The Intraocular Pressure Lowering Effect of a Dual Kinase Inhibitor (ITRI-E-(S)4046) in Ocular Hypertensive Animal Models

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Purpose. The purpose of this study was to develop a preclinical compound, ITRI-E-(S)4046, a dual synergistic inhibitor of myosin light chain kinase 4 (MYLK4) and Rho-related protein kinase (ROCK), for reducing intraocular pressure (IOP).

Methods. ITRI-E-(S)4046 is an amino-pyrazole derivative with physical and chemical properties suitable for ophthalmic formulation. In vitro kinase inhibition was evaluated using the Kinase-Glo Luminescent Kinase Assays. A comprehensive kinase selectivity analysis of ITRI-E-(S)4046 was performed using the KINOMEscan assay from DiscoverRx. The IOP reduction and tolerability of ITRI-E-(S)4046 were assessed in ocular normotensive rabbits, ocular normotensive non-human primates, and ocular hypertensive rabbits. In vivo studies were conducted to assess drug concentrations in ocular tissue. The adverse ocular effects of rabbit eyes were evaluated following the OECD405 guidelines.

Results. ITRI-E-(S)4046 showed highly selective kinase inhibitory activity against ROCK1/2, MYLK4, and mitogen-activated protein kinase kinase kinase 19 (MAP3K19), with high specificity against protein kinase A, G, and C families. In ocular normotensive rabbits and non-human primates, the mean IOP reductions of 0.1% ITRI-E-(S)4046 eye drops were 29.8% and 28.5%, respectively. In hypertonic saline-induced and magnetic beads-induced ocular hypertensive rabbits, the mean IOP reductions of ITRI-E-(S)4046 0.1% eye drops were 46.9% and 22.0%, respectively. ITRI-E-(S)4046 was well tolerated with only temporary and minor signs of hyperemia.

Conclusions. ITRI-E-(S)4046 is a novel type of highly specific ROCK1/2 and MYLK4 inhibitor that can reduce IOP in normotensive and hypertensive animal models. It has the potential to become an effective and well-tolerated treatment for glaucoma.

Keywords: dual kinase inhibitor, new glaucoma medications, intraocular pressure, trabecular meshwork, animal models

Glaucoma affects the optic nerve, leading to the progressive death of retinal ganglion cells (RGCs) and subsequent irreversible loss of vision.1 Primary open-angle glaucoma is the most common form of the disease, in which the patient has an elevated intraocular pressure (IOP) due to increased resistance to aqueous outflow facility in the trabecular meshwork (TM).2 Recently, a new class of glaucoma treatment has been developed to reduce IOP by inhibiting Rho-related protein kinase (ROCK). The ROCKs are protein serine/threonine kinases, which mediate RhoA-induced actin cytoskeletal changes and smooth muscle contraction by myosin light chain (MLC) phosphorylation.

ROCK inhibitors, Ripasudil (K-115) and netarsudil (AR-13324), have been developed into ophthalmic formulations for glaucoma treatment,3,4 but were associated with hyperemia and subconjunctival hemorrhage.5,6 Netarsudil (AR-13324) is an ROCK inhibitor, as a reference listed drug (RLD), with a highly potency active metabolite.7,8 One of the ways for ROCK inhibitors to treat glaucoma is to inhibit the

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phosphorylation of MLC by MLC phosphatase. Phosphorylation of MLC would otherwise lead to synthesis and remodeling of the extracellular matrix to decrease conventional outflow and permeability of the Schlemm’s canal in the TM. The balance between ROCK and MLC phosphatase plays an important role in controlling the contraction of the TM.9

Through another pathway, myosin light chain kinase (MYLK) also participates in TM contraction by triggering signaling pathways, including Ca2+-calmodulin binding and kinase-dependent phosphorylation. MYLK and ROCK are a part of two main pathways which lead to MLC phosphorylation in vitro and in vivo. Honjo et al. discovered that after administration of ML-9, a specific MYLK inhibitor, the IOP of rabbit eyes decreased in a dose-dependent manner, possibly through cell contraction and dissociation, destruction of actin bundles, and TM cell adhesion.10

The purpose of this study was to find a novel compound with high selectivity to both MYLK and ROCK. Among the 71 compounds synthesized, we investigated ITRI-E-(S)-4046, which successfully lowered IOP in both normotensive and hypertensive (IOP >30 mm Hg) animal models with limited hyperemia.

