A Highly Selective Rho-Kinase Inhibitor (ITRI-E-212) Potentially Treats Glaucoma Upon Topical Administration With Low Incidence of Ocular Hyperemia

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Purpose. The purpose of this study was to investigate the IOP-lowering effects of the ITRI-E-212, a new Rho-associated protein kinase (ROCK) inhibitor. ITRI-E-212 improved fluid outflow through the trabecular meshwork and reduced IOP with transient and mild conjunctival hyperemia. ITRI-E-212 can potentially be developed into new antiglaucoma agents.

Methods. ITRI-E-212 was selected from more than 200 amino-isoquinoline structures because of its adequate solubility and drug-loading percentage in eye drops. ITRI-E-212 has less than 50% inhibitory concentration (IC50) against ROCK2. The in vitro kinase inhibition was evaluated using the ADP-Glo kinase assay. A comprehensive analysis of the kinase inhibitor selectivity of ITRI-E-212 was performed using the KINOMEscan methodology. The IOP-lowering effect and tolerability of ITRI-E-212 were investigated in normotensive and ocular hypertensive rabbits. The pharmacokinetics study was performed in vivo in the aqueous humor (AH), and hyperemia was assessed.

Results. ITRI-E-212 showed high in vitro inhibitory activity against ROCK2 and high specificity against AGC kinases. The mean IOP-lowering effect of ITRI-E-212 in normotensive and ocular hypertensive models was 24.9% and 28.6%, respectively; 1% ITRI-E-212 produced notable reductions in IOP that were sustained for at least 6 hours after each dose once per day. Only transient, mild hyperemia was observed. The compound extracted from the AH reached 78.4% ROCK2 kinase inhibition at 1 hour after dose administration and was sustained for 4 hours.

Conclusions. ITRI-E-212 is a novel and highly specific ROCK2 inhibitor with the ability to lower IOP in animal models. It has favorable pharmacokinetic and ocular tolerability profiles with only minimal conjunctival hyperemia.

Keywords: IOP-lowering effect, ROCK inhibitors, hyperemia, glaucoma, primary open-angle glaucoma (POAG)

Glaucoma, the primary cause of blindness, is a disease that affects the optic nerve head, characteristically leading to the progressive death of retinal ganglion cells (RGCs) and the subsequent irreversible loss of visual field.1–3 Primary open-angle glaucoma (POAG), the most common subset of glaucoma, develops due to chronically elevated IOP, which is caused by an abnormally high resistance to aqueous humor (AH) drainage through trabecular meshwork (TM) outflow pathways.4–6 This resistance appears to be associated with the juxtacanalicular portion of the TM-mediated extracellular matrix and endothelial-lined Schlemm’s canal, which is responsible for the conventional outflow.7–8 Pharmacologic lowering of IOP has been demonstrated to reduce the risk of POAG progression.9,10

Conventional antiglaucoma medications act by suppressing AH production by inhibiting the synthesis of cyclic adenosine monophosphate (cAMP) in the ciliary epithelium, resulting in an increase in the uveoscleral and conventional outflow. Cholinergic agents, such as pilocarpine, increase the conventional outflow by widening Schlemm’s canal and reducing IOP by targeting the ciliary muscle.11 Because IOP is sensitive to this conventional pathway,11 a drug that targets this pathway may prevent more IOP spikes compared with drugs that target AH production or the uveoscleral outflow. A novel class of drugs, Rho-kinase or Rho-associated protein kinase (ROCK) inhibitors, appear to directly reduce IOP by addressing the diseased TM.13–19 ROCK is a serine/threonine kinase that serves as an essential downstream effector of Rho-GTPase. ROCK activates
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over the actomyosin system and is responsible for smooth muscle contraction. The actomyosin system is present in the juxtanaculcular portion of the TM and Schlemm’s canal. ROCK inhibitors modulate myosin light chain phosphorylation (pMLC) and reduce the contractility of the TM and Schlemm’s canal cells, thereby facilitating trabecular outflow. ROCK inhibitors are also promising therapeutic targets for increasing ocular blood flow, improving the RGC survival rate, and promoting RGC axon regeneration.

