**Development of a Novel Intraocular-Pressure-Lowering Therapy Targeting ATX**

Norimichi Nagano,*a Megumi Honjo,a Mitsuyasu Kawaguchi,b Hiroshi Nishimasu,c Osamu Nureki,c Kuniyuki Kano,d,e Junken Aoki,d,e Toru Komatsu,f Takayoshi Okabe,g Hirotatsu Kojima,g Tetsuo Nagano,*a and Makoto Aihara*ga

*Department of Ophthalmology, Graduate School of Medicine, The University of Tokyo; 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–8655, Japan; b Graduate School of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya 467–8603, Japan; c Graduate School of Science, The University of Tokyo; 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan; d Graduate School of Pharmaceutical Sciences, Tohoku University; 6–3 Aoba, Aramaki, Aoba-ku, Sendai 980–8558, Japan; e Core Research for Evolutional Science and Technology (CREST); 1–7–1 Otemachi, Chiyoda-ku, Tokyo 100–0004, Japan; f Drug Discovery Initiative, The University of Tokyo; 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan; and g Drug Discovery Initiative, The University of Tokyo; 7–3–1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan.

Received July 9, 2019; accepted August 13, 2019

Elevated intraocular pressure (IOP) is the major cause of glaucoma, which is the second leading cause of blindness. However, current glaucoma treatments cannot completely regulate IOP and progression of glaucoma. Our group recently found that autotaxin (ATX) activity in human aqueous humor (AH) was positively correlated with increased IOP in various subtypes of glaucoma. To develop new IOP-lowering treatments, we generated a novel ATX inhibitor as an ophthalmic drug by high-throughput screening, followed by inhibitor optimization. Administration of the optimized ATX inhibitor (Aiprenon) reduced IOP in laser-treated mice exhibiting elevated IOP and higher level of ATX activity in AH and normal mice *in vivo*. The stimulation of ATX induced outflow resistance in the trabecular pathway; however, administration of Aiprenon recovered the outflow resistance *in vitro*. The *in vitro* experiments implied that the IOP-lowering effect of Aiprenon could be correlated with the altered cellular behavior of trabecular meshwork (TM) and Schlemm’s canal (SC) cells. Overall, our findings showed that ATX had major impact in regulating IOP as a target molecule, and potent ATX inhibitors such as Aiprenon could be a promising therapeutic approach for lowering IOP.

**Key words** high-throughput screening; autotaxin; ocular hypertension; intraocular pressure

**INTRODUCTION**

Elevated intraocular pressure (IOP) is thought to be a major factor in triggering axonal damage of the optic nerve and retinal ganglion cell death, leading to blindness in glaucomatous eyes.1,2) There are two routes of aqueous humor (AH) outflow, involving the trabecular and uveoscleral pathways. The major AH drainage pathway from the anterior chamber is the trabecular pathway, which drains approximately 80% of the AH from the anterior chamber in human eyes, and decreased AH outflow through the trabecular pathway is considered the primary cause of increased IOP in glaucoma patients.3) The resistance to AH outflow occurs mainly in the juxtaanaculiclar region of the trabecular meshwork (TM) and the inner wall of Schlemm’s canal (SC).4–7) Histological analysis indicates that the extracellular matrix (ECM) accumulates and increases resistance in the trabecular pathway.8,9)

Glaucoma is classified into two major types: open-angle glaucoma (OAG) and angle-closure glaucoma (ACG). The latter indicates an anatomically closed angle and, therefore, a surgical procedure is performed prior to medical treatment for IOP reduction. The former, OAG, indicates an anatomically open angle, but shows higher resistance to aqueous outflow caused by accumulation of ECM. We call the subtype of OAG without specific ocular diseases primary OAG (POAG), and those with past or current ocular diseases, secondary OAG (SOAG).

Elevated IOP is currently the major and sole clinically treatable risk factor, and it is the only target of current glaucoma treatments. A number of drugs exhibit their IOP-lowering effects through suppression of AH production or enhancement of the outflow facility.10) Numerous ophthalmic drugs such as prostaglandin analogs, β-blockers, carbonic anhydrase inhibitors, a2-agonists, and Rho kinase inhibitors have been clinically approved to lower IOP. However, combined use of these medications cannot completely regulate IOP and progression of glaucoma.

Autotaxin (ATX) is a secreted enzyme with lysophospholipase D (lysoPLD) activity, which converts lysophospholipids such as lysophosphatidylcholine (LPC) to lysophosphatic acid (LPA).11,12) ATX produces most of the extracellular LPA.11,12) LPA plays roles in embryogenesis, vasculogenesis, and tissue remodeling with cytoskeletal changes, as well as ECM accumulation and wound repair.13,14) We recently reported that ATX and LPA concentrations in human AH were significantly higher in all subtypes of glaucoma patients, and that the ATX and LPA concentrations in human AH were correlated with IOP and glaucoma subtypes.15) This implies that the ATX–LPA pathway is commonly activated and increases outflow resistance by tissue remodeling in the outflow pathway.
in various glaucoma subtypes. The ATX–LPA pathway may therefore be a promising target for reducing IOP, based on the mechanism of outflow disturbance. Animals with elevated IOP and simultaneously higher ATX activity in the AH are indispensable for developing topically available drugs. However, there have been no reports describing mice with both elevated IOP and simultaneously higher ATX activity in the AH.