METHODS

Design of Dual Kinase Inhibitors for Lowering IOP

ITRI-E-4046 is an amino-pyrazole derivative (Fig. 1). Subsequent modifications were carried out using fragment-based approaches and molecular modeling methods. We used the ChiralPak AD-H column (250 × 30 mm, 10 μm) to separate ITRI-E-(S)-4046 and ITRI-E-(R)-4046, and tested these two stereoisomers. All inactive ingredients and excipients in used in the ITRI-E-(S)-4046 ophthalmic solution were approved by the US Food and Drug Administration, and in used in the ITRI-E-(S)-4046 ophthalmic solution were two stereoisomers. All inactive ingredients and excipients in this study were approved by the US Food and Drug Administration, and in this study were two stereoisomers. All inactive ingredients and excipients in this study were approved by the US Food and Drug Administration, and in this study were two stereoisomers. All inactive ingredients and excipients in this study were approved by the US Food and Drug Administration, and in used in the ITRI-E-(S)-4046 isomers and AR-13324 for 6 hours. The cells were lysed in radio immunoprecipitation assay buffer with protease and phosphatase inhibitors on ice and centrifuged at 14,000 × g at 4°C for 10 minutes. The lysate was resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis for Western blotting. Antibodies used include pMLC2 (Ser18/Thr19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Danvers, MA, USA).

KINOMEscan Kinase Profiling of ITRI-E-(S)-4046

We use the KINOMEscan screening platform (DiscoverRx Corporation, Fremont, CA, USA) to analyze ITRI-E-(S)-4046, which was applied to a set of 468 kinases, covering protein kinase A, G, and C families (AGC), Ca2+/calmodulin-dependent protein kinase (CAMK), cyclin-dependent kinase (CDK), mitogen-activated protein kinase, glycogen synthase kinase, CDK-like kinase (CMGC), casein kinase 1 (CK1), and some atypical kinase families. The strength and relative specificity of kinase-binding interactions were evaluated online using the TREESpot software (DiscoverRx Corporation). In a visualization graph, the larger the red circle, the higher the binding affinity of kinases. The binding interactions are reported as a percentage of the control (%Ctrl), where %Ctrl = [(positive control signal – test compound signal)/ (positive control signal – negative control signal)] × 100. DMSO was used as the negative control.

IOP-Lowering Effect of ITRI-E-(S)-4046 in Ocular Normotensive New Zealand White Rabbits

Male New Zealand White (NZW) rabbits, weighing 1.5 to 3.0 kg, were used in this study. The study was conducted in accordance with the ophthalmic research guidelines of the Association of Research in Vision and Ophthalmology (ARVO) for the use of animals. The Animal Care and Use Committee of the Industry Technology Research Institute (ITRI-IACUC-2020-017) officially legalized animal research in this study. The experimental procedure involved the instillation of ITRI-E-(S)-4046 eye drops once a day for 3 consecutive days. A 35 μL eye drop of vehicle (0.05% boric acid, 4.7% mannitol, and 0.125% nonoxynol-9 in double distilled

buffer (0.05 M Trizma hydrochloride buffer [pH 7.5] containing 0.1 M KCl, 0.01 M MgCl2, 0.1 mM EGTA, 30 mmol/L long S6 kinase substrate, and 1 mmol/L ATP) at room temperature for 90 minutes. The SSBK-LanthaScreen binding assay against MYLK4 and the SSBK-Z’-LYTE kinase-binding assay against mitogen-activated protein kinase kinase kinase 19 (MAP3K19 and YSK4) were both used with the manufacturer’s protocol (ThermoFisher Scientific, Waltham, MA, USA). To calculate half-maximal inhibitory concentration (IC50), nonlinear regression, sigmoidal dose-response (variable slope) curves were plotted using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Assessment of MLC Phosphorylation Inhibition by Western Blot