Preclinical data have demonstrated the therapeutic potential of ROCK inhibitors, such as Y-27632, Y-39983, and fasudil, which alter the contractility of TM cells and reduce IOP by facilitating conventional outflow. However, one of the adverse effects of ROCK inhibitors, as potent vasodilators, is conjunctival hyperemia. At higher concentrations, ROCK inhibitors may affect other protein kinases in the body, such as protein kinases A and C and myosin light chain kinase. The narrow therapeutic time window limits the application of existing ROCK inhibitors and leads to the development of novel ROCK inhibitors. Given the risks associated with kinase inhibitors, studies have focused on drugs that are not only potent but also highly selective for ROCK2. Over the last few years, topical ROCK inhibitors used for the treatment of glaucoma were ripasudil (also known as K115 precursor, Kowa, Aichi, Japan; IC50 = 19 nM for ROCK2) and netarsudil (also known as AR-13324, Aerie Pharmaceuticals, Inc., Durham, NC, USA; IC50 = 1.1 nM for ROCK). Netarsudil also inhibits the norepinephrine transporter (NET) and belongs to a new class of ROCK/NET inhibitors that are used to reduce episcleral venous pressure. To prevent systemic risks, drugs such as AM0076 have been designed to remain active only in the AH. As a soft ROCK inhibitor, AM0076 did not cause significant hyperemia at a dose sufficient for IOP reduction in New Zealand White (NZW) rabbits.

The goal of this study was to create a new aminoisoquinoline ROCK inhibitor compound through homology modeling, molecular docking studies, and fragment-based drug discovery. This new compound should have excellent IOP-lowering effects and corneal penetration, be easily manufactured, and cause less conjunctival hyperemia. We aimed to investigate compounds with highly specific ROCK inhibitions in vitro and less adverse effects in vivo. More than 200 ROCK inhibitors were designed and synthesized, from which eight compounds were selected to be formulated into eye drops based on high solubility (>50 μM, 7 mg/mL) and high loading yield (>2, 1%). These compounds (IC50 < 250 nM) were administered topically to reduce IOP in ocular normotensive and hypertensive NZW rabbit models. The most effective compound for lowering IOP was ITRI-E-212, which demonstrated superior inhibitory activity and high relative selectivity for ROCK2.

METHODS

Test Articles

More than 200 patentable chemical structures of ITRI-E-212 and their analogs were chemically modified from aminoisoquinoline in a single process that applied insights from molecular modeling/docking models and fragment-based drug discovery. Our technical platform proposes concepts for developing a series of ROCK inhibitors. The substitution of urea on aminoisoquinoline derivatives provides an opportunity to fine-tune pharmaceutical properties such as the aqueous solubility, kinase inhibitory activity, IOP-lowering effect, and pharmacokinetic profile.

In Vitro Kinase Inhibitory Assay

The luciferase-based ADP-Glo kinase assay, purchased from Promega (Madison, WI, USA), was used to measure kinase activity by quantifying the amount of ADP produced during a kinase reaction (see ADP-Glo kinase assay technical manual). The measured luminescence signal was proportional to the amount of ADP, with ADP representing kinase activity. The ADP-Glo kinase assay was performed in two steps. First, the kinase cocktail reaction occurred for 30 minutes at 30°C (20 μL/well), and an equal volume of ADP-Glo reagent was added at room temperature and allowed to equilibrate for 1 hour to terminate the kinase reaction and deplete the remaining ATP. Subsequently, the kinase detection reagent (80 μL) was added at room temperature and allowed to equilibrate for 1 hour to convert ADP to ATP and allow the newly synthesized ATP to be measured using an Orion II Microplate luminometer (Titertek Berthold, Pforzheim, Germany). Relative luminescence units (RLUs) were obtained, and the RLU was set as 100% (control value) without a test compound and at 0% (normal value) without an enzyme in a compound. The reaction rate (percentage of control) was then calculated from the RLU with each addition of different concentrations of test compounds. The 50% inhibitory concentration (IC50) was determined using logistic regression analysis.

Myosin Light Chain Phosphorylation

The function of pMLC was assessed in rat aortic smooth muscle cell lines using a cell-based assay. A7r5 cells (American Type Culture Collection, Manassas, VA, USA) were plated at 5000 cells per well in 96-well plates, and a direct ELISA technique was used after the cells had been serum starved for 3 hours. The pMLC status was quantified from the phospho-levels of MLC-Thr18/Ser19, which was probed with goat polyclonal anti-phospho MLC (Thr18/Ser19, diluted 1:2,000 and kept overnight at 4°C in a blocking buffer; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). This cell-based pMLC assay in 96-well plates allowed for rapid screening of novel Rho-kinase inhibitors that exhibited a decrease in the phosphorylated content of the myosin light chain. After adding the stop solution of 1 N HCl and establishing the optical density as 450 nm, pMLC was used to rapidly generate quantitative cell-based IC50 values of Rho-kinase inhibitors.

