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Figure Legends

Fig. 1. Visual field defects are classified by Goldmann perimetry.

Fig. 2. Corrected visual acuity before and after surgery at 2 months in 49 eyes. One outlier (solid circle enclosed by another circle) shows an eye with severe hyphema. In this case, vitreous hemorrhage developed from hyphema because this eye was aphakic. However, visual acuity was recovered to pre-surgery levels when the vitreous hemorrhage disappeared.

Fig. 3. Kaplan-Meier survival curve of intraocular pressure (IOP) control, with or without anti-glaucoma medication.

Fig. 4. Corrected visual acuity before surgery and at final visit in 49 eyes. Eleven outliers (solid circle enclosed by another circle) show the eyes with progression of cataract during the follow up period. Two outliers (solid circle enclosed by a square) represent an eye with central visual field loss during the follow up period. Two outliers with arrow show an eye with BRVO. BRVO = branch retinal vein occlusion

Effects of Topical Administration of Y-39983, a Selective Rho-Associated Protein Kinase Inhibitor, on Ocular Tissues in Rabbits and Monkeys

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ABSTRACT

Purpose. To elucidate the intraocular pressure (IOP)-lowering effects and associated characteristics of Y-39983, a selective Rho-associated coiled coil-forming protein kinase (ROCK) inhibitor derived from Y-27632, in animal eyes.

Methods. Y-39983 was compared with Y-27632 for selectivity of ROCK inhibition by biochemical assay. The IOP was monitored by pneumatonometer in albino rabbits and cynomolgus monkeys that were topically administered Y-39983. The total outflow facility and uveoscleral outflow were measured by two-level constant pressure perfusion and perfusion technique using fluorescein isothiacyanate-dextran, respectively, at 2 hours after topical administration of Y-39983 in albino rabbits. The ocular toxicological effects of topical administration of Y-39983 were observed in albino rabbits and cynomolgus monkeys.

Results. Biochemical assay showed that Y-39983 inhibited ROCK more potently than Y-27632. In rabbits, topical administration of Y-39983 significantly increased conventional outflow by 65.5%, followed by significant, dose-dependent reduction in IOP. Maximal IOP reduction was 13.2 $\pm$ 0.6 mmHg (mean $\pm$ standard error) at 0.1% Y-39983 in rabbits. In monkeys, at 3 hours after topical administration of 0.05% Y-39983, maximal reduction of IOP was 2.5 $\pm$ 0.8 mmHg. No serious side effects were observed in ocular tissues except sporadic punctate subconjunctival hemorrhage during long-term topical administration of Y-39983 4 times a day (at 2-hours intervals) in rabbits or monkeys. However, punctate subconjunctival hemorrhage was not observed with administration twice a day (with a 6-hour interval) or 3 times a day (at 5-hour intervals).

Conclusion. Y-39983 causes increased outflow facility followed by IOP reduction. Y-39983 ophthalmic solution may be a candidate drug for lowering of IOP since it increases

conventional outflow and produces relatively few side effects.

INTRODUCTION

Because the small GTPase Rho plays critical roles in signaling pathways that lead to formation of actin stress fibers and focal adhesions,[1-4] it regulates various cellular behaviors including cytoskeletal rearrangement,[5,6] cell morphology,[7] cell motility,[8] cytokinesis,[9] and smooth muscle contraction.[10,11] These effects of Rho are mediated by downstream Rho effectors such as Rho-associated coiled coil-forming protein kinase (ROCK) and mDia. The GTP-bound forms of Rho activate these Rho effectors that control the actin cytoskeleton, resulting in changes in morphology and adhesion of fibroblasts and epithelial cells. [12-16] Inhibitors of ROCK have been developed because of their potential for use in treating metastasis and axon injury. Among these inhibitors, Y-27632 is the first identified specific inhibitor of the ROCK family of protein kinases.[17] In our previous studies, Y-27632 was found to lower intraocular pressure (IOP) in rabbit eyes. [18,19] Our previous studies also revealed that Y-27632 altered the contractility of the trabecular meshwork (TM) cells and ciliary muscle (CM). Recently, alteration in contractility, focal adhesion, and stress fiber formation in Schlemm's canal (SC) cells, TM cells and CM have been proposed to lower IOP.[18-29] ROCK inhibitors are thus considered candidates for novel IOP lowering anti-glaucoma drugs.[18,19,23,25,26]

In addition, it has been reported that other protein kinase inhibitors such as H-7 and HA-1077 have ROCK inhibitory activity, though their specificity for ROCK is less than that of Y-27632.[17] H-7 and HA-1077 also reduce IOP by increasing conventional outflow by altering the contractility of TM and the cellular behavior of TM cells.[27-30] These inhibitors may also have potential for development as anti-glaucoma drugs to lower IOP.

