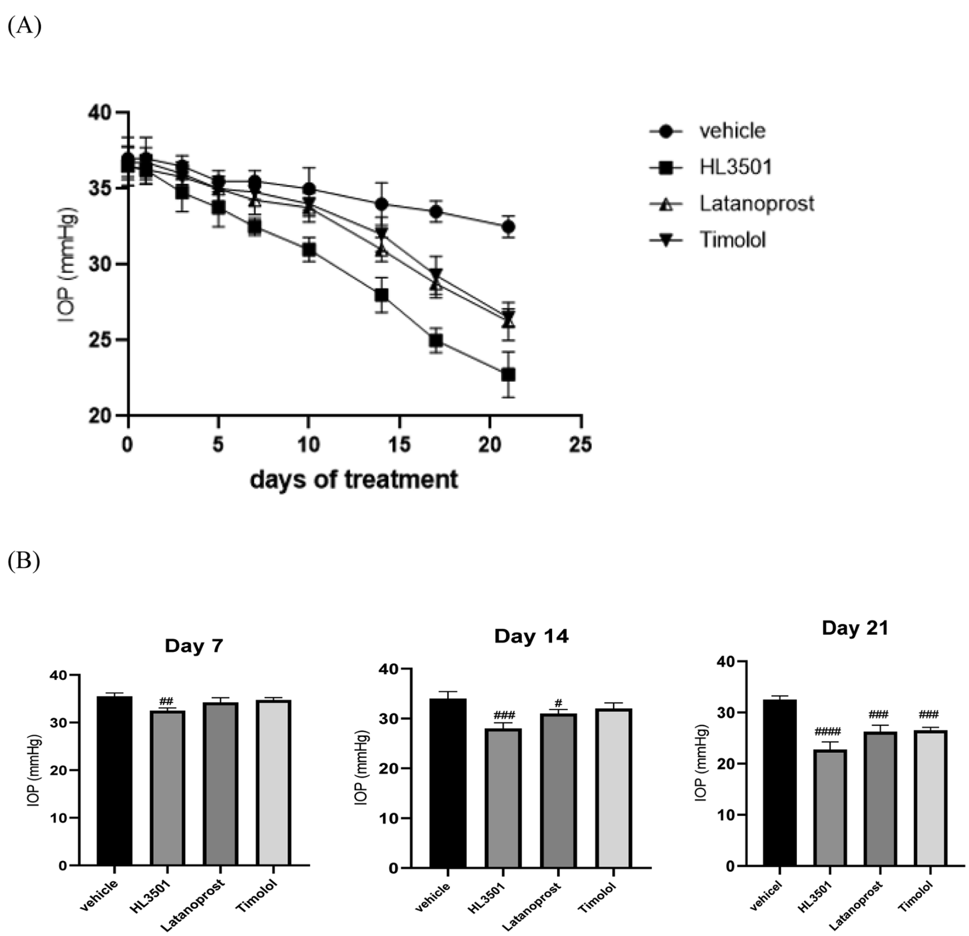

Figure 1. The entire circumference of the TM was injured with a laser, which resulted in an increased IOP. HL3501 showed IOP-lowering effects in the laser-induced OHT rabbit model. (**A**) The IOP of NZW rabbits over 3 weeks of treatment with vehicle, HL3501 (0.02%), latanoprost (0.005%), or timolol (0.5%). (**B**) The IOP of NZW rabbits on days 7, 14, and 21. All data are expressed as mean ± SD ($n = 4$ rabbits per drug-treated group; $n = 2$ rabbits per vehicle-treated group). #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$; ####$P < 0.0001$ versus vehicle by one-way ANOVA with post hoc Dunnett's test.

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Table 3. Rabbit IOP Levels for Each Treatment in the Laser-Induced OHT Rabbit Model

Rabbit IOP (mmHg)	Mean ± SD Day 0	Day 7	Day 14	Day 21	Change Value (mmHg) vs. Vehicle on Day 21
Vehicle	37.0 ± 1.4	35.5 ± 1.4	34.0 ± 1.4	32.5 ± 0.7	—
HL3501	36.5 ± 1.3	32.5 ± 0.6*	28.0 ± 1.5**	22.8 ± 1.5***	9.7↓
Latanoprost	36.8 ± 1.0	34.3 ± 1.0	31.0 ± 0.8*	26.2 ± 1.3**	6.3↓
Timolol	36.5 ± 1.3	34.8 ± 0.5	32.0 ± 1.2	26.5 ± 0.6**	6.0↓

*$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$ versus vehicle by one-way ANOVA with post hoc Dunnett's test.