DiscoveRx Kinase Profiling of ITRI-E-212

“Kinome” has been catalogued in the catalytic domain according to sequence similarity. The inhibitors of the protein kinase family (including tyrosine, serine, threonine, and histone kinases) are classified into four types based on their action mechanism. A set of 468 kinase inhibitory tests was performed on ITRI-E-212 by using the scanMAX kinase assay panel of KINOMEscan assay from the DiscoveRx Corporation (San Diego, CA, USA; http://www.discoverx.com). In this study, the kinase-binding maps of ITRI-E-212 demonstrated the strength and relative specificity of kinase-binding interactions. Results are reported as the percentage of the control (%Ctrl), where %Ctrl = [(positive control signal – test compound signal)/(positive control signal – negative control signal)] × 100. Dimethyl sulfoxide (DMSO) was used as the negative control. Lower values of %Ctrl indicated a stronger interaction between ITRI-E-212 and kinases. TRESspot was generated online using the TRESspotTM Software Tool and was reprinted with permission from KINOMEscan, a division of DiscoveRx Corporation (http://www.discoverx.com/services/drug-discovery-development-services/treespot-data-analysis). A large red circle indicated higher-affinity binding of numerous kinases. 
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**Animals and Study Design**

Male NZW rabbits aged 12 to 14 weeks and weighing 2.5 to 3.0 kg were used in this study. The animal study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Care and Use Committee of National Defense Medical Center. All animals were kept in a 12-hour day/night cycle in a pathogen-free condition at the animal center of National Defense Medical Center, Taipei, Taiwan. The animals were fed a chow diet and water ad libitum. As AH sampling is known to interfere with IOP, the animals studied were stratified into two groups, one of which was assigned to pharmacokinetic sampling and the other to IOP measurement.

**IOP-Lowering Effect of ITRI-E-212 in Ocular Normotensive NZW Rabbits**

A pneumatometer (Model 30 Classic; Reichert Ophthalmic Instruments, Depew, NY, USA) was used to monitor IOP, with an average of five recordings per eye. The eyes of NZW rabbits were anesthetized using a topical instillation of 0.4% oxybuprocaine (Unicare; Thea Pharma, Schaffhausen, Switzerland) before every IOP measurement. Compounds were instilled topically into the right eyes of NZW rabbits at ITRI-E-212 concentrations of 0.3% and 1.0%, and their left eyes received a control blank vehicle, 0.5% polyethylene glycol 400 (PEG-400) in PBS. IOP was measured immediately prior to treatment and 0, 1, 2, 4, and 6 hours after each compound administration, and changes in IOP were calculated to a value of \( n = 12 \). In the 10-day repeated dose experiment performed to examine drug safety and tolerability, we observed the effect of ITRI-E-212 when it was administered on the morning of IOP measurements. To examine the continual IOP-lowering effect, 1.0% ITRI-E-212 (\( n = 10 \)) was instilled at 10:00 AM, and IOP was measured at 0, 1, 2, 4, 6, and 24 hours after instillation each day, with the final measurement being taken at 222 hours (10 days). The contralateral eyes of ITRI-E-212-treated animals received the blank control vehicle 0.5% PEG 400 in PBS. No conjunctival hyperemia occurred during the 10-day repeated dose experiment.

**IOP-Lowering Effect of ITRI-E-212 on Ocular Hypertensive NZW Rabbits**

Before inducing ocular hypertension, the IOP of both the eyes was measured. Subsequently, rabbits were anesthetized with an intramuscular injection of 50 mg/mL ketamine (Ketalar; Pfizer, Ann Arbor, MI, USA) and 2% Rompun (Bayer Healthcare, Pittsburgh, PA, USA). Ocular hypertension was induced by injecting 0.3 mL 0.4% PreviscAid Ophthalmic Viscoelastic (Maxigen Biotech, Taoyuan City, Taiwan) and then instilling 1% Pred Forte (Allergan, Inc., Irvine, CA, USA) once a day for 3 days. On the third day, the steroid was instilled, and the rabbits were randomly assigned to their groups. Before the injection of Maxigen, a temporal paracentesis was created using a sharp 26-gauge needle (Terumo Europe N.V., Leuven, Belgium). After the injection, the paracenteses were hydrated with saline to prevent AH reflux. Once a day for 3 days at zero hours (10:00 AM), ITRI-E-212 (1.0%) was instilled topically in the right eye (OD), and the vehicle was instilled topically in the left eye (OS). IOP was measured 0, 2, 4, and 6 hours after every instillation of the test compounds (\( n = 12 \)).

