Effects of the New Ethacrynic Acid Derivative SA9000 on Intraocular Pressure in Cats and Monkeys

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To evaluate the pharmacological characteristics of the new ethacrynic acid (ECA) derivative SA9000, we examined its ocular hypotensive effects in cats and cynomolgus monkeys, its corneal toxicity in rabbits, and its binding affinities for forty-three receptors, ion channels, and second messenger systems. A 20 μl injection into the anterior chamber of eye (intracameral injection) of 0.1 mM SA9000 significantly reduced intraocular pressure (IOP) 3.8 mmHg in cats. A 10 μl intracameral injection of 1 mM SA9000 significantly reduced IOP 7 mmHg in living monkeys without evidence of in vivo or in vitro toxicity. The ocular hypotensive effect of SA9000 in monkeys was greater than that of ECA. The morphology of corneal endothelial and epithelial cells in rabbit eyes after intracameral injection of SA9000 was observed using electron microphotography. SA9000 at 2 mM did not induce any abnormalities, indicating that it has no corneal toxicity at a concentration higher than the minimum needed for an ocular hypotensive effect (1 mM). SA9000 at 0.01 mM showed negligible binding affinity for, or inhibition of, forty-three different receptors, ion channel proteins, and second messenger systems. These findings indicate that SA9000 has the potential to be both effective and safe as an ocular hypotensive drug, although the mechanism of action remains unclear.

Key words SA9000; ethacrynic acid derivative; ocular hypotensive effect; intraocular pressure; European cat; cynomolgus monkey

In glaucoma, the second most common cause of blindness, an elevated intraocular pressure (IOP) is one of the risk factors for damage to the optic nerve. Currently, application of ocular hypotensive drugs is the mainstream approach to glaucoma therapy, and extensive efforts have been made to develop anti-glaucoma drugs that lower IOP. These drugs are intended either to modulate aqueous humor outflow at sites in the trabecular meshwork or ciliary muscle,1–5 or to inhibit the production of aqueous humor by the ciliary body. Aqueous humor outflow consists of conventional trabecular meshwork outflow and unconventional uveoscleral outflow. It has been proposed that TM plays the major role in the regulation of normal aqueous humor outflow to maintain normal range of IOP.6–9

At present, many ocular hypotensive agents that modulate uveoscleral outflow (such as the prostaglandin derivatives) are in use as anti-glaucoma drugs. In contrast, there are no drugs that act directly on the trabecular meshwork to increase conventional outflow, even though this constitutes approximately 90% of the normal eye’s total aqueous outflow.10 Thus, an effective modulator of conventional outflow might be capable of exerting a powerful ocular hypotensive effect and be of great value as a next-generation ocular hypotensive drug.

Ethacrynic acid (ECA) is well known as a sulfhydryl (SH) reactive diuretic and a Na+/K+ /Cl− co-transport system inhibitor,11,12 and it has been observed to increase aqueous humor outflow and lower IOP. ECA has been reported to increase aqueous humor outflow facility in enucleated animal and human eyes and in living monkey eyes,13–17 and these effects have been correlated with changes in human trabecular meshwork cell shape and cytoskeleton in vitro.18 IOP lowering also has been observed in living human eyes with glaucoma.19 ECA thus seems to have the potential to become an ocular hypotensive agent by its targeting of the trabecular meshwork, a site at which it seems to cause reversible changes in both cell shape and attachment.20 Nevertheless, because of its possible ocular side effects, there is a need for derivatives of ECA with even greater ocular safety16,17 and corneal penetration.20 To broaden the therapeutic index, which is shown as the ratio of doses between the IOP reducing effects and potential side effects, may lead to find the clinically useful ocular hypotensive drugs. We, therefore, synthesized thirty-two compounds and evaluated ECA derivatives that might retain the strong cytoskeletal modulating activity of ECA while having a lower cytotoxicity potential, i.e. a broader therapeutic index. The structural modifications of ECA examined involved both the phenoxycetic acid and the acryloyl moieties. Among these, we found a new ECA derivative, SA9000, which appeared to have a broader therapeutic index. In the present study, we examined the effects of SA9000 on IOP in cats and monkeys, and also evaluated changes in corneal endothelial and epithelial morphology as surrogates for possible side effects.