($P < 0.01$) compared with latanoprost and timolol (Fig. 1B). On both days 14 and 21, HL3501 showed the highest IOP-lowering effect among all of the medications (Fig. 1B). Table 3 shows the mean IOP values.

HL3501 Showed an IOP-Lowering Efficacy in the Chronic Dex-Induced OHT Mouse Model

In addition to the IOP-lowering effect of HL3501 in a laser-induced glaucoma rabbit model, we further investigated whether HL3501 produced a similar IOP-lowering effect in other glaucoma models such as the Dex-induced OHT mouse model.[17] Prolonged glucocorticoid (GC) therapy induces ocular side effects such as IOP elevation. It has been suggested as the link between open-angle glaucoma and GC-induced glaucoma.[18] Dex–OHT mouse models were created, and Dex was administered topically for 2 weeks. IOP elevation was relatively rapidly induced by blocking the conventional outflow pathway. The Dex-treated vehicle groups revealed phenotypes that mimic glaucoma, such as high IOP, a reduction of b-wave amplitude, and increased fibronectin and α-SMA in the TMs in mouse eyes in comparison with normal controls. The IOPs of all mice were measured on days 0, 1, 3, 5, 7, 10, 14, 17, and 21. As shown in Figure 2A, HL3501, latanoprost, and HL3501 + latanoprost showed a consistent IOP-lowering effect for 3 weeks. Notably, the HL3501 + latanoprost combination–treated group exhibited a significant reduction ($P < 0.001$) in IOP on day 1. This result suggests that HL3501 also has an IOP-lowering effect in the GC-induced glaucoma mouse model, similar to that in a laser-induced glaucoma rabbit model.

ERG is mainly used for the measurement of retinal function, which reflects the electrical response of the light-sensitive retinal cells.[19] ERG was performed on day 21, and the results showed that the b-wave values of the vehicle significantly decreased compared with those of normal animals. The b-wave values of HL3501 or latanoprost alone did not decrease as much. They also were not at a normal level. However, the b-wave values of the HL3501 + latanoprost combination–treated group increased compared with the vehicle-treated group (Fig. 2B, Supplement information Figure S2). These results suggest that the HL3501 + latanoprost combination treatment could improve the b-wave response in a Dex-induced OHT model.

Excessive GC treatment leads to profibrotic changes in the AH outflow pathway via the TM.[20] The pathogenesis of open-angle glaucoma is fibrosis of the TM, which induces ECM accumulation.[21–23] In the Dex-induced OHT mouse model, the profibrotic proteins, fibronectin (green color), and α-SMA (red color) of the vehicle-treated group were elevated in the TM compared with those of the normal group. As shown in Figure 2C, HL3501, HL3501 + latanoprost combination, and latanoprost alone suppressed fibronectin and α-SMA expression in the TM compared with the vehicle treatment. We further examined whether HL3501 could inhibit RGC loss in a Dex-induced OHT mouse model. Figure 2D shows the representative images exhibiting the histological appearance of retinal cross-sections following hematoxylin and eosin staining. In the vehicle group, the loss of RGCs was observed compared with the normal group. However, in Dex-induced OHT mice, the HL3501 group was less affected by RGC loss, which was also observed in the HL3501 + latanoprost combination-treated and latanoprost alone groups.

In glaucoma, IOP elevation is mainly caused by AH outflow resistance. This resistance is generated in the TM, where the ECM is continuously being remodeled by the MMP family. In glaucoma, abnormal changes in MMP/TIMP along with an abnormal decrease and increase in MMP-2, MMP-9, TIMP-1, and TIMP-2, respectively, lead to the imbalance of ECM degradation, which contributes to disease progression.[24] We examined the retinal expression of MMP-2, MMP-9, TIMP-1, and TIMP-2 and their relationship with ECM remodeling in mouse eyes through western blot analysis (Supplement information Figure S1). TIMP-2 selectively inhibits MMP-2, whereas TIMP-1 selectively inhibits MMP-9. In the Dex-induced OHT mouse model, the MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios of the vehicle-treated