In this study, we first developed a novel ATX inhibitor as an ophthalmic drug by high-throughput screening in a large chemical library, followed by inhibitor optimization. We identified a potent and selective ATX inhibitor scaffold using a sensitive and selective ATX fluorescence probe, TokyoGreen-methylenecyclohexyldimethyldimethoxy monophosphate (TG-mTMP) which we have previously reported, and then developed novel ATX inhibitors derived from the scaffold. We named the most potent ATX inhibitor as an ophthalmic drug Aiprenon. Second, we investigated in vivo and in vitro therapeutic efficacy of the optimized ATX inhibitor (Aiprenon). We performed laser peripheral iridotomy (LPI) to create mice with elevated IOP. LPI in clinical practice involves laser procedure to prevent acute glaucoma attacks. However, we sometimes see clinical cases where LPI acutely and transiently causes increased IOP. We used these mice with transient LPI-induced ocular hypertension to examine the IOP-lowering effects of Aiprenon. To investigate the effects of higher ATX activity on the trabecular pathway in vitro, differentiation to myofibroblasts of human trabecular meshwork (HTM) cells was evaluated by examining changes in filamentous actin (F-actin) and α-smooth muscle actin (αSMA) in immunocytochemistry and Western blotting. αSMA expression is a hallmark of myofibroblast generation and the fibrogenic reaction of cells. F-Actin organization was visualized by rhodamine-phalloidin. Furthermore, the barrier function of confluent monkey Schlemm’s canal endothelial (MSC) cells was assessed by means of transendothelial electrical resistance (TEER) and fluorescein permeability measurements. TEER measurement is a valuable non-invasive technique that can monitor live cells and quantify the integrity of tight junctions in cell culture models. We also examined ATX-induced intracellular localization of ZO-1 and β-catenin using immunocytochemistry to reveal the participation of ATX in cytосkeletal organization and mobilization of proteins required for cell adhesion in MSC cells. ZO-1 is a tight-junction-related protein that interacts with multiple other tight-junction protein and moves from cytoplasm to cell boundary in association with their intercellular adhesion, resulting in the localization of ZO-1. β-Catenin is an adaptor protein related to adherence junctions. HTM and MSC cell viabilities were assessed using a cell proliferation assay (WST-1 assay) in vitro to confirm the safety of Aiprenon. Overall, we showed that topical administration of potent ATX inhibitors such as Aiprenon can be a promising therapeutic approach for lowering IOP.

MATERIALS AND METHODS

High-Throughput Screening of ATX Inhibitor High-throughput screening was conducted in the large chemical library containing 81600 compounds in Drug Discovery Initiative, The University of Tokyo using our previously reported approach. Briefly, 2 mM small molecules in dimethyl sulfoxide (DMSO) stock solution from library plates were diluted with buffer (200 mM Tris–HCl, pH 9.0, 200 mM NaCl and 10 mM MgCl₂) to 30 μM solution with 1.5% DMSO using a Multi-Dispenser EDR 384 (BioTec, Japan). And then, 5 μL of 3 μM TG-mTMP was dispensed into each well of the assay plates, and the 5 μL prepared compound solution was also added to the wells. Finally, 5 μL of ATX solution was dispensed into the plates. The plates were incubated for 2 h at room temperature. After the incubation, the fluorescence was measured using a microplate reader with an excitation wavelength of 490 nm and an emission wavelength of 510 nm.

Preparation and Administration of ATX Inhibitors Nine compounds were selected from derivatives of the detected ATX inhibitory scaffold (AI scaffold) considering their strong ATX inhibitory activity and a wide range of molecular weight (MW) and lipophilicity. They were obtained from Shionogi & Co., Ltd. (Osaka, Japan) under a materials transfer agreement. The compounds were dissolved in sterile autoclave-distilled water with 0.5% Polysorbate 80 (Wako, Osaka, Japan). DMSO could not be used to dissolve these compounds because topical administration required millimolar concentrations. A single drop with 3 μL of 5 mM ATX inhibitor was topically applied twice.

Mouse Experiments Male C57BL/6J mice aged 8–10 weeks old were bought from Japan SLC (Hamamatsu, Japan). The mice were bred and housed in clear cages covered loosely with air filters. The cages contained white chip bedding. The environment was kept at 21°C with a 12 h:12 h light-dark cycle. All mice were fed ad libitum, and every effort was made to minimize pain and suffering during experiments.

LPI and Subgroups We created LPI-treated mice by mimicking acute and transient elevation of IOP in human eyes after LPI. LPI was performed for 8- to 10-week-old C57BL/6J male mice using a 532-nm diode laser at 100 mM and 50 μm spot size for 50 ms. Ten laser burns were delivered to the peripheral zone of the eye. A single 3-μL drop of 5 mM ATX inhibitor was topically applied twice, at 1 h before and immediately after LPI. AH samples were then collected at 1 h after LPI. We put sterile filtered water with 0.5% Polysorbate 80 on control eyes of different mice as a sham procedure. We measured lysoPLD activity of the mice AH samples and IOP using the microneedle method at 1 h after LPI.