**Pharmacokinetics Study in AH**

The ITRI-E-212 concentration in AH is a surrogate marker for ROCK inhibition in the TM. The ocular pharmacokinetic (PK) models in NZW rabbits were set up and validated to evaluate the ocular penetration of ITRI-E-212 (doses administered at 1, 4, and 6 hours). Liquid chromatography and tandem-mass spectrometry (LC-MS/MS) analyses were performed in accordance with Good Laboratory Practice. AH samples (50 μL) from NZW rabbits were briefly deproteinized using 300 μL acetonitrile (1:6). These samples were centrifuged (13,000 rpm for 5 minutes), and the supernatants were evaporated using a GeneVac EZ-2 plus Speed Vac Concentrator (Marshall Scientific, Hampton, NH, USA). The concentrations of reconstituted samples were determined through LC/MS/MS (4000 QTRAP; Applied Biosystems, Foster City, CA, USA). These results provided information regarding the on-target effect of ITRI-E-212. The extracted ITRI-E-212 from the AH of the other NZW rabbit group was resolved in the PBS buffer to determine in vitro ROCK2 inhibitory activity at different times (1 and 4 hours).

**Hyperemia Scoring**

NZW rabbits were used to investigate hyperemia after a single dose of 0.3% and 1% ITRI-E-212. Eyes were photographed using a digital camera (RX100 Advanced Camera with 1.0-inch Sensor; Sony, Inc., Los Angeles, CA, USA). Hyperemia was assessed using the Organization for Economic Co-operation and Development (OECD) 405 “Acute Eye Irritation/Corrosion” Guideline and was recorded 1, 2, and 4 hours after the topical instillation of the compound. Two scores were assigned to ocular irritation. One number was given to the conjunctiva redness, which refers to palpebral and bulbar conjunctiva, excluding the cornea and iris: 0, normal; 1, presence of some hyperemic blood vessels; 2, diffused crimson color where individual vessels were not easily discernible; 3, diffused beefy red color. Another number was given to the swelling of the eyelids or nictitating membranes: 0, normal; 1, slight swelling; 2, obvious swelling with partial eversion of the eyelids; 3, swelling with eyelids about half closed; 4, swelling with eyelids more than half closed.

**Statistical Analysis**

The data are expressed as the mean ± SD and were evaluated using a 1-way or 2-way ANOVA followed by Tukey’s post hoc test with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**In Vitro Inhibitory Activity by ITRI-E-212**

Our synthesized technical platform had more than 200 amino-quinoline derivatives that allowed the development of a series of novel ROCK inhibitors against ROCK2 and the downregulation of pMLC in A7r5 cells. Eight compounds exhibited higher solubility (>50 μM, 7 mg/mL) in PBS (Table 1). ITRI-E-212 was found to have the highest drug-loading percentage (2.1%) in PBS (pH 6) among the selected compounds. The kinase inhibitory activity of the selected compounds (IC\(_{50}\)) against ROCK2 was demonstrated using an ADP-Glo kinase assay. ITRI-E-212 was found to have the lowest IC\(_{50}\) (0.250 ± 0.08 μM) of all the compounds (Table 2). ITRI-E-212 also downregulated pMLC in A7r5 cells (62.0 ± 13% inhibition at 10 μM pMLC) with an IC\(_{50}\) of 8.71 ± 1.08 μM (Table 2). These findings demonstrate that ITRI-E-212 exhibited a high inhibitory activity against ROCK2 and the highest drug-loading yield.
The in vitro kinase selectivity of ITRI-E-212 was comprehensively evaluated using the kinase-binding assays against a panel of 468 distinct kinases by using the KINOMEscan screening platform (DiscoverX) with a single-point binding at 1 μM ITRI-E-212. The KINOMEscan uses a novel active site-directed competitive binding assay to quantitatively measure interactions between ITRI-E-212 and 468 kinase assays. Lower values of %Ctrl indicated a stronger interaction between ITRI-E-212 and kinases. Based on KINOMEscan profiling results, rho-associated protein kinase 1 (ROCK1), rho-associated protein kinase 2 (ROCK2), protein kinase X-linked (PRKX), protein kinase G (PKG) 2 (PRKG2), protein kinase, cAMP-dependent, catalytic, alpha (PKAC-α), protein kinase G (PKG) 1 (PRKG1), protein kinase N1 (PKN1), and protein kinase, cAMP-dependent, catalytic, beta (PKAC-β) were found to possess highly selective S (10) scores of 0.05, 0.35, 2.3, 2.9, 4.9, 5.5, 7.8, and 8.2, respectively (defined as having elicited more than 90% inhibition at 1 mM; Fig. 1). An analysis of interaction patterns provides an explanation for the class of “group-selective” inhibitors that are broadly active against a single subfamily of kinases. Our data revealed that AGC inhibitors, as a class, were more selective than other inhibitors. For example, the relative binding percentage of ROCK2 to control was 0.35% in the presence of 1.0 μM ITRI-E-212. An efficient dissociation constant (K_d) of 17 nM in ROCK2 was confirmed using the KINOMEscan system. At 1 μM, ITRI-E-212 exhibited high specificity against AGC kinases in vitro without off-target effects.