Human trabecular meshwork cells (HTMCs) were purchased from ScienCells (Carlsbad, CA, USA) and were cultured according to the manufacturer’s instructions. The cells were seeded in 6-well plates overnight. Serum-starved HTMCs (24 hours) were treated with various concentrations of ITRI-E-(S)-4046 isomers and AR-13324 for 6 hours. The cells were lysed in radio immunoprecipitation assay buffer with protease and phosphatase inhibitors on ice and centrifuged at 14,000 × g at 4°C for 10 minutes. The lysate was resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis for Western blotting. Antibodies used include pMLC2 (Ser18/Thr19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Danvers, MA, USA).
water) was instilled in the left eye, and ITRI-E-(S)-4046 of 0.01%, 0.03%, or 0.1% concentration was instilled into the right eye of each rabbit. IOP was measured without sedation in both eyes before instillation, at 1, 2, 4, 6, 8, 12, and 24-hour time points for 3 consecutive days using a rebound tonometer (Icare TONOVIEW Plus, Vantaa, Finland). Results were compared to the experiment sample’s contralateral eye at each time point.

**IOP-Lowering Effect of ITRI-E-(S)-4046 in Ocular Normotensive Non-Human Primates**

We evaluated the efficacy of ITRI-E-(S)-4046 for lowering IOP in a normotensive primate model (*Macaca cyclopis*). This study was conducted in accordance with the ophthalmic research guidelines of the ARVO for the use of animals and was approved by the Animal Care and Use Committee of the National Defense Medical Center (MDMC-IACUC-20-068). A pneumatic tonometer, Reichert Model 30 Pneumotonometer (Reichert Technologies, Depew, NY, USA), was used to measure IOP. None-human primates (NHPs) were anesthetized with intramuscular ketamine 5 mg/kg for each IOP measurement. IOP measurements were obtained 10 to 15 minutes after anesthetization, because IOP levels become stabilized then. Three independent IOP measurements were obtained and averaged from the instilled eye at each time point. The experimental groups were 0.03% and 0.1% ITRI-E-(S)-4046, whereas 0.02% AR-13324 was administered as the reference control and then one drop of 35 μL was instilled topically on each animal’s right eye once a day for 3 consecutive days; whereas the left eye as the negative control was instilled with saline. IOP was measured before instillation (0 hours), and at 2, 4, 8, 24, 48, 52, 56, and 72-hour time points using a pneumotonometer (Model 30 Classic; Medtronic, Jacksonville, FL, USA). The NHPs were sedated systemically with intraperitoneal ketamine for the measurements. The IOP change (∆IOP mm Hg) at each time point compared to time zero was recorded (Mean IOP<sub>0h</sub>: 20.5 ± 0.3 mm Hg, mean ± standard error of the mean [SEM]).

**Hyperemia Scoring**

The eyes of the NZW rabbits were photographed using a digital camera (RX100 Advanced Camera with 1.0-inch Sensor; Sony Inc., Culver City, CA, USA). Hyperemia was assessed using the Organization for Economic Cooperation and Development (OECD) 405 “Acute Eye Irritation/Corrosion” guideline. Cornea, iris, and conjunctiva examinations were performed 0, 1, and 8 hours after instillation of 0.1% ITRI-E-(S)-4046, and recorded for 5 consecutive days.

**Statistical Analysis**

One-way or two-way analysis of variance followed by the Tukey’s post hoc test was conducted for statistical analyses using GraphPad Prism 5 (GraphPad Software). The t-test and P values < 0.05 were considered statistically significant.