Measurement of IOP The IOPs of mouse eyes were measured using the microneedle method, as previously described. Briefly, a microneedle made of borosilicate glass (75–100 μm tip diameter and 1.0 mm outer diameter; World Precision Instruments, Sarasota, FL, U.S.A.) was connected to a pressure transducer (model BLPR; World Precision Instruments). The system pressure detected by the transducer was recorded by a data acquisition and analysis system (PowerLab; AD Instruments, Colorado Springs, CO, U.S.A.). The microneedle was placed in the anterior chamber and the conducted pressure was recorded immediately after anesthesia.

Collection of the AH Mouse AH was collected from C57BL/6J mice. The mice were first sacrificed by cervical dislocation and then a microneedle was carefully inserted into the anterior chamber of the eye to withdraw the AH. The AH was collected and stored at −80°C until the lysoPLD assay.

Measurement of LysoPLD Activity LysoPLD activity was assessed based on the amount of choline released with the use of LPC as the substrate. The reactions were performed in 20-μL aliquots; the AH (2 μL) was incubated with 2 mM of 3,7-dimethyl-1,2,3,7-tetramethylnitrospiropyran (DMSO) stock solution from library plates.
added to the wells and incubated for 10 min at room temperature. The liberated choline was measured at 550 nm in an absorption spectrometer (ARVO X; PerkinElmer, Inc., Japan, Tokyo, Japan), with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Western Blot Analysis HTM cells were first starved by incubation for 24 h in serum-free medium. The starvation medium was changed to media with or without Aiprenon and then, 30 min later, ATX was added at 100 nmol/mL/h. After a 24-h incubation, the cells were collected in RIPA Buffer (Thermo Fisher Scientific K.K., Kanagawa, Japan) containing protease inhibitors (Roche Diagnostics, Basel, Switzerland), sonicated, and centrifuged. Protein concentrations in the supernatant were determined by a BCA assay using a BCA Protein Assay Kit (Thermo Fisher Scientific K.K.). Proteins were boiled in sample buffer (Thermo Fisher Scientific K.K.). The same amount of protein was loaded onto 4–20% precast polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) and the membranes were immersed in Tris-buffered saline with Tween 20 (TBST) containing the first antibody. After washing, the membranes were immersed in TBST containing the second antibody and reacted with ECL substrate (Thermo Fisher Scientific K.K.). Protein bands were detected by ImageQuant LAS 4000 Mini (GE Healthcare, Chicago, IL, U.S.A.). Primary antibodies were anti-α-SMA (1:1000; Sigma-Aldrich) and anti-β-tubulin (1:1000; Wako). Horseradish peroxidase-conjugated secondary antibody (1:2000) was purchased from Thermo Fisher Scientific (Waltham, MA, U.S.A.). The bands were quantified with ImageJ, version 1.51 (National Institutes of Health).

Measurement of Monolayer Transendothelial Electron Resistance (TEER) MSC cells were seeded at 2 × 10⁴ cells/well and grown until confluent on polycarbonate membrane inserts (0.4-μm pore size and 6.5-mm-diameter inserts; Transwell; Corning, Corning, NY, U.S.A.) in DMEM supplemented with 10% FBS and antibiotics at 37°C in 5% CO₂. The volume of the apical side was 0.5 mL and that of the basal side was 1.5 mL. Two weeks after seeding, samples were serum-starved overnight. After washing, Aiprenon was added at 10 μM beforehand. Then, 30 min later, ATX was added for the TEER experiments. The TEER was measured using an electrical resistance system (Millicell ERS-2; EMD Millipore, Billerica, MA, U.S.A.) according to the manufacturer’s instructions, at 1, 4, 6, and 24 h after treatment with ATX, and recorded as net values (Ωcm²). Time-dependent changes after the treatment were monitored and compared as increased fold changes from baseline values.

Measurement of Monolayer Cell Permeability MSC cells were prepared using the same method for TEER measurements as described above. A tracer, 4kDa FITC-dextran (Sigma-Aldrich), was simultaneously applied at 50 μM to the basal compartment of the wells. The medium was collected from the apical side for fluorescence measurements at 4, 6, and 24 h. The concentration of FITC-dextran in the collected medium was measured using a multimode plate reader (ARVO X; PerkinElmer, Inc. Japan, Tokyo, Japan), with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Measurement of Cell Toxicity of the ATX Inhibitor Cellular viability was evaluated using a cell proliferation assay.
assay (WST-1 assay; Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer’s protocol. HTM and MSC cells were cultured in DMEM with 10% FBS and antibiotics. After the cells reached confluence, Aiprenon was applied for 24h followed by WST-1 reagent in phenol red-free DMEM for 1h at 37°C, and the dye was measured at 450 nm by a multimode plate reader (ARVO X; PerkinElmer, Inc.). The results were compared with those of the sham-treated controls.