Effectively Lowered IOP in Normal NZW Rabbits

To evaluate the IOP-lowering effect of ITRI-E-212, IOP measurements were taken every hour (1 to 6 hours) immediately before and after the administration of ITRI-E-212 eye drops in normotensive NZW rabbits. ITRI-212 reduced IOP in a dose-dependent manner at concentrations between 0.3% and 1% (Fig. 2). The maximum

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**Table 1.** Solubility and Loading Percentage of ROCK Inhibitors as Derived From Amino-Isoquinoline in an Optimal Eye Drop Formulation (50% PEG 400 Under Different pH PBS)

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Solubility in PBS Buffer, μM</th>
<th>Drug Loading in Eye Drop, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITRI-E-185</td>
<td>7.0</td>
<td>&gt;100</td>
<td>0.28</td>
</tr>
<tr>
<td>ITRI-E-187</td>
<td>7.0</td>
<td>50–100</td>
<td>0.56</td>
</tr>
<tr>
<td>ITRI-E-194</td>
<td>6.1</td>
<td>50</td>
<td>0.1</td>
</tr>
<tr>
<td>ITRI-E-211</td>
<td>8.0</td>
<td>&gt;100</td>
<td>0.25</td>
</tr>
<tr>
<td>ITRI-E-212</td>
<td>6.0</td>
<td>50–100</td>
<td>2.1</td>
</tr>
<tr>
<td>ITRI-E-226</td>
<td>7.8</td>
<td>&gt;100</td>
<td>0.4</td>
</tr>
<tr>
<td>ITRI-E-227</td>
<td>7.4</td>
<td>50–100</td>
<td>0.17</td>
</tr>
<tr>
<td>ITRI-E-239</td>
<td>7.0</td>
<td>&gt;100</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Table 2.** Relative In Vitro Potencies (IC50 Value, μM) for ROCK2 Inhibition and Diphospho-Myosin Light Chain Assay (Modulation of Cellular Contraction/Relaxation) in A7r5 (Rat Aortic Smooth Muscle Cells) for ITRI-212

<table>
<thead>
<tr>
<th>Compound</th>
<th>ROCK2 IC50, μM</th>
<th>pMLC Inhibition at 10 μM</th>
<th>pMLC IC50, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITRI-E-185</td>
<td>3.54 ± 0.15</td>
<td>43.1 ± 1.5</td>
<td>–</td>
</tr>
<tr>
<td>ITRI-E-187</td>
<td>1.96 ± 0.02</td>
<td>67.9 ± 7.3</td>
<td>–</td>
</tr>
<tr>
<td>ITRI-E-194</td>
<td>1.05 ± 0.32</td>
<td>39.8 ± 0.4</td>
<td>–</td>
</tr>
<tr>
<td>ITRI-E-211</td>
<td>2.39 ± 1.86</td>
<td>61.7 ± 7.8</td>
<td>9.58</td>
</tr>
<tr>
<td>ITRI-E-212</td>
<td>0.25 ± 0.08</td>
<td>62.0 ± 13.0</td>
<td>8.71 ± 1.08</td>
</tr>
<tr>
<td>ITRI-E-226</td>
<td>0.99 ± 0.18</td>
<td>48.0 ± 7.5</td>
<td>–</td>
</tr>
<tr>
<td>ITRI-E-227</td>
<td>0.85 ± 0.08</td>
<td>54.8 ± 5.3</td>
<td>–</td>
</tr>
<tr>
<td>ITRI-E-239</td>
<td>0.36 ± 0.09</td>
<td>44.3 ± 3.5</td>
<td>–</td>
</tr>
</tbody>
</table>