**RESULTS**

**Optical Isomerism of ITRI-E-4046**

Immunoblotting was used to determine the inhibition of MLC phosphorylation (pMLC) of the R and S isomers of
ITRI-E-(S)4046 in HTM cells. We found that ITRI-E-(S)4046 had a 10-fold higher inhibitor potency (IC\textsubscript{50} = 50.4 nM) than ITRI-E-(R)4046 (IC\textsubscript{50} = 513.6 nM). ITRI-E-(S)4046 also demonstrated nearly two-fold higher drug potency compared to ITRI-E-(S)4046 (Table 1). In normotensive rabbits, 0.03% ITRI-E-(S)4046 decreased IOP more than 0.03% ITRI-E-(R)4046 (Table 2). A dose-dependent effect of ITRI-E-(S)4046 was observed (Table 3). Thus, ITRI-E-(S)4046 was selected for further investigation.

### In Vitro Kinase Selectivity Profile of ITRI-E-(S)4046

To evaluate the kinase selectivity of ITRI-E-(S)4046 in vitro, the kinase panel test was used. According to the KINOME scan results, ROCK 1/2, MYLK4, and YSK4 were found to have lower %Ctrl values, indicating that ITRI-E-(S)4046 had a strong interaction with these kinases. The spot map showed a large concentration of dots in the ROCK domain of the AGC protein kinase subgroups (Fig. 3). The smaller red spots marked MYLK4, which belongs to the CAMK subfamily. This narrow distribution of the affected kinases may make it less likely for ITRI-E-(S)4046 to cause side effects.

### Efficacy of IOP Reduction in Normotensive Animals

In our study, the IOP-lowering effect in normotensive NZW rabbits was evaluated at different time points (0, 1, 2, 4, 6, 8, 12, and 24 hours) for 3 consecutive days after daily administration of ITRI-E-(S)4046 eye drops. As shown in Table 3 and Supplementary Figure S2, different concentrations (0.01%, 0.03%, and 0.1%) of ITRI-E-(S)4046 were administered. ITRI-E-(S)4046 lowered IOP in a concentration-dependent manner. IOP reduction was noticed from the first hour after administration and lasted for 6 to 8 hours. Six hours after

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**Table 1. The IC\textsubscript{50} for Kinases (ROCK, YSK4, and MYLK4), and pMLC2 were Examined After 72 Hours in HTM Cells for the Inhibitory Activity of ITRI-E-(4046 Isomers**

| Compound          | ROCK1 IC\textsubscript{50} (nM) | YSK4 IC\textsubscript{50} (nM) | MYLK4 IC\textsubscript{50} (nM) | pMLC IC\textsubscript{50} (nM) |
|-------------------|---------------------------------|-------------------------------|-------------------------------|-------------------------------|
| ITRI-E-(4046) (S): (R) 50%/50% | 5.9 ± 0.4                       | 2600                          | 61.9                          | 93.3                          |
| ITRI-E-4046 (S)    | 10.1                            | 760                           | 30.1                          | n.a.                          |
| ITRI-E-(S)4046     | 37.6 ± 5.5                      | 4180                          | 196                           | n.a.                          |
| ITRI-E-(R)4046     | n.a.                            | 25.6                          | 50.4                          | n.a.                          |

n.a., not applicable.