Statistical Analysis  Statistical analyses were performed using JMP Pro 13 software (SAS Institute, Cary, NC, U.S.A.). Student’s t-test was used for comparisons of two groups. Differences in the data among the multiple groups were analyzed by parametric tests (one-way ANOVA and Tukey’s test) or nonparametric tests (Kruskal–Wallis test and Steel–Dwass test) as a post hoc test. p-Values less than 0.05 were regarded as statistically significant.

Study Approval  All animals used in this study were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the rules outlined by the local Animal Use Committee of the University of Tokyo. Human cells were managed in compliance with the Declaration of Helsinki. The informed consent was obtained from all subjects or their legal guardians. The Ethics Committee for Animal Experiments at University of Tokyo approved all experimental procedures.

RESULTS

The ATX Inhibitory Scaffold and Structure–Activity Relationship (SAR) Analysis of Optimized ATX Inhibitors  To identify novel and potent inhibitors targeting ATX as an ophthalmic drug, we conducted high-throughput screening in a large chemical library containing 81600 chemical structures, using our previously reported approach.16) We identified a novel ATX inhibitory compound with an 8-substituted imidazopyrimidinone, in addition to previously reported compounds24) (Fig. 1A). The novel compound showed the highest ATX-inhibitory activity of all hits: an IC₅₀ of 110 nM. To predict the binding mode of the compound to ATX, a docking study was performed based on X-ray crystal-structure analysis of ATX.25,26) The simulation showed that the compound featured an ATX inhibitory scaffold bound to the lipid-binding hydrophobic pocket of ATX. We termed the ATX inhibitory scaffold with the 8-substituted imidazopyrimidinone AI scaffold (Fig. 1A). We then optimized AI scaffold to create more potent derivatives of AI scaffold. SAR analyses were performed to measure the inhibitory activities of the derivatives. We selected nine promising types of ATX inhibitors from optimized analogs of AI scaffold, and evaluated their activities both in vitro and in vivo (Fig. 1B).

Effect of LPI and Optimized ATX Inhibitors on LysoPLD Activity in the Mouse AH  LysoPLD activity levels in control eyes and LPI-treated eyes were 68.1 ± 3.1 nmol/mL/h and 80.8 ± 2.4 nmol/mL/h (p < 0.05) (Fig. 2A). LPI significantly elevated lysoPLD activity in the mouse AH by 18.6%. The ATX inhibitory potency of the selected ATX inhibitors as an ophthalmic drug was also assessed by measuring the lysoPLD activity. The results indicated that compounds 1, 2, 4, 5, 6, and 7 significantly decreased lysoPLD activity after instilla-
similar inhibitory activity in the AH. Compound 2, which was an optical isomer of compound 1, induced a significant but weak decrease of 22.8% compared with that of LPI eyes ($p < 0.05$). Considering the higher activity in the AH of LPI-treated mice, we determined the ATX activity in the following in vitro experiments at 100 nmol/mL/h.

**Effect of ATX Inhibitors on LysoPLD Activity in Normal Mouse AH without LPI** LysoPLD activity levels in control eyes, compound-1-treated eyes, and compound-2-treated eyes were $68.1 \pm 3.14$, $6.7 \pm 0.57$, and $51.3 \pm 3.24$ nmol/mL/h, respectively (control vs. compound 1, $p < 0.001$; control vs. compound 2, $p < 0.01$; $n = 10–12$) (Fig. 2B). Topical administration of compound 1 significantly decreased lysoPLD activity in the normal mouse AH by 90.2%, while compound 2 significantly but less markedly decreased activity by 24.7%. Based on these results, we confirmed that compound 1 had the most potent inhibitory activity as an ophthalmic drug, so we decided to use compound 1 in the following in vivo and in vitro experiments, and termed compound 1 Aiprenon (Fig. 1B).

**Effect of LPI and Aiprenon on IOP** To investigate the effect of LPI and Aiprenon on IOP, IOP was measured at 1 h after LPI and instillation of Aiprenon. The IOPs of the control eyes, LPI eyes, and Aiprenon-treated eyes with LPI were $11.4 \pm 0.50$ mmHg, $14.3 \pm 0.62$ mmHg, and $12.2 \pm 0.45$ mmHg, respectively (control vs. LPI eyes, $p < 0.05$; LPI eyes vs. Aiprenon-treated eyes with LPI, $p < 0.05$ $n = 18–21$), (Fig. 2C). LPI significantly increased IOP by 25.2% compared with that of control eyes. Ocular instillation of Aiprenon significantly decreased IOP by 14.7% compared with that of LPI eyes.

**The IOP-Lowering Effect of Aiprenon on Normal Mouse Eyes** We also investigated the IOP-lowering effect of Aiprenon on normal eyes without LPI. We instilled 5 mM Aiprenon twice at an interval of 1 h, and measured the change in IOP at 1 h after instillation. The IOPs in the control eyes and Aiprenon-treated eyes were $11.8 \pm 0.40$ mmHg and $10.3 \pm 0.37$ mmHg, respectively ($p < 0.05$, $n = 12–14$) (Fig. 2D). Topical administration of 5 mM Aiprenon therefore significantly decreased IOP in normal eyes by 12.7%.