Thus, inhibition of the Rho-ROCK signaling pathway is a new target for glaucoma

treatment. In the present study, we show that a novel selective ROCK inhibitor, Y-39983, inhibits Rho-ROCK signaling more potently than Y-27632, and that topical administration of it facilitates aqueous conventional outflow, resulting in lowering of IOP. We also examined the toxicological effects of topical administration to evaluate the possibility of clinical use of Y-39983.

MATERIALS AND METHODS

Animals

In pharmacological studies (measurements of IOP and aqueous outflow), adult male Japanese white (albino) rabbits weighing 2.0 to 2.8 kg and adult male cynomolgus monkeys (*Macaca fascicularis*) weighing 6.0 to 8.9 kg were used. In this experiment, adult cynomolgus monkeys were trained for measurement of IOP in conscious condition (without systemic anesthesia). In toxicological studies, adult male Japanese white rabbits weighing 1.8 to 2.7 kg and adult male and female cynomolgus monkeys weighing 2.2 to 3.5 kg were used. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

For IOP measurements in rabbits and monkeys, the eyes were anesthetized by topical instillation of 0.04% and 0.4% oxybuprocaine hydrochloride, respectively.

Chemicals, drug preparation

Y-27632 (M.W. 338.3) and Y-39983 (M.W. 316.8) were synthesized by Mitsubishi Pharma Corporation (Osaka, Japan). The structures of Y-27632 and Y-39983 are shown in Figure 1. Staurosporine, a non-specific protein kinase inhibitor, was purchased from Wako Pure

Chemical (Osaka, Japan). In the topical administration experiments, Y-39983 was used as an ophthalmic solution containing preservative for clinical use. In addition, 0.005 % latanoprost (Xalatan; Tokyo, Pfizer) was used as a comparator in examination of IOP-lowering effects.

Measurement of inhibition of ROCK, Protein kinase C and Calmodulin-dependent protein kinase II

Recombinant ROCK (ROK α/ROCK II) and purified protein kinase C (PKC : mixture of α, β, γ isoforms) were purchased from Upstate Biotechnology (MA, USA). Recombinant calmodulin-dependent protein kinase II (CaMK II) was purchased from Daiichi Pure Chemical (Tokyo, Japan). ROCK (0.2 units/ml) was incubated with 1 μM [γ-^{32}P] ATP and 10 μg/ml histone as substrates in the absence or presence of various concentrations of Y-27632, Y-39983, or staurosporine at room temperature for 20 minutes in 20 mM MOPS buffer (pH 7.2) containing 0.1 mg/ml bovine serum albumin (BSA), 5 mM DTT, 10mM β-glycerophosphate, 50 μM Na_3VO_4, and 10 mM $MgCl_2$ in a total volume of 100 μl. PKC (10 ng/ml) was incubated with 1 μM [γ-^{32}P] ATP and 20 μM PKC substrate (Peptide Institute, Osaka, Japan) in the absence or presence of various concentrations of Y-27632, Y-39983, or staurosporine at room temperature for 30 minutes in 20 mM MOPS buffer (pH 7.5) containing 0.1 mg/ml BSA, 10 mM DTT, 10 mM β-glycerophosphate, 50 μM Na_3VO_4, 2 mM $CaCl_2$, 20 μg/ml phosphatidyl-L-serine, and 10 mM $MgCl_2$ in a total volume of 100 μl. CaMK II (125 units/ml) was incubated with 1 μM [γ-^{32}P] ATP, 10 μM calmodulin and 20 μM CaMK II substrate (Daiichi Pure Chemical) in the absence or presence of various concentrations of Y-27632, Y-39983, or staurosporine at room temperature for 30 minutes in 20 mM MOPS buffer (pH 7.5) containing 0.2 mg/mL BSA, 0.5 mM DTT, 0.1 mM