**Table 2. The Effect of Lowering IOP was Conducted by Administration of 0.03% of ITRI-E-(S)4046 (N = 4) and ITRI-E-(R)4046 (N = 4) in Ocular Hypertensive Rabbits. \( \Delta \text{IOP} = (\text{IOP}_\text{OR} - \text{IOP}_\text{OS}) \); \( t \)-test, \( \ast: P < 0.05 \) for 0.1% ITRI-E-(S)4046 vs. 0.03% ITRI-E-(R) 4046

| Time (hour) | Mean | SEM | 0.03% ITRI-E-(S) 4046 (n = 4) | Mean | SEM | 0.03% ITRI-E-(R) 4046 (n = 4) |
|-------------|------|-----|-------------------------------|------|-----|-------------------------------|
| 0           | 0.3  | 0.2 | -1.3                          | 0.5  |      |                               |
| 1           | -0.6 | 0.3 | -1.0                          | 0.7  |      |                               |
| 2           | -1.8 | 0.8 | -2.5                          | 0.5  |      |                               |
| 4           | -2.1 | 0.5 | -2.3                          | 0.5  |      |                               |
| 6           | -3.0 | 0.8 | -1.8                          | 0.3  |      |                               |
| 8           | -4.9 | 0.6 | -1.3                          | 0.5  |      |                               |

**Table 3. The Percent of E\text{\textsubscript{max}} \( \Delta \text{IOP}_\text{day} \) of Ocular Normotensive Rabbits were Assessed after Treating with 0.01%, 0.03%, and 0.1% ITRI-E-(S)4046 Once Daily for 3 Consecutive Days (at 0, 24, and 48 hours). The Instillation of Medication was Performed onto the Experimental Right Eyes (OR), whereas Vehicle Treatment was Performed on the Control Left Eyes (OS). \( \Delta \text{IOP\%} = (\text{IOPOS} - \text{IOP}_\text{OR}) / \text{IOP}_\text{OS} \times \% \)

| ITRI-E-(S)4046 | Day 1 | Day 2 | Day 3 |
|----------------|-------|-------|-------|
| 0.1%          | -28.6±2.3| -28.8±2.8| -29.8±1.8 |
| 0.03%         | -15.9±1.3| -18.1±2.0| -18.3±2.0 |
| 0.01%         | -13.1±2.1| -17.8±2.9| -18.1±2.4 |

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**FIGURE 2.** The 0.1% ITRI-E-(S)4046 in human trabecular meshwork (HTM) cells did not show significant cytotoxicity (for 72 hours) until 5 μM. The percentage of cell viability was normalized according to the MOCK control group.
administration of the first dose of 0.1% ITRI-E-(S)4046, the maximum IOP-lowering percentage (maximum effect \( E_{\text{max}} \) \( \Delta \text{IOP} = 28.6 \pm 1.3\% \text{ at 6 hours} \)) was observed \((N = 14)\). The maximum IOP reduction in the groups that received 0.01% ITRI-E-(S)4046 \((N = 10)\) and 0.03% ITRI-E-(S)4046 \((N = 10)\) was 13.1 \(\pm 2.1\% \) (at 2 hours) and 15.9 \(\pm 1.3\% \) (at 6 hours), respectively. Interestingly, on the third day of ITRI-E-(S)4046 instillation, we discovered a dose-escalation effect, that is, the drug lowered the IOP more effectively than on day 1. The maximum IOP-lowering percentage of 0.1% ITRI-E-(S)4046 \((N = 14)\), 0.01% ITRI-E-(S)4046 \((N = 10)\), and 0.03% ITRI-E-(S)4046 \((N = 10)\) was 29.8 \(\pm 1.8\% \) (at 4 hours), 18.1 \(\pm 2.4\% \) (at 2 hours), and 18.3 \(\pm 2.0\% \) (at 4 hours), respectively. In normotensive NHPs, ITRI-E-(S)4046 reduced IOP more effectively than AR-13324 after 3 consecutive days of drug use (Fig. 4). In ocular normotensive animals, 0.1% ITRI-E-(S)4046 showed significant IOP-lowering and dose-dependent effect.