**Immunochemical Characterization of the Effects of ATX and Aiprenon on αSMA, F-Actin, and the Cytoskeleton of Cultured Human TM Cells** We further investigated the role of ATX in modulating fibrogenic activity in HTM cells, because fibrogenic activity of TM cells is involved in the resistance of the trabecular pathway.\(^{27}\) We evaluated the expression of αSMA and phalloidin in HTM cells. In an immunohistochemical study, ATX treatment induced assembly of αSMA and phalloidin-positive stress fibers in HTM cells, while untreated serum-starved cells showed weak staining (Fig. 3A). However, 10μM Aiprenon prevented ATX-induced expression of αSMA and phalloidin and its incorporation of actin stress fibers (Fig. 3A).

**The Effect of ATX and Aiprenon on αSMA Expression**
We also performed Western blotting to examine how ATX affected HTM cell differentiation into myofibroblasts. After ATX treatment for 24 h, the levels of αSMA were significantly increased compared with those of the control \((p < 0.01)\) (Figs. 3B, 3C). However, treatment with Aiprenon significantly decreased ATX-induced αSMA expression \((p < 0.05)\) (Figs. 3B, 3C).

Localization of Molecules Associated with Intercellular Adhesion in MSC Cells after ATX Treatment We evaluated the expression of ZO-1 and β-catenin in MSC cells. Immunostaining revealed that ZO-1 and β-catenin were localized at the boundary of the ATX-treated MSC cells, when compared with controls (Fig. 4A). The results showed that ATX treatment resulted in the formation of intact tight-junction complexes between MSC cells. However, we observed partial disruptions of ZO-1 and β-catenin localizations in ATX- and Aiprenon-treated cells, indicating the focal loss of intercellular attachment (Fig. 4A).

Effect of ATX and Aiprenon on MSC Cell Monolayer Permeability The effect of ATX on the barrier function of the confluent MSC cell monolayer was quantified and evaluated by measuring TEER and fluorescein permeability. The TEER of the MSC cell monolayer after administration of ATX was significantly higher than that of controls at 4, 6, and 24 h \((p < 0.05)\) (Fig. 4B). However, treatment with 10 μM Aiprenon and ATX significantly decreased the ATX-induced increases in TEER at 1, 4, 6, and 24 h \((1 \text{ and } 4 \text{h}, p < 0.001; 6 \text{h}, p < 0.05;\)
Fig. 4. The Effects of ATX and Aiprenon (Compound 1) on Intercellular Adhesion in Monkey Schlemm’s Canal Endothelial (MSC) Cells

(A) MSC cells were immunostained for molecules related to intercellular adhesion involving ZO-1 (green) and β-catenin (red). Cell nuclei were counterstained with DAPI; blue. The right image of each figure is a merged image. Cells were treated for 24 h and immunostained as described in Materials and Methods. Arrows indicate regions where ZO-1 and β-catenin were detected. Scale bar: 50 µm. (B) Transendothelial electrical resistance (TEER) was measured in the MSC cell monolayer. MSC cells were treated with autotaxin (ATX) for 1, 4, 6, and 24 h. Baseline is the relative TEER in MSC cells before the treatment. Time-dependent changes after the treatment were compared as the increased fold changes from each baseline value. Data are represented as the mean ± S.E.M. (*p < 0.05; **p < 0.01; ***p < 0.001, n = 7). (C) The MSC cell monolayer permeability of 4kDa fluorescein isothiocyanate-dextran was measured. MSC cells were treated for 4, 6, and 24 h. Data are represented as the mean ± S.E.M. (*p < 0.05; n = 7). (Color figure can be accessed in the online version.)
The WST-1 assay was performed to confirm cell viability after treatment with Aiprenon. The results indicated the percentage of live or viable cells as compared to the viability of cells not exposed to Aiprenon. There was no significant difference in cell viability among the treatment groups. Data are expressed as the mean ± S.E.M. (n = 7–8). See also Supplementary Fig. S1. (Color figure can be accessed in the online version.)

Table 1. ADME Profile of Aiprenon

| Metabolic stability (%) | CYP<sup>α</sup> inhibitory activity (µM) | Polarity | Solubility (µM) |
|-------------------------|----------------------------------------|----------|----------------|
| Human liver microsomes  | Rat liver microsomes                   | CYP1A2   | CYP2C19        | CYP2C9        | CYP2D6        | CYP3A4        | tPSA<sup>β</sup> (Å<sup>2</sup>) | Number of HBA<sup>γ</sup> | Number of HBD<sup>δ</sup> | JP1<sup>ε</sup> | JP2<sup>ε</sup> |
| 96.2                    | 88.1                                   | >20      | >20            | >20           | >20           | 78.67         | 7                           | 1                           | 1.3                        | >50                        |

24h, p < 0.01).

In addition, the permeability assays using a flux of FITC-dextran showed that treatment with ATX decreased the concentration of FITC-dextran in the apical side at 6 and 24h, compared to that of controls (p < 0.05) (Fig. 4C). Treatment with 10 µM Aiprenon and ATX significantly increased the ATX-induced decrease in concentrations of FITC-dextran at 4, 6, and 24h compared with those of ATX alone (p < 0.05).