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**Figure 1.** Comprehensive analysis of the kinase inhibitor selectivity of ITRI-E-212 using the KINOMEscan methodology, which is a quantitative analysis method using %Ctrl as a potency threshold to describe and compare the selectivity of different compounds. The TREESpot compound profile demonstrated that ITRI-E-212 possesses a highly selective profile of the AGC kinase inhibitor.
IOP reduction (ΔIOP) observed 4 hours after the administration of 0.3% and 1% ITRI-E-212 eye drops (solid line) in one eye and the vehicle control in the other eye (dotted line) in NZW rabbits. (A) IOP measured prior to experiments and at 1, 2, 4, and 6 hours after administration. (B) Change in IOP (ΔIOP) at 1, 2, 4, and 6 hours after administration. (C) Percentage (%) of ΔIOP in ocular normal NZW rabbits. (D) IOP-lowering effect of ITRI-E-212 in ocular hypertensive NZW rabbits; vehicle-only (dotted line) and 1.0% (solid line) ITRI-E-212 eye drops for 3 days. (E) The % of ΔIOP and comparison between normal and hypertensive rabbits obtained at 1, 2, 4, and 6 hours after the topical administration of 1% ITRI-E-212 eye drops. All data are presented as mean ± SEM (n = 12), *P < 0.05 compared with the control (paired t test).
group). To further test the IOP-lowering effect of ITRI-E-212, we used an ocular hypertensive rabbit model, which was developed using a single intracameral injection of hyaluronic acid, followed by continual administration of a topical steroid for 3 days. An increase in IOP to 22 mm Hg indicated the establishment of an acute ocular hypertensive rabbit model. We instilled 1.0% ITRI-E-212 eye drops once a day into the experimental eye of acute ocular hypertensive rabbit models. The IOP-lowering effects observed 0, 2, 4, 6, and 24 hours after the drug administration were measured for 3 days. The IOP-lowering effects of topically administered 1% ITRI-E-212 on the eyes of ocular hypertensive NZW rabbits were compared with those on the control eyes (n = 6 in each group, P < 0.05; Fig. 2D). The maximum reduction of 5.92 mm Hg (28.6%) was measured at 6 hours and reached statistical significance (P < 0.001) after the administration of 1% ITRI-E-212 in ocular hypertensive models compared with the normotensive model (Fig. 2E). These results demonstrated that the effect of 1% ITRI-E-212 on IOP was significant in both ocular normotensive and acute ocular hypertensive NZW rabbit models.

Tolerability and Conjunctival Hyperemia Scores in Normotensive NZW Rabbits

Topical ROCK inhibitors induce mild to severe transient conjunctival hyperemia. The dose-dependent IOP-lowering ability and tolerability of ITRI-E-212 were evaluated in normotensive NZW rabbits by measuring IOP and conducting ocular examinations. The 1.0% ITRI-E-212 eye drops exerted a potent IOP-lowering effect, with statistically significant IOP reduction at every measured time point in comparison with the contralateral control eye (P < 0.05, P < 0.001, and P < 0.0001). The maximum reduction of 20% (ΔIOP, %) was achieved at 4 hours after the administration of 1% ITRI-E-212 eye drops (Fig. 3). Hyperemia in the 1.0% ITRI-E-212–treated eye and control eye (without ITRI-E-212) was assessed on a scale from 0 to 3 (none to severe), for 10 days. Tolerability was evaluated by considering the conjunctival hyperemia scoring system and other severe adverse effects. Typically, mild hyperemia (+1) persisted for 1 to 4 hours after dose administration, and no severe ocular complications were noted. These results demonstrated that the topical administration of ITRI-E-212 was well tolerated and effective in lowering IOP in rabbit models and thus has potential clinical applications in long-term glaucoma treatment. Mild conjunctival hyperemia was observed in both 0.3% and 1% ITRI-E-212–treated eyes in normotensive NZW rabbits (Fig. 4A). We evaluated the degree of hyperemia after a single topical administration of 0.3% and 1% ITRI-E-212 before administration and at 1, 2, and 4 hours after administration and assessed the acute eye irritation/corrosion level at 6 hours after ITRI-E-212 administration in accordance with procedures described in the OECD Guideline 405. The results revealed that the total scores decreased with time (Fig. 4B). The 0.3% and 1% ITRI-E-212–induced conjunctival hyperemia did not reach statistical significance at 2, 4, or 6 hours compared with control rabbit models.