**Efficacy of IOP Reduction in Hypertensive Animals**

Further research was conducted on rabbits with ocular hypertension, induced using either hypertonic saline or magnetic beads, to continue assessing the IOP-lowering effects of ITRI-E-(S)4046. The increased mean IOP was measured to be above 40 mm Hg and 30 mm Hg, in the hypertonic saline and magnetic beads models, respectively. In hypertonic saline-induced OHT rabbits, the IOP at different time points \((0, 0.5, 1, 1.5, 3, \text{and 5 hours})\) after administrating ITRI-E-(S)4046 were measured. As shown in Figure 5, 0.1% ITRI-E-(S)4046, saline, or 0.02% AR-13324 was administered topically immediately after the hypertonic saline injection, and their effects were evaluated 30 minutes after instillation of eye drops. The maximum percentage of IOP reduction with 0.1% ITRI-E-(S)4046 was 46.9%, and the mean IOP decreased to 26.3 \(\pm 2.8\text{ mm Hg} \) was compared to saline 49.5 \(\pm 2.7\text{ mm Hg} \) at 1 hour after instillation. Moreover, 0.1% ITRI-E-(S)4046 executed a better IOP-reducing effect and a statistically significant difference than saline group \((P < 0.05)\). \( E_{\text{max}} \) of IOP reduction of 0.1% ITRI-E-(S)4046, 23.2 \(\pm 2.8\text{ mm Hg} \) at 1 hour, was better than 0.02% AR-13324, 12.1 \(\pm 0.9\text{ mm Hg} \) at 1.5 hours, \(P < 0.05\).

In the acute magnetic bead-induced OHT rabbits, after instillation of the medication, IOP was measured at times points 0, 2, 4, 6, and 8 hours for 2 consecutive days. Different concentrations of ocular medicine were instilled in the rabbit models. In the magnetic bead-induced long-term OHT rabbit model, the IOP decreased within the first 2 hours of administration of both 0.03% and 0.1% ITRI-E-(S)4046 eye drops (Fig. 6). The 0.1% ITRI-E-(S)4046 showed a better IOP-lowering effect than the 0.03% eye drops. Most importantly, after 2 days of use, 0.1% ITRI-E-(S)4046 demonstrated almost 1.5 times more IOP reduction than 0.02% AR-13324. The maximum IOP reduction at 30 hours by 0.03% and 0.1% ITRI-E-(S)4046 was 5.3 \(\pm 0.6\text{ mm Hg} \) and 8.0 \(\pm 1.6\text{ mm Hg} \), respectively, with reference to baseline IOP. This indicates that 0.1% ITRI-E-(S)4046 was able to reduce the IOP in animal models of normotension, acute hypertension, and long-term hypertension (IOP >30 mm Hg).
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**Figure 4.** The 0.03%, 0.1% ITRI-E-(S)4046, and 0.02% AR-13324 were topically administered once daily for 3 consecutive days (at 0, 24, and 48 hours) into ocular normotensive *Macaca cyclopis* monkeys. The IOP was assessed at times 0, 4, 8, 24, 48, 52, 56, and 72 hours. The $\Delta$IOP (mm Hg) was deducted from time zero IOP ($IOP_{0h}$). The $t$-test, *: $P < 0.05$ for 0.1% ITRI-E-(S)4046 vs. 0.03% ITRI-E-(S)4046 (mean $IOP_{0h}$: 20.5 ± 0.3 mm Hg, mean ± SEM, $n = 9$).

**Figure 5.** Saline, 0.1% ITRI-E-(S)4046, and 0.02% AR-13324 were topically administered at 0 hours in a 5% hypertonic saline-induced hypertensive rabbit model. The IOP was assessed at times 0, 0.5, 1, 1.5, 3, and 5 hours. The $t$-test, *: $P < 0.05$ for 0.1% ITRI-E-(S)4046 vs. saline; #: $P < 0.05$ for 0.1% ITRI-E-(S)4046 vs. AR-13324 (max $IOP_{1h}$: 49.5 ± 1.5 mm Hg, mean ± SEM, $n = 4$).