β-glycerophosphate, 50 μM Na_3VO_4, 1 mM $CaCl_2$, and 5 mM $MgCl_2$ in a total volume of 100 μl. Incubation was terminated by the addition of 100 μl of 0.7 % phosphoric acid. A 160 μl portion of the mixture was transferred to Multiscreen^{TM}-PH plate (Millipore, MA). A positively charged phospho-cellulose filter absorbed the substrate which ^{32}P was bound using Multiscreen^{TM}-Vacuum manifold (Millipore). The filter was washed with 300 μl of 0.5% phosphoric acid and then twice with purified water, and then dried. The radioactivity of dried filter was measured with a liquid scintillation counter (LS6500; Beckman Instruments, CA). Values are presented as 50 % inhibitory concentrations and 95 % confidence intervals (CI).

IOP measurements

Pneumotonometers (Alcon, TX or Medtronic Solan, FL) were used to monitor IOP. In the experiments on single topical administration using rabbits and monkeys, 50 μl of Y-39983 at concentrations of 0.003 to 0.1 % (0.1% in rabbits only) was topically administered to one eye. In addition, 0.005% latanoprost was topically administered as a comparator to one eye in monkeys. Saline was topically administered to the contralateral eyes in both species. IOPs were measured before topical administration and at 1, 2, 3, 5, 7, 9, and 12 hours (12 hours in monkeys only) after it. In the experiments on repeated topical administration using rabbits, 50 μl of 0.03 % Y-39983 was topically administered to one eye 4 times a day (10:00, 13:00, 16:00, and 19:00, at 3-hour intervals) for 28 days. The contralateral eyes were not treated. IOPs were measured at maximal reduction (2 hours after topical administration in the morning) at 7, 14, 21, and 28 days after administration. The vehicle of Y-39983 was used as a control. IOPs were calculated from the difference between results for Y-39983 or its vehicle-treated eyes and the contralateral saline-treated or non-treated eyes at each time point.

Measurement of total outflow facility and uveoscleral outflow

Total outflow facility was determined by two-level constant pressure perfusion (25 and 35 mmHg) at 2 hours after topical administration of 50 μl of 0.05 % Y-39983 in one eye and its vehicle in the contralateral eye, according to the method of Bárány.[31] Briefly, the anterior chambers of rabbits anesthetized with 40% urethane were perfused with mock aqueous humor (Opeguard MA, Senju Pharmaceutical, Osaka, Japan) with a constant pressure of either 25 or 35 mmHg, which was alternately applied for 10-minute intervals. During each 10-minute period, fluid flow was measured for 8 minutes, beginning 2 minutes after pressure change.

Uveoscleral outflow was determined with a perfusion technique using fluorescein isothiacyanate-dextran (FITC-dextran, mean M.W.=71,200, Sigma, St. Louis, MO)[32,33] at 2 hours after topical administration of 50 μl of 0.05 % Y-39983 in one eye and its vehicle in the contralateral eye. Rabbits were anesthetized with 40 % urethane, and two 23-gauge needles connected to a pair syringes were inserted into the anterior chamber of each eye of each rabbit. The pair of syringes was controlled by an infusion/withdrawal pump (Model 55-1382; Harvard Apparatus, MA), and the infusion syringe was filled with 10^{-4} M FITC-dextran. One milliliter of the FITC-dextran solution was washed through the anterior chamber using the syringes at a rate of 0.5 ml/min. The IOP level was then set to 20 mmHg. The FITC-dextran solution was perfused continuously through the anterior chamber at a rate of 10 μl/min for 30 minutes. The anterior chamber was washed with 2 ml of PBS at a rate of 0.5 ml/min. Each eye was then enucleated and dissected into the following sample groups: anterior uvea, anterior sclera, posterior sclera plus posterior uvea, and posterior segment fluid plus vitreous.