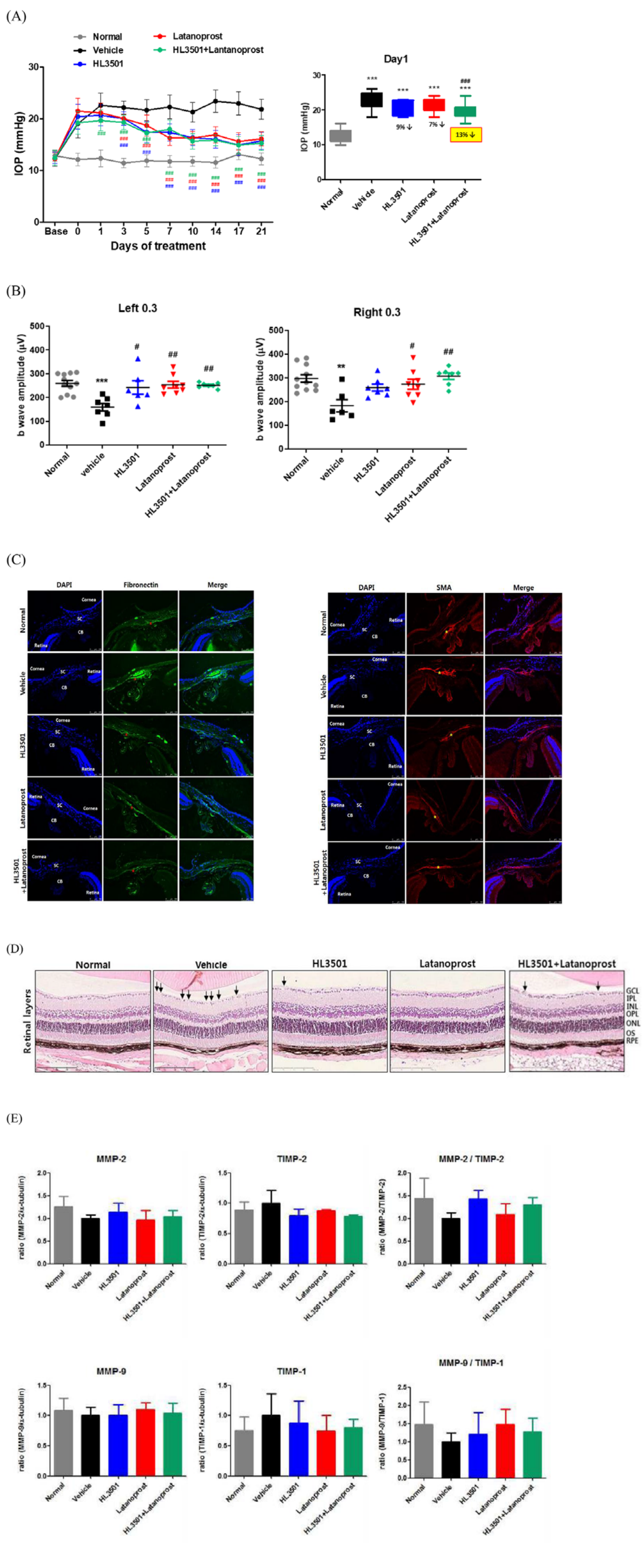

Figure 2. HL3501 showed anti-glaucoma effects in a Dex-induced OHT mouse model. (**A**) IOP of mice over 3 weeks of treatment after topical Dex treatment for the induction of OHT; the IOP of the mouse on day 1 is shown. The mean value of the IOP was estimated. Base (basal IOP, before Dex treatment) ***$P < 0.001$ versus normal group; ###$P < 0.001$ versus vehicle-treated group. (**B**) ERG of mice (day 21). **$P < 0.01$, ***$P < 0.001$ versus normal group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus vehicle-treated group. (**C**) Effect of HL3501 on fibronectin expression in the TM of murine GC-induced glaucoma. The paraffin-embedded retinal tissue was immunolabeled with fibronectin antibody (*green*) and SMA antibody (*red*) and observed under a fluorescence microscope focusing on the TM (*red* and *yellow asterisks*). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). *Scale bar:* 100 mm. CB, ciliary body; SC, Schlemm's canal. (**D**) The effect of HL3501 on RGCs in murine GC-induced glaucoma. The arrows indicate the loss of RGCs. *Scale bar:* 200 mm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segment; RPE, retinal pigment epithelia. (**E**) Effect of HL3501 on MMP-2, MMP-9, TIMP-1, and TIMP-2 protein expression in the GC-induced glaucoma model. Retinal extracts underwent western blot analysis to measure the expression of MMPs and TIMPs. Figure shows the quantification of each band normalized to α-tubulin when evaluating the amount of MMPs and TIMPs separately. Topical ocular application of HL3501 (0.04%, BID), latanoprost (0.005%, QD), or vehicle following IOP elevation induced by Dex treatment for 2 weeks. The date of measurement of the IOP of the mouse model was recorded for 21 days. Electroretinography was performed on both eyes of dark-adapted anesthetized mice on days 0 and 21. Each group had eight animals. #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$ versus vehicle; **$P < 0.01$; ***$P < 0.001$ versus normal.