PK in AH of Rabbit Eyes

We examined the penetration of ITRI-E-212 into the AH of NZW rabbits and assayed the mean concentrations of ITRI-E-
212 in AH samples after a single dose of ITRI-E-212 eye drops by using a validated LC-MS/MS method. The mean concentration profile of ITRI-E-212 in the AH is shown in Figure 5. We briefly observed the ITRI-E-212 concentration (Cmax) in the AH after 1 hour (Tmax). The ITRI-E-212 concentration in the AH was proportional to the administration of different doses of ITRI-E-212 eye drops. The effect of 1.0% ITRI-E-212 eye drops persisted for more than 4 hours in drug concentrations of more than 100 ng/mL (the concentration closest to the IC50 of ITRI-E-212, 250 nM). The highest IOP-lowering effect in vivo was observed at 4 hours, and the Cmax of 1% ITRI-E-212 was 900 ng/mL at Tmax (1 hour). These results demonstrated that ITRI-E-212 has a rapid onset of action for inhibiting ROCK2. The maximum efficacy of ITRI-E-212 was sustained for at least 4 hours after administration. The ITRI-E-212 compound of the amino-quinoline series demonstrated good corneal penetration. The percentage of 1% ITRI-E-212 in AH was 0.08% at Cmax after the volume correlation. The AH samples of ROCK2 inhibition revealed that ITRI-E-212 exerted an ideal efficacy in vitro pharmacology studies. The extracted ITRI-E-212 from the AH was inspected for in vitro ROCK2 kinase inhibition and showed 78.4% inhibition 1 hour after dose administration, with the inhibition being maintained for 4 hours. ITRI-E-212 between 0.3% and 1% yielded the maximum drug concentrations in the AH after 1 hour, with high specificity for the ROCK2 kinase.

**DISCUSSION**

The results of this preclinical study demonstrated the significant IOP-lowering ability of ITRI-E-212, as a potent ROCK inhibitor, in ocular hypertensive and normotensive NZW rabbit models. ITRI-E-212 is highly specific and well tolerated, with only transient, mild conjunctival hyperemia. In vitro biochemical assays revealed that ITRI-E-212 is highly effective at inhibiting ROCK2 in comparison with other compounds, and the IC50 of 0.25 μM was 10 times lower than that of common ROCK inhibitors, such as Y-27632 and Fasudil.
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(2 μM). However, ITRI-E-212 is still not as active as the ROCK inhibitors K-115 (IC50 = 19 nM) and AR-13324 (IC50 = 1.1 nM).30,33,34 Studies have demonstrated that ROCK inhibitors may reduce IOP by modulating actin contraction fibers, resulting in the relaxation of ciliary muscles.35 In our study, the in vitro potency of ITRI-E-212 appeared to downregulate pMLC in A7r5 cells. ITRI-E-212 resulted in 62.0 ± 13% inhibition of 10 μM pMLC, and pMLC leads to the inactivation of the ROCK signal pathway. These data suggest that a reduction in IOP may be associated with an increased number of inter trabecular pores in vivo and consequently decreased resistance to AH outflow through the TM. The in vitro kinase selectivity, evaluated using the KINOMescan system, represented the highly specific AGC subfamily kinase-binding affinity of ITRI-E-212, including its affinity for ROCK 1/2. ITRI-E-212 was selected to be formulated into eye drops because of its high specificity for ROCK inhibition in vitro and favorable physicochemical properties. ITRI-E-212 had the highest water solubility at 7 mg/mL and an adequate drug-loading yield (2.1% in ophthalmic formulation). ITRI-E-212 was found to be the most effective compound for IOP reduction and with high selectivity for ROCK2.

IOP-Lowering Effect in a Normotensive and Hypertensive Model

ROCK inhibitors that are currently available, including SNJ-1656 (Y39983; Senju, Tokyo, Japan), Ripasudil (K-115; Kowa, Aichi, Japan), PHP-201 (AMA-0076; Amakem, Diepenbeek, Belgium), and Netarsudil (AR-13324; Aerie Pharmaceuticals, Inc.), have emerged as promising and efficacious therapeutic molecules in clinical trials for the treatment of glaucoma and ocular hypertension. A study including healthy volunteers reported that ROCK inhibitors reduced IOP through a unique combination of action mechanisms, including increased trabecular outflow and decreased episcleral venous pressure in both the proximal and distal portions of the conventional outflow pathway. Variability in the roles of AH suppression and uveoscleral outflow enhancement requires further investigation.35