The Cytotoxicity of Aiprenon Cell viability was examined in HTM and MSC cells using a nonradioactive colorimetric assay (WST-1), according to the manufacturer’s protocol. There was no significant difference among the groups in posttreatment viability (Fig. 5). As the concentration of Aiprenon increased from 1 to 10 µM, the percentages of HTM and MSC cell deaths did not increase. Treatment with 10 µM Aiprenon was not cytotoxic to HTM or MSC cells. We decided, therefore, to administer Aiprenon at a concentration of 10 µM in the in vitro assays described above (see also supplementary Fig. S1).

ADME Profile of Aiprenon We also evaluated the ADME (absorption, distribution, metabolism, and excretion) of Aiprenon. The data of metabolic stability in human and rat liver microsomes, CYP inhibitory activity, polarity and solubility in the first fluid of the powder solubility test (JP1) and that in the second fluid (JP2) of Aiprenon are listed in Table 1.24)

DISCUSSION

We conducted this research to develop a novel IOP-lowering therapy targeting ATX. We examined IOP-lowering effect of the ATX inhibitor on mouse eyes, rather than one of some other species, to develop effective ATX inhibitors for both mice and humans, because the amino acid sequence for mouse ATX (mATX) shows 93% identity to that of human ATX (hATX), and residues in the active site are highly conserved in mice and humans.28) We used mice with higher IOP by performing LPI (Fig. 2C). Notably, the ATX activity in the AH of LPI-treated mice was significantly increased (Fig. 2A). To the best of our knowledge, this is the first report describing mice with both higher ATX activity in AH and elevated IOP simultaneously. The use of LPI-treated mice enabled us to find ATX was a target molecule in the regulation of IOP, to screen therapeutic agents against ATX, and to examine the IOP-lowering effect of Aiprenon. Considering the higher activity in the AH of LPI-treated mice, we determined the ATX activity in in vitro experiments at 100 nmol/mL/h (Fig. 2A).

ATX treatment increased the expression of αSMA and phallloidin in HTM cells, which could lead to their fibrogenic activity. In addition, it induced localizations of ZO-1 and β-catenin, increased TEER, and decreased the permeability of FITC-dextran in MSC cells, which implied that ATX could decrease the aqueous outflow facility of eyes (Figs. 3A–3C, 4A–4C). These changes contributed to the increased resistance of the trabecular outflow and led to the elevation of IOP. Overall, these results showed that higher levels of ATX could trigger elevation of IOP, and further corroborated our clinical study in which ATX-dependent IOP elevation was observed in the human aqueous fluid of various subtypes of glaucoma.15) We concluded, therefore, that ATX could be a target molecule in the regulation of IOP, and began developing a novel therapy targeting ATX.

Some inhibitors targeting ATX have been developed because of the importance of ATX. Several compounds including BrP-LPA, S32826, GWJ-A23, HA130, and PF-8380 have been reported to be ATX inhibitors.29–34) There is also a report that the chemical ATX inhibitor, S32826, inhibits ATX activity in AH aspirated from rabbit eyes in vitro.35) However, there
have been no ATX inhibitors that lower ATX activity in the AH by means of instillation in vivo. Here, we generated a novel, highly potent ATX inhibitor, Aiprenon, as an ophthalmic drug by means of in vitro screening in a large chemical library and optimization of detected AI scaffold. These inhibitors selectively and potently inhibited ATX. Aiprenon had the strongest ATX inhibitory activity in vivo (Fig. 2A). Compound 2 was an optical isomer of Aiprenon, so compound 2 had exactly the same MW and SlogP value as Aiprenon (Fig. 1B). When comparing Aiprenon and compound 2, we showed that one order nM of IC$_{50}$ was required to be effective as an ophthalmic drug. Consistent with this observation, instillation of compound 3 (IC$_{50}$ = 26 nM) did not inhibit ATX activity in the AH as much as did Aiprenon (Figs. 1B, 2A). The MW and polarity of compounds affect corneal penetration. When compounds have a molecular weight $\geq 500$, their permeability is low. It is reasonable that instillation of compounds 6, 7, 8, and 9, which had molecular weights $\geq 500$, did not decrease ATX activity as much as did Aiprenon, regardless of their low IC$_{50}$ values (Figs. 1B, 2A). In addition, the corneal epithelium is lipoidal in nature and has a great deal of resistance to permeation of topically administered hydrophilic agents. However, more hydrophilic agents are associated with better permeability in the corneal stroma, which includes water as a major component, indicating that the permeability in the corneal stroma is opposite to that in the corneal epithelium. The balance of the lipid and water solubility of compounds is therefore essential to allow them to pass through both the corneal epithelium and stroma. The appropriate lipophilicity can result in better delivery of inhibitors into the AH. Compounds 5 and 6 also had low IC$_{50}$ values (Fig. 1B). However, instillation of compounds 5 and 6 did not decrease the ATX activity of the mouse eyes as much as did Aiprenon, presumably because of the low SlogP values of the former compounds, indicative of high-level water solubility (Figs. 1B, 2A). We consider that Aiprenon has the strongest ATX inhibitory potency as an ophthalmic drug because of its low IC$_{50}$ value, relatively small MW, and suitable lipophilicity (Fig. 1B). Compound 2, which exhibited an IC$_{50}$ value approximately 10-fold that of Aiprenon, significantly but less markedly than Aiprenon reduced lysoPLD activity in normal mice and LPI-treated mice exhibiting the higher ATX activity (Figs. 1B, 2A, B). We concluded that we should use Aiprenon in further experiments in both in vivo and in vitro experiments.