All samples were homogenized, centrifuged, and then each volume was measured. The supernatant was measured to determine FITC-dextran concentration using a fluorophotometer. Uveoscleral outflow (Fu) was calculated as follows:

$$Fu\ (\mu l/min) = \frac{\sum (a \times b)\ (ng)}{C(ng/\mu l) \times T\ (min)}$$

a: Volume of each sample (ml)

b: Concentration of FITC-dextran in each sample (ng/ml)

c: Concentration of FITC-dextran in the perfusion fluid (10^{-4} M=7120 ng/ml)

T: Time of perfusion (30 minutes)

Ocular toxicology

Ocular toxicological properties of Y-39983 were evaluated in rabbits and monkeys. In the study with administration 4 times a day (QID), performed to test severe conditions, 100 μl of Y-39983 (0.003% to 0.03%) or saline as a control was topically administered to both eyes of rabbits 4 times a day (at 2-hour intervals) for 4 weeks (n=5). In addition, 50 μl of Y-39983 (0.003% to 0.05%) or its vehicle was topically administered 4 times a day (at 2-hour intervals) for 26 weeks (n=8). In slit lamp examinations, the cornea (epithelial defects revealed by fluorescein biostaining, opacity, and neovascularization), conjunctiva (hyperemia, swelling), anterior chamber (flare), and iris (hyperemia, swelling) were observed. The lens, vitreous, and retina were observed under mydriasis using a slit lamp and binocular indirect ophthalmoscope. Tear quantity was measured by phenol red thread (Zonequick, Menicon,

Nagoya, Japan). The electroretinogram (ERG) was measured to evaluate retinal safety. In darkness and under mydriasis with systemic anesthetization, a contact lens-type electrode was fitted to the eye. Results of light stimulation and the ERG were recorded using a veterinary ERG system. Amplitudes of the a- and b-waves and the peak latency were determined. In addition, histological examination was performed after the last observation using the usual method. The tissues observed were the eye including the palpebral and bulbar conjunctiva, and optic nerve, lacrimal glands, internal organs including the liver, gallbladder, kidneys, spleen, heart, aorta, gullet, stomach, intestines, lungs, and bronchial tube, sex organs, brain, bone, muscle, skin, and other tissues.

Additionally, to investigate the safety of Y-39983 at various frequencies of administration, administration 2 and 3 times a day (BID and TID) was performed in rabbits and monkeys. In rabbits in the BID administration study, 0.05% or 0.1% Y-39983 or saline was topically administered to both eyes twice a day (with a 6-hour interval) for 4 weeks (n=3). In rabbits in the TID administration study, 0.1% Y-39983 or saline was topically administered to both eyes 3 times a day (at 5-hour intervals) for 2 weeks (n=5). In monkeys in the BID administration study, 0.05%, 0.1%, or 0.2% Y-39983 or saline was topically administered to both eyes twice a day (with a 6-hour interval) for 4 weeks (n=3). Ocular tissues were observed in the same fashion as for administration 4 times a day.

Effects on cultured human umbilical venous endothelial cells

Human umbilical venous endothelial cells (HUVECs) were purchased from Dainippon Pharmaceutical (Osaka, Japan). HUVECs were cultured in CS-C medium (Dainippon Pharmaceutical) and maintained in a 95 % air-5 % CO_2 atmosphere at 37 $^{\circ}C$ and passaged

using the trypsin-EDTA method. HUVECs were seeded into 24-well plates. After seeding, HUVECs were incubated in medium containing 1 μM Y-39983 for 15 or 30 minutes, and observed by phase-contrast microscopy. Medium was then removed and HUVECs were incubated in medium without Y-39983 for 1 hour to evaluate recovery from the morphological changes induced by Y-39983.

RESULTS

Selective inhibitory effect of Y-39983 on ROCK activity

Results are summarized in Table 1. The 50 % inhibitory concentration (IC_{50}) of Y-27632 for ROCK, 0.11 μM (95 % CI, 0.074 – 0.17 μM), was 30.6 times that of Y-39983, 0.0036 μM (95 % CI, 0.0025 – 0.0051 μM). On the other hand, in the examination of inhibition of PKC and CaMKII, the IC_{50} values of Y-27632 and Y-39983 for PKC were 9.0 μM (95 % CI, 7.1 – 11 μM) and 0.42 μM (95% CI, 0.36 – 0.49 μM), respectively, while the IC_{50} values of Y-27632 and Y-39983 for CaMKII were 26 μM (95 % CI, 21 - 32 μM) and 0.81 μM (95 % CI, 0.67 – 0.97 μM), respectively. The IC_{50} values of Y-27632 and Y-39983 for PKC were 82 and 117 times those for ROCK, respectively, while the IC_{50} values of Y-27632 and Y-39983 for CaMKII were 236 and 225 times those for ROCK, respectively. In addition, the same experiments were performed as controls using staurosporine, a non-specific protein kinase inhibitor. Staurosporine exhibited the same inhibitory effects on all three kinases, ROCK, PKC, and CaMKII, as shown in Table 1. These findings indicate that Y-39983 more potently inhibits ROCK than Y-27632 and has the same selectivity for ROCK as Y-27632. In addition, Y-39983 was more selective for ROCK than was staurosporine.