**Conjunctival Hyperemia Scores in Normotensive NZW Rabbits**

Although the efficacy profile of ROCK inhibitors is promising, the most commonly reported side effect of ROCK inhibitors is conjunctival hyperemia. The adverse ocular effects of rabbit eyes were evaluated according to the OECD405 guidelines. Trace-to-mild hyperemia (0–1) was noticed at the first hour after topical 0.1% ITRI-E-(S)4046 administration when compared with the contralateral eye (without ITRI-E-(S)4046; Fig. 7). ITRI-E-(S)4046 has a short half-life and would be eliminated within 60 minutes of topical administration. No further complications of the eye tissue or toxicity of TM cells were investigated. No further
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FIGURE 6. The 0.03%, 0.1% ITRI-E-(S)4046, and 0.02% AR-13324 were topically administered to each rabbit once daily for 2 days (at 0 and 24 hours) in magnetic beads induced hypertensive NZW rabbits. The IOP was assessed at times 0, 2, 4, 6, 8, 24, 26, 28, 30, and 32 hours. The △IOP (mm Hg) was deducted from time zero IOP. The *t-test, *: P < 0.05 for 0.1% ITRI-E-(S)4046 vs. 0.03% ITRI-E-(S)4046 (mean IOP0h: 36.0 ± 1.3 mm Hg, mean ± SEM, n = 5).

complications of the eye tissue or toxicity of TM cells were investigated.

DISCUSSION

In this study, we synthesized an amino-pyrazole derivative, ITRI-E-(S)4046, which demonstrated a strong synergistic inhibition of ROCK and MYLK4. ITRI-E-(S)4046 lowered IOP in animal models with only minimal hyperemia. Consistent with previous studies, the decrease in IOP by ROCK inhibitors is largely mediated by increased AH outflow facility via relaxation of the TM in the conventional outflow pathway. In human TM cells, ITRI-E-(S)4046 decreases MLC phosphorylation (Supplementary Fig. S3). Our results demonstrated that ITRI-E-(S)4046 has the same effect as other ROCK inhibitors at decreasing pMLC levels. The concentration-time profile of ITRI-E-(S)4046 in the AH at 8 hours after administration of 0.1% ITRI-E-(S)4046 was higher than ROCK1 90% inhibitory concentration (IC90; 10.9 ng/mL) and fairly similar to MYLK4 IC90 (81.6 ng/mL). Topical use of 0.1% ITRI-E-(S)4046 resulted in a peak concentration (3.0 ng/mL) of the active drug in plasma at 2 hours. The plasma protein binding was 90.3% in rabbits and the calculated unbound drug was 0.29 ng/mL, which was lower than ROCK1 IC50 = 1.2 ng/mL and MYLK4 IC50 = 9.7 ng/mL in the systemic circulation (Fig. 8, Supplementary Fig. S4). From our liver microsomal stability study, ITRI-E-(S)4046 gave an average intrinsic clearance value of 8.56 μL/min/mg, 15.0 μL/min/mg, 7.82 μL/min/mg, 45.4 μL/min/mg, 4.21 μL/min/mg, and 16.9 μL/min/mg in humans, rats, dogs, mice, monkeys, and rabbits, respectively.