Our results revealed that Aiprenon treatment inhibited fibrogenic activity and the higher resistance of the trabecular pathway caused by ATX in vitro (Figs. 3A–C, 4A–C). We observed focal disruptions of ZO-1 and $\beta$-catenin in ATX and Aiprenon-treated MSC cells, which indicated a loss of intercellular adhesion (Fig. 4A). These results imply that Aiprenon induced paracellular permeability through changes in the tight junctions. In brief, Aiprenon could recover the outflow facility decreased by ATX, resulting in a significant fall in IOP. However, further studies will be needed to elucidate the origin of the higher levels of ATX in eyes with higher IOPs. Further studies focusing on the mechanisms of higher ATX in AH would facilitate an understanding of how ATX affects IOP. It is our firm belief that this newly developed ATX inhibitor, Aiprenon, will be a useful tool and will allow researchers to investigate more fully the origin and roles of ATX in regulating IOP in future experiments.

It is essential to examine the safety and ADME profile of any compound before its clinical application. In the present study, at least 10$\mu$M of Aiprenon did not affect cell viability (Fig. 5). Metabolic stability, solubility, and CYP inhibitory activity were also confirmed (Table 1). From these results, we consider that Aiprenon has promising clinical applications.

A limitation of this study should be acknowledged. We could not compare changes over time in lysoPLD activity and IOP in the same eyes, because we measured IOP using the microneedle method. The microneedle method provides precise measurement of IOP; however, the measurement cannot be repeated at short intervals because of its invasiveness. In addition, we need to evaluate the IOP-lowering effect of Aiprenon for a longer period of time in our next study. Even though more experiments on the physiological pathway involving ATX in regulating IOP are required, the present study showed that a topical anti-ATX agent had the potential to lower IOP by instillation, and this newly developed inhibitor, Aiprenon, is expected to be a useful tool for investigating the roles of ATX in eye disorders caused by excessive ATX expression in AH, including LPI-induced ocular hypertension and glaucoma.

In conclusion, we found that ATX was a target protein in the regulation of IOP and potent ATX inhibitors can be used as an alternative treatment strategy to lower IOP. Therefore, our newly developed ATX inhibitor, Aiprenon, can be a promising candidate as an anti-ATX therapeutic agent for lowering IOP.

Acknowledgments The authors thank the staff at Shionogi & Co., Ltd. (Osaka, Japan) for their help with the chemical and molecular biological experiments and T. Fujimoto (Kumamoto University) for providing monkey Schlemm’s canal endothelial cells. N.N. was supported by a UT Grant for a Ph.D. Researcher Program. This study was supported by the Japan Society for the Promotion of Science, Grant number 15K10854 (M.H.). This research was also supported by the Platform Project for Supporting Drug Discovery and Life Science Research from AMED under Grant Number JP17am0101086 (T.O., H.K., and T.N.). The English in this document has been checked by at least two professional editors, both native speakers of English. M.A. and T.N. conceived the project and designed the experiments. N.N. performed the cellular and molecular biological experiments and data analyses. M.K., T.K., H.K., and T.O. directed the chemical experiments. M.H., N.N., O.N., K.K., and J.A. directed the cellular and molecular biological experiments. M.A., T.N., and N.N. wrote the manuscript.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Kaufman PL, Gabelt BT, Cynader M. Introductory comments on neuroprotection. Surv. Ophthalmol. 43(Suppl. 1), S89–S90 (1999).
2) Wiederholt M, Bielka S, Schweig F, Lutjen-Drecoll E, Lepley-Wien, hues A. Regulation of outflow rate and resistance in the perfused
anterior segment of the bovine eye. *Exp. Eye Res.*, **61**, 223–234 (1995).

3) Gabelt BT, Kaufman PL. Changes in aqueous humor dynamics with age and glaucoma. *Prog. Retin. Eye Res.*, **24**, 612–637 (2005).

4) Grant WM. Experimental aqueous perfusion in enucleated human eyes. *Arch. Ophthalmol.*, **97**, 783–801 (1963).

5) Rosenquist R, Epstein D, Melamed S, Johnson M, Grant WM. Outflow resistance of enucleated human eyes at two different perfusion pressures and different extents of trabeculotomy. *Curr. Eye Res.*, **5**, 1233–1240 (1989).

6) Mâepea O, Bill A. Pressures in the juxtasacanalicular tissue and Schlemm's canal in monkeys. *Exp. Eye Res.*, **54**, 879–883 (1992).

7) Johnson M, Shapiro A, Ethier CR, Kamm RD. Modulation of outflow resistance by the pores of the inner wall endothelium. *Invest. Ophthalmol. Vis. Sci.*, **33**, 1670–1675 (1992).