IOP measurements in rabbit eyes

In rabbits, Y-39983 lowered IOP in dose-dependent fashion, as shown in Figure 2A. Statistically significant IOP-lowering effects were found at concentrations of Y-39983 equal to or higher than 0.01% at 2 hours after topical administration. IOP reduction was maximal between 2 and 3 hours after administration of 0.01% to 0.1% Y-39983. Maximal IOP reduction (mean $\pm$ standard error; SE) was 2.5 $\pm$ 0.8 ($p = 0.28$ vs. vehicle-treated eyes, William's test, one-side), 7.0 $\pm$ 1.6 ($p = 0.0009$), 11.0 $\pm$ 1.0 ($p < 0.0001$), 12.1 $\pm$ 1.5 ($p < 0.0001$), and 13.2 $\pm$ 0.6 mmHg ($p < 0.0001$) at 0.003 %, 0.01 %, 0.03 %, 0.05 %, and 0.1 % Y-39983, respectively (Figure 2B). This result demonstrated the potent IOP-lowering effects of Y-39983 in rabbit eyes.

In addition, repeated topical administration of 0.03% Y-39983 (4 times a day) was performed in rabbit eyes. Figure 3 shows the time course of changes in peak IOP reduction over 28 days. Mean reduction of IOP was between 7.0 and 9.6 mmHg during the 28-day period, demonstrating that maintenance of IOP reduction is obtained with repeated administration.

IOP measurements in monkey eyes

In monkeys, Y-39983 dose-dependently lowered IOP, as shown in Figure 4A. Compared with vehicle-treated eyes, 0.05% Y-39983-treated eyes in particular exhibited significant reduction of IOP between 2 and 7 hours after topical administration ($p < 0.05$, Dunnett's test, one-side). The reduction of IOP was maximal 3 hours after administration of 0.05 % Y-39983. The reductions of IOP (mean $\pm$ SE) at 2, 3, 5, and 7 hours after administration of 0.05% Y-39983

were 1.9 ± 0.3, 2.5 ± 0.4, 1.7 ± 0.3, and 0.8 ± 0.2 mmHg, respectively. Maximal IOP reduction (mean ± SE) was 0.4 ± 0.1, 0.4 ± 0.2, 1.4 ± 0.3 ($p < 0.05$ vs. vehicle-treated eyes, William's test, one-sided), and 2.5 ± 0.8 mmHg ($p < 0.05$) at 0.003%, 0.01%, 0.03%, and 0.05% Y-39983, respectively (Figure 4B). Statistically significant reduction of IOP was obtained at concentrations of Y-39983 equal to or greater than 0.03 %. Administration of 0.005% latanoprost lowered IOP by 2.5 ± 0.2 mmHg ($p < 0.001$ vs. vehicle-treated eyes, t-test, one-sided), demonstrating that the IOP-lowering effect of 0.05 % Y-39983 was similar to that of 0.005% latanoprost.

Measurements of total outflow facility and uveoscleral outflow

Outflow facility was measured 2 hours after topical administration of 0.05 % Y-39983, when maximal IOP reduction was observed. As summarized in Table 2, outflow facility (mean ± SE) in eyes treated with Y-39983 (0.168 ± 0.018 μl/min/mmHg) was approximately 1.7 times (+65.5%) that in the contralateral, vehicle-treated eyes (0.111 ± 0.014 μl/min/mmHg). This difference was significant ($p < 0.001$, paired t-test, one-sided). In contrast, there were no significant differences in uveoscleral outflow between eyes treated with Y-39983 and those treated with vehicle.