The therapeutic potential of ITRI-E-212 was demonstrated when the IOP of treated eyes showed significant reduction at 4 and 6 hours after administration in normotensive and hypertensive NZW rabbit models. The onset and peak of the IOP-lowering effects of ITRI-E-212 were similar to those of existing agents, such as Y-39983, AR-13324, and AMA-0076.16,36,37 The maximum IOP reductions were 25.3% and 28.4% in normotensive and hypertensive models, respectively. These results were comparable to those obtained from other ROCK inhibitors. The data from the present study suggested that ITRI-E-212 could maintain lowered IOP for at least 6 hours. Y-39983 was no longer effective after 8 hours.38,39 and AR-13324 exhibited IOP-lowering efficacy for 24 hours after dose administration. The higher efficacy and prolonged duration of IOP lowering could be attributed to the inhibitory activity of Y-39983 against NET in addition to ROCK inhibition.38 K-115, used as an adjunctive therapy in glaucoma, demonstrated peak IOP reduction at 2 hours after dose administration and required two doses a day to maintain efficacy throughout the day.40 Within the IOP-lowering therapeutic window, ROCK inhibitors reduce blood pressure and vascular resistance, leading to side effects in the event of systemic exposure.38 Clinical studies have focused on drug candidates that are not only potent but also highly selective ROCK inhibitors over other protein kinases for the treatment of glaucoma. The results of the present study revealed that 1% ITRI-E-212 is one of the most specific ROCK 2 inhibitors, with potent IOP-lowering effects. Viscoelastic-induced ocular hypertension was a pre trabecular experimental model that has been demonstrated to repair ocular damage in the actual course of glaucoma.41,42 Cellular and particulate matter in the AH may have appeared suspended and almost immobile, with viscoelastic remnants in the anterior chamber, and the particulate occlusion of the TM after injection provided an effective method for the induction of experimental glaucoma.43 The injected viscoelastic caused an elevation in IOP which consequently injured a part of the TM tissue. The 1% ITRI-E-212 reached a significant IOP reduction in the ocular hypertensive model and targeted the TM. In acute ocular hypertensive models, abrupt elevation of IOP sometimes impaired the functionality of the TM. In this case, the potency of ITRI-E-212 would, at times, decrease because of the inefficient targeting of the contractile tone of tissues. After administration of 1% ITRI-E-212 eye drops, a maximum IOP reduction of 28.4% was attained at 6 hours in the ocular hypertensive model, and in the normotensive model, a maximum IOP reduction of 25.3% was attained at 4 hours. The significant difference in IOP reduction was not observed until 6 hours after the administration of 1% ITRI-E-212 in the normotensive and hypertensive groups. This result may be explained by the potential inhibition of the outflow facilitated by the TM under ocular hypertensive conditions, resulting in a delay in ITRI-E-212 action.

PK in AH

Numerous ophthalmic drugs have been developed as ester prodrugs to reduce ocular side effects and increase corneal penetration. Ester prodrugs can be converted back to active parent drugs by esterases present in the eyes.44 AMA-0076 was designed to rapidly convert to its functionally inactive metabolite to prevent off-target activity and reduce conjunctival hyperemia. A substitution on the urea site of aminoisoquinoline series resulted in a compound, ITRI-E-212, which displayed superior corneal penetration potency, a rapid onset profile, and potent ROCK2 kinase inhibition that could be sustained for at least 6 hours. When the parent drug is lipophilic in nature, a crucial parameter to consider is the aqueous solubility of the prodrug.44 In the present study, chemical substitution on the urea site provided an opportunity to improve bioavailability, aqueous solubility, and PK properties. Similar to other topical antiglaucoma medications, ITRI-E-212 demonstrated a rapid onset after instillation followed by a time lag before achievement of peak IOP. The in vitro inhibitory activity of ITRI-E-212 on ROCK 2 kinase in the AH demonstrated its potency, IOP-lowering efficacy, and specificity. The in vitro ROCK2 kinase inhibition of extracted ITRI-E-212 from the AH reached 78.4% 1 hour after dose administration and was sustained for up to 4 hours. ITRI-E-212 maintained ROCK2 inhibitory activity after penetrating the cornea.

Conjunctival Hyperemia and Tolerability in the Normotensive Model

The topical administration of ROCK inhibitors for the treatment of ocular diseases remains limited by the attribution of moderate conjunctival hyperemia development to smooth muscle cell relaxation in conjunctival blood vessels. For this reason, the development of such inhibitors for the treatment of ocular hypertensive drugs has been suspended.45,46 In our testing of ITRI-E-212 on animals, the severity and duration of conjunctival hyperemia were minimal and virtually unobservable within 6 hours after instillation. No significant difference was noted between the hyperemia scoring of controls and eyes treated with various concentrations of ITRI-E-212, and no severe adverse effects or profound conjunctival injections were observed in the present study. The ocular tolerability scale was evaluated in normotensive rabbit models over the course of 10
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consecutive days and revealed sustained, significant IOP-lowering efficacy, suggesting that if ITRI-E-212 is instilled in the evening, hyperemia would likely resolve overnight and be minimized the following day.

CONCLUSIONS

The present study demonstrates that ITRI-E-212 is a novel selective ROCK inhibitor with potential clinical application. The administration of ITRI-E-212 was well tolerated, and only transient and mild hyperemia was observed in NZW rabbits. The preclinical data, which include biochemical assays, in vitro inhibition activity and selectivity profiles, IOP-lowering ability in both hypertensive and normotensive rabbit models, PK, and improved ocular tolerability, verify that ITRI-E-212 is a promising, new pharmacologic agent for hindering the progression of glaucoma.

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