group were decreased when compared with those of the normal group. As shown in Figure 2E, HL3501 increased the MMP-2/TIMP-2 ratio, similar to that in the normal control. Moreover, HL3501 tended to increase the MMP-9/TIMP-1 ratio, and the HL3501 + latanoprost combination and latanoprost alone increased the MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios.

Discussion

The selective activation of A3AR can increase AH production, and consequently IOP, via a mechanism that involves the activation of Cl^- channels in the nonpigmented ciliary epithelia.[12] It was reported that the baseline IOP in A3AR knockout mice was lower than that of wild-type mice. The A3AR is known as a therapeutic target for glaucoma treatment. In the present study, we have confirmed that HL3501 binds only to the hA3 receptor and not to the hA1 or hA2a receptors. In the functional assay, HL3501 has characteristics similar to those of an A3 antagonist. These results suggest that HL3501 is a selective adenosine A3 antagonist.

We assessed the IOP-lowering effects of HL3501 in laser-induced glaucoma rabbit and Dex-induced OHT mouse models. In a laser-induced glaucoma rabbit model, HL3501 lowered the IOP more than latanoprost and timolol (Fig. 1A). HL3501 reduced the IOP by 9.7 mmHg compared with the vehicle treatment on day 21. Meanwhile, latanoprost and timolol reduced the IOP by 6.3 and 6.0 mmHg, respectively. Moreover, the Dex-induced OHT mouse model was used to evaluate the effects of HL3501 alone and the HL3501 + latanoprost combination treatment. In the Dex-induced OHT model, HL3501 consistently decreased the IOP for 3 weeks, whereas latanoprost and HL3501 + latanoprost also had similar effects. Notably, on day 1, the HL3501 + latanoprost combination treatment showed a significant IOP-lowering effect, which is suggestive of the additive effect of these compounds.

ERG was performed to measure retinal function. On day 21, the ERG results revealed that the vehicle group showed lower b-wave values than the normal group did. In comparison, the b-wave values of the HL3501 group were shown to be higher than those of the vehicle group but not higher than normal. This result suggests that the b-wave values of the HL3501 group are less affected than those of the vehicle group. However, the b-wave values of the HL3501 + latanoprost combination-treated group were higher than those of the vehicle-treated group (Fig. 2B). These results suggest that HL3501 may have the benefit of ameliorating damaged retinal function when coadministered with latanoprost.

In correlation with the ERG results, the loss of RGC in the vehicle-treated group was present compared with the normal group. However, HL3501 tended to mitigate the RGC loss compared with the vehicle treatment. In the vehicle-treated group, prolonged dexamethasone treatment induced the accumulation of profibrotic proteins, such as fibronectin and α-SMA in the TM. However, HL3501 decreased the expression of fibronectin and α-SMA. These results suggest that HL3501 is likely to reduce fibrosis and, consequently, stiffness of the TM. AH outflow resistance occurs when the ECM remodeling process is disturbed, resulting in glaucoma. MMPs/TIMPs are related with ECM degradation. Additionally, we confirmed a reduction of the MMP/TIMP ratio in the vehicle group in comparison with that in the normal group. However, HL3501 increased the MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios in the normal group.

To date, selective A3AR antagonists have been found to lower IOP in mice. HL3501 is a new compound and structurally different from those compounds. In the present study, HL3501 decreased the IOP in both the rabbit and mouse glaucoma models. It has also been found to relieve the fibrosis in the TM and RGC loss in a Dex-induced mouse model. One of the advantages of co-administering HL3501 with glaucoma drugs of other classes, such as latanoprost, is that they increase uveoscleral outflow and sometimes outflow facility. Therefore, HL3501 could be used as a novel approach to treat glaucoma. It may also provide a new treatment option to fill the unmet needs of many glaucoma patients.

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