Ocular toxicological effects of topical administration of Y-39983

Ocular toxicological properties were evaluated for long-term topical administration of Y-39983. In the QID administration study, rabbit eyes were examined with 0.003% to 0.03% Y-39983 4 times a day (at 2- hour intervals) for 4 weeks, and monkey eyes with 0.003% to 0.05% Y-39983 4 times a day (at 2-hour intervals) for 26 weeks. In neither species were

significant abnormalities of the corneal surface, anterior chamber, lens, vitreous, or retina observed on slit lamp examination, nor were significant findings of toxicity detected on histological examination. ERG analysis revealed no abnormalities in eyes treated with Y-39983 of either species. At week 4, thread wetting values (mean ± SE) for rabbits determined by the phenol red thread method were 29.4 ± 1.7, 29.4 ± 0.9, 28.8 ± 1.0, and 29.0 ± 1.1 mm with saline, 0.003%, 0.01%, and 0.03% Y-39983, respectively. At week 25, the thread wetting values in monkeys were 30.0 ± 1.0, 28.5 ± 1.0, 31.0 ± 0.7, 28.9 ± 1.1, and 29.1 ± 0.8 mm with vehicle, 0.003%, 0.01%, 0.03%, and 0.05% Y-39983, respectively. There were no differences in thread wetting values between the groups in rabbits and monkeys. However, conjunctival hyperemia and punctate subconjunctival hemorrhage were observed in eyes with topical administration of Y-39983 in rabbits (Figure 5A) and monkeys (Figure 5B), and punctate subconjunctival hemorrhage was sporadic during the administration period in both species. In the QID administration study, punctate subconjunctival was observed in 4/5 rabbits receiving 0.03% Y-39983 and 2/8 monkeys receiving 0.05% Y-39983. The hemorrhage recovered during the administration period. However, in the BID and TID administration studies, punctate subconjunctival hemorrhage was not observed in eyes treated with Y-39983, though conjunctival hyperemia was observed in eyes treated with Y-39983 in both species. To elucidate the mechanisms responsible for the punctate subconjunctival hemorrhage, we examined morphological changes in cultured HUVECs following the addition of Y-39983. In medium containing 1 μM Y-39983, HUVECs exhibited contraction (Figures 6A, 6B, 6C). The morphological changes in HUVECs were reversible and had nearly recovered by 1 hour after removal of Y-39983 (Figure 6D).

DISCUSSION

The present study demonstrated that topical administration of a selective inhibitor of the ROCK/ROK family of protein kinases, Y-39983, can significantly reduce IOP in rabbit and monkey eyes. A number of clinical investigations have revealed that elevated IOP is a major factor that causes glaucomatous optic neuropathy.[34-36] Because they potently lower IOP in mammalian eyes, ROCK inhibitors have been considered potential drugs for the treatment of glaucoma.[18,19] In this study, we examined the efficacy and safety of Y-39983 for potential clinical application.

Although our previous study revealed significant IOP-lowering effects of Y-27632 in animal eyes, [18,19] for potential clinical use, this compound has the disadvantage of poor stability in solution (data not shown). A series of modifications of molecular structure was therefore conducted to develop a more suitable form and more potent ROCK inhibitory activity for clinical use. Among the forms obtained, Y-39983 exhibits potent inhibition of ROCK activity and has acceptable stability even in solution. Furthermore, we found that the ratio of IC_{50} for inhibition of ROCK/PKC for Y-39983 was 117 while that for Y-27632 was 82, suggesting that Y-39983 has the same specificity for ROCK as Y-27632. Also, the inhibition of ROCK by Y-39983 was 30 times that by Y-27632. These *in vitro* findings suggested that Y-39983 was a more useful candidate for an anti-glaucoma drug than Y-27632. In our previous study,[18] Y-27632 at concentrations of 0.34 % to 3.4% reduced IOP by 7 to 12 mmHg in rabbit eyes under the same conditions of administration as in this study. Reduction of IOP ($\Delta IOP \geq 10$ mmHg) was observed with a lower dose of Y-39983 (0.03 % to 0.1 %). In addition, the degree of reduction of IOP ($\Delta IOP = 5.3$ mmHg at maximum) obtained with 0.1 % Y-27632, as determined in a previous study,[19] was observed with 0.01 % Y-39983

(ΔIOP = 6.6 mmHg at the peak). These findings together suggest that the reduction of IOP by Y-39983 is about 10 times more potent than that byY-39983. These findings appear to agree with our *in vitro* result that the ROCK inhibitory activity of Y-39983 is 30 times that of Y-27632.