8) Vranka JA, Kelley MJ, Acott TS, Keller KE. Extracellular matrix in the trabecular meshwork: intraocular pressure regulation and dysregulation in glaucoma. *Exp. Eye Res.*, **183**, 112–125 (2015).

9) Torrejon KY, Papke EL, Halman JR, Bergkvist M, Danis J, Sharfstein S1, Xie Y. TGFBeta2-induced outflow alterations in a bioengineered trabecular meshwork are offset by a rho-associated kinase inhibitor. *Scientific Reports*, **6**, 38319 (2016).

10) Quigley HA. Open-angle glaucoma. *N. Engl. J. Med.*, **328**, 1097–1099 (1993).

11) Umezu-Goto M, Kishi Y, Taiza A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J, Arai H. Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J. Cell Biol.*, **158**, 227–233 (2002).

12) Tokumura A, Majima E, Kariya Y, Tominaga K, Kogure K, Yasuda K, Fukuzawa K. Identification of human plasma lysophosphatase D, a lysophosphaticidase-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J. Biol. Chem.*, **277**, 39436–39442 (2002).

13) van Meeteren LA, Moolenaar WH. Regulation and biological activities of the autotaxin-LPA axis. *Prog. Lipid Res.*, **46**, 145–160 (2007).

14) Nakagawa K, Hama K, Aoki J. Autotaxin—an LPA producing enzyme with diverse functions. *Biochem.*, **148**, 13–24 (2010).

15) Honjo M, Igarashi N, Kurano M, Yatomi Y, Igarashi K, Kano K, Aoki J, Weinreb RN, Aihara M. Autotaxin–lysophosphaticidase acidic pathway in intraocular pressure regulation and glaucoma subtypes. *Invest. Ophthalmol. Vis. Sci.*, **59**, 693–701 (2018).

16) Kawayaguchi M, Okabe T, Okudaira S, Nakagawa K, Hama K, Aoki J, Tanaka N, Yaginuma K, Arai H, Kawahara A, Nishina H, Aoki J. Autotaxin regulates vascular development via multiple lysophosphatidic acid (LPA) receptors in zebrafish. *J. Biol. Chem.*, **286**, 43972–43983 (2011).

17) Zhang H, Xu X, Gajewiak J, Tsukahara R, Fujiwara Y, Liu J, Fells JJ, Perygin D, Parrill AL, Tigyi G, Prestwich GD. Dual activity lysophosphatidic acid receptor pan-antagonist/autotaxin inhibitor reduces breast cancer cell migration in vivo and causes tumor regression in vivo. *Cancer Res.*, **69**, 5441–5449 (2009).

18) Ferry G, Mouhitarat N, Pradere JP, Desos P, Aigenti GG, Toumelin M, Bertrand M, Saulnier-Blache JS, Tucker GC, Cordis M, Boutin JT, S2826, a nanomolar inhibitor of autotaxin: discovery, synthesis and applications as a pharmacological tool. *J. Pharmacol. Exp. Ther.*, **327**, 809–819 (2008).

19) Park GY, Lee YG, Berdyshiev E, et al. Autotaxin production of lysophosphatidic acid mediates allergic asthmatic inflammation. *Am. J. Respir. Crit. Care Med.*, **188**, 928–940 (2013).

20) Oikonomou N, Mouratis MA, Tzouvelakis K, Kafes E, Valavanis C, Vilagas G, Karameris A, Prestwich GD, Bouros D, Aidinis V. Pulmonary autotaxin expression contributes to the pathogenesis of pulmonary fibrosis. *Am. J. Respir. Cell Mol. Biol.*, **47**, 566–574 (2012).

21) Alberts HM, Dong A, van Meeteren LA, Egan DA, Sunnaka M, van Tilburg EW, Schuurman K, van Tellingen O, Morris AJ, Smyth SS, Moolenaar WH, Ovaan H. Boronic acid-based inhibitor of autotaxin reveals rapid turnover of LPA in the circulation. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 7257–7262 (2010).

22) Gierse J, Thorarensen A, Beltey K, Bradshaw-Pierce E, Cortes-Burgos L, Hall J, Johnston A, Murphy M, Nemirovsky O, Ogawa S, Pegg L, Pel C, Prinsen M, Schnute M, Wendling J, Weine S, Weinberg R, Wittwer A, Zweifel B, Masferrer J. A novel autotaxin inhibitor reduces lysophosphaticidic acid levels in plasma and the site of inflammation. *J. Pharmacol. Exp. Ther.*, **334**, 310–317 (2010).

23) Lyyer P, Lalane R 3rd, Morris C, Challa P, Vann R, Rao PV. Autotaxin–lysophosphaticidic acid axis is a novel molecular target for lowering intraocular pressure. *PLOS ONE*, **7**, e42627 (2012).

24) Gaudana R, Ananthula HK, Parenky A, Mitra AK. Ocular drug delivery. *AAPS J.*, **12**, 348–360 (2010).

25) Wildman SA, Crippen GM. Prediction of physicochemical parameters by atomic contributions. *J. Chem. Inf. Comput. Sci.*, **39**, 868–873 (1999).