Synergistic Effect of Dual-Target Kinase Inhibitor

Compared with other ROCK inhibitors, ITRI-E-(S)4046 had better IOP-lowering effects, especially in the OHT models. The strong MYLK4 inhibition proposed to be synergistic with ROCK inhibition resulting in maximum cell relaxation of the TM. Some studies have claimed that OHT can induce MYLK expression, however, it is unknown whether ROCK inhibition decreases MYLK4 expression in HTM cells. We found that the expression of pMLC2 had mildly decreased, but MYLK4 did not change in the rabbit TM cells treated with ROCK inhibitor (Supplementary Figs. S5, S6A). Previous studies that identified the cell types that express genes implicated in glaucoma in humans showed MYLK gene expression in the ciliary muscle, especially in the uveoscleral outflow pathway. Several studies have successfully demonstrated a better IOP reduction by a combination therapy with drugs from different classes. ML-9, an inhibitor of MYLK, demonstrated significant IOP lowering and increase in AH outflow in rabbit eyes. The mechanism by which ML-9 is reported to reduce IOP involve disrupting actin bundles and impairing focal adhesion formation in the TM. According to the KINOME scan screening platform, in addition to ROCK1/2, ITRI-E-(S)4046 strongly interacts with MYLK-4 target genes. ITRI-E-(S)4046 inhibits MYLK4 with an IC50 of 25.6 nM. The expression of
MYLK4 in the TM, detected by immunohistochemical (IHC) staining, was commonly found in NZW rabbits with higher IOP, but not in native rabbits (shown in Supplementary Fig. S5, Fig. S6A). We believe that the overexpression of MYLK4 plays a major role in increasing IOP in OHT models. ITRI-E-(S)-4046 demonstrated better IOP reduction than AR-13324 in two ocular hypertension models (magnetic beads and hypertonic saline induced). With this information, we propose that ITRI-E-(S)-4046 can modulate IOP in a ROCK and MYLK4-dependent manner via the actomyosin contractile machinery.

Conjunctival Hyperemia and Tolerability in the Animal Model

Conjunctival hyperemia is a commonly reported side effect of ROCK inhibitors. In clinical references, we found that commercial ROCK inhibitors, ripasudil and netarsudil, are associated with >50% hyperemia. Ripasudil was reported to cause hyperemia in the majority of patients. In clinical data regarding ripasudil, conjunctival hyperemia occurred at various time points and concentrations and resolved spontaneously within 4 hours. In our evaluation...
of ITRI-E-(S)4046 in rabbits, mild conjunctival hyperemia was observed at 1 hour in the normotensive rabbit model after administration of 0.1% ITRI-E-(S)4046, but recovered rapidly. In the drug ocular distribution evaluation of normotensive NZW rabbits, the conjunctiva revealed a lower concentration of ITRI-E-(S)4046 among anterior ocular tissues. After topical administration of 0.1% ITRI-E-(S)4046, there was no corneal opacity in slit lamp examinations. However, further drug safety studies should be conducted. In this study, ITRI-E-(S)4046 is effective at lowering IOP, with limited systemic effects and few ocular side effects observed. It is a potential drug for the treatment of glaucoma.

**MYLK as a Target**

MYLK is a serine/threonine-specific protein kinase that specifically phosphorylates the regulatory light chain of myosin II. Four isoforms of MYLK have been identified, of which MYLK4 is thought to be a major kinase. Not very much is known about the specific functional characteristics of MYLK4. ITRI-E-(S)4046 is a dual kinase inhibitor, specific for MYLK4 inhibition. An experiment was conducted to determine the distribution of MYLK4 in the anterior ocular tissue in rabbits with OHT induced using magnetic beads. Rabbits with high IOP show remarkably higher MYLK4 levels in the TM. When it comes to other tissues in this model, the myocardium expresses the most MYLK4, followed by the aorta. A small amount of MYLK4 expression is found in smooth muscle and skeletal muscle tissues (see Supplementary Fig. S6B). Therefore, possible effects on the cardiovascular system should be considered. Further studies are needed to determine whether ITRI-E-(S)4046 will interfere with other kinases, calcification markers, autocrine, and paracrine growth factors. Further insight should be considered regarding MYLK4 inhibition to enable future drug development.

**CONCLUSION**

A novel, selective dual kinase inhibitor of ROCK and MYLK4, ITRI-E-(S)4046, is a promising topical treatment for glaucoma. ITRI-E-(S)4046 significantly reduced IOP, especially in an acute super-elevated IOP (>30 mm Hg) animal model. The only side effect observed was mild hyperemia. Thus, ITRI-E-(S)4046 shows potential as a future treatment for glaucoma in clinical practice.

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