In considering clinical application of Y-39983, the next important question is whether Y-39983 is also effective in lowering IOP in primate eyes, since primate eyes have a modality of aqueous outflow different from that of lower mammalian eyes. The present study revealed that, even in monkey eyes, 0.05% Y-39983 induces significant IOP reduction almost equal to that obtained with 0.005% latanoprost. The IOP-lowering effect of Y-39983 in monkey eyes suggests the possibility of clinical use of this compound.

In this study, we examined the IOP-lowering effects of Y-39983 in rabbits and monkeys. In 0.05% Y-39983, maximal reductions of IOP were 12.1 $\pm$ 1.5 (mean $\pm$ SE) and 2.5 $\pm$ 0.2 mmHg in rabbits and monkeys, respectively, and the magnitude of effect of Y-39983 in monkeys was much less than that in rabbits. This difference between species may be explained by the difference in baseline IOP values, which were 22.0 $\pm$ 0.6 and 17.5 $\pm$ 0.2 mmHg in rabbits and monkeys, respectively. In fact, it has been reported that the IOP-lowering effects of H-7 and prostaglandin analogues in monkeys, which have low baseline IOP values, are weaker than that in rabbits, which have high baseline IOP values.[30,37] In a preliminary pharmacokinetics study after the topical administration of Y-39983, the disappearance of Y-39983 in tears of monkeys was faster than that in rabbits, and this difference in pharmacokinetics may be due to differences in frequency of blinking in these species. This difference in pharmacokinetics may also have resulted in the difference in IOP-lowering effect in rabbits and monkeys in this study.

There are two routes of aqueous humor outflow, that via the conventional (trabecular) and that via the unconventional (uveoscleral) pathway.[20] In primate and human eyes, conventional outflow is considered the main route, and is believed to be regulated by the cellular behavior and contractility of TM cells.[38,39] Our previous study showed that Y-27632 increased conventional outflow by altering the contractility of TM cells.[18,19] In addition, Y-27632 has been shown to increase conventional outflow by inducing cellular relaxation and loss of cell-substratum adhesion in TM and SC cells.[23] Our outflow measurements suggest that Y-39983 may also affect the contractility of TM and SC cells, resulting in increased conventional outflow. Consistent with this, it has been demonstrated that TM exhibit higher levels of mRNAs for ROCK and ROCK substrates than CM in human and monkey eyes, suggesting that TM is one of the major sites of regulation of IOP by ROCK.[25]

The IOP-lowering mechanism of H-7, a broad-spectrum inhibitor of serine-threonine kinases including ROCK, has been investigated.[27,28,30] H-7 also increases conventional outflow by altering the shape, actin cytoskelton, and cell-cell adhesion of TM and SC cells, as Y-27632. Thus, alterations of TM and SC cells affect conventional outflow, and compounds causing cytoskeletal change in TM and SC cells may potentially be useful as anti-glaucoma drugs.

In our toxicological study, no serious side effects were observed in ocular tissues of rabbits and monkeys except sporadic punctate subconjunctival hemorrhage. This type of hemorrhage was observed after frequent administration of Y-39983 (4 times a day at 2-hour intervals). The side effects may be explained by impairment of barrier function or morphological changes in vascular endothelial cells, as shown in the experiments using

HUVECs. The morphological changes in HUVECs observed following the addition of Y-39983 suggest the possibility of impairment of barrier function in vascular endothelial cells in the retina, since the Rho-ROCK signaling pathway is considered ubiquitous. However, our animal experiments revealed no cases of detectable hemorrhage from the iris-ciliary body or retina-choroid, suggesting that the concentration at which lowering of IOP is elicited may not be high enough to induce hemorrhage in the ocular fundus. Also, subconjunctival hemorrhage, which was encountered with frequent administration (4 times a day), was not observed with administration 2 or 3 times per day. It is possible that no side effects will be induced in the conjunctiva with clinical usage of Y-39983 if excessively frequent instillation is avoided.

In summary, the present study showed that Y-39983, a selective and potent ROCK inhibitor, reduced IOP and increased outflow. Selective inhibition of the Rho/ROCK signaling pathway may be a useful new strategy for the treatment of glaucoma, and Y-39983 ophthalmic solution may be a candidate drug since it lowers IOP by increasing aqueous conventional outflow and produces fewer side effects.