MATERIALS AND METHODS

Animals and Anesthesia European cats (IFFACREDO, Lyon, France), cynomolgus monkeys (KEARI Co. Ltd., Osaka, Japan), and Japanese white rabbits (KITAYAMA LABES Co. Ltd., Nagano, Japan) weighing approximately 3.5—5.0 kg, 5.0—7.0 kg, and 3.0—4.0 kg, respectively, were used in this study. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For IOP measurements, all animals’ eyes were anesthetized by topical instillation of 0.4% oxybuprocaine hydrochloride solution (Benoxil® 0.4% solu-
tion; Santen Pharmaceutical Co. Ltd., Osaka, Japan). Cats, which were pre-treated with an intramuscular injection of ketamine (Ketal FL; Sankyo Co., Tokyo, Japan; 10 mg/kg), were anesthetized through out the experiment with a continuous infusion of pentobarbital in Ringer's solution (approximately 12.5 μg/kg/min, i.v.). Monkeys were anesthetized by intramuscular injection of ketamine (10 mg/kg) at 10 min before each IOP measurement.

**Chemicals, Drug Preparation, and Drug Administration** SA9000, 4-(2-phenyl acryloyl) cinnamic acid, was synthesized by Santen Pharmaceutical Co. Ltd., while ECA, [2,3-dichloro-4-(2-methylenecetyl) phenoxyl] acetic acid, was obtained from Sigma (St. Louis, MI, U.S.A.). Their structures are shown in Fig. 1. Injection into the anterior chamber of eye (intracameral injection) was chosen as an administration route for first screening to examine the potency of compounds without affects according to abilities of corneal penetration. The doses chosen for intracameral injection were 0.3 to 3 mM (by reference to the effective dose, 3 mM, of ECA on monkey IOP). Intracameral injections were given via a microsyringe (Hamilton, Reno, NV, U.S.A.) fitted with a 30-gauge needle. Cats were injected with 20 μl of 0.1 mM SA9000. Cynomolgus monkeys were injected with 10 μl of either ECA or SA9000 at concentrations 0.3, 1, and 3 mM.

**Intraocular Pressure Measurement** A calibrated pneumatic tonometer (Model 30 Classic; Mentor Co., Norwell, MA, U.S.A.) was used to monitor IOP. In the cat study, IOP was measured one hour before and just before the injection of an ECA derivative or its vehicle, and at 2, 4, 6, and 8 h after the injection. In the monkey study, IOP was measured one hour before and just before the injection of an ECA derivative or its vehicle, and at 4, 8, 12, and 24 h after the injection. The IOP value was normalized by subtracting the IOP of the contralateral non-treated eye from that of the treated eye (in order to minimize the influence of anesthesia etc.). The change in the IOP value from the pre-dosing IOP value obtained at 0 h were calculated for each animal.

**Electron Microphotography** Each rabbit was sacrificed by an overdose of pentobarbital sodium 24 h after intracameral injection of 20 μl of 2 mM SA9000 or its vehicle, and both eyes were enucleated and fixed in glutaraldehyde (2.5%) in 0.1 M phosphate buffer. The corneas, destined for scanning electron microscopy (SEM) (2 mM SA9000 or vehicle; each n = 2), were processed in the usual fashion (dehydration, critical point drying, and sputter coating with gold). These specimens were examined using an electron microscope (JSM-840A; JEOL Ltd., Tokyo, Japan) at 15 kV.

**Receptor Binding and Biological Assays** The following incubation conditions were used for the various assays. GABA: [3H] GABA in 50 mM Tris–HCl buffer (pH 7.4) at 22 °C for 60 min with A7r5 cells; V1: [3H] AVP in DMEM (pH 7.4) at 22 °C for 60 min with A7r5 cells; V2: [3H] AVP in 50 mM phosphate buffer (pH 7.4) at 4 °C for 180 min with LLC-PK1 cells; gluocorticoid: [3H] triamcinolone in 10 mM Hepes buffer (pH 7.2) at 4 °C for 240 min with L-929 cells (cytosol); estrone: [3H] estradiol in 50 mM Hepes buffer (pH 7.5) at 4 °C for 20 h with calf uterus (cytosol); progesterone: [3H] R 5020 in 20 mM Tris–HCl, 1 mM EDTA, 12 mM monothioglycerol, 8% glycerol, and 20 mM NaMoO4 buffer (pH 7.5) at 4 °C for 20 h with calf uterus (cytosol); androgen: [3H] mibolerone in 20 mM Tris–HCl buffer (pH 7.5) at 4 °C for 24 h with rat prostate (cytosol); TH: [3H] T3 in 50 mM NaCl, 10% glycerol, 2 mM EDTA, 5 mM 2-mercaptoethanol, and 20 mM Tris–HCl buffer (pH 7.6) at 4 °C for 18 h with rat liver; Ca2+ channel (L, verapamil site): [3H] (+) D 888 in 50 mM Hepes buffer (pH 7.4) at 22 °C for 60 min with rat cerebral cortex; K+ATP channel: [3H] glibenclamide in 50 mM Tris–HCl buffer (pH 7.4) at 22 °C for 60 min with rat cerebral cortex; Na+/K+Cl– cotransport: 86Rb in Earle's salt solution (pH 7.4) at 37 °C for 10 min with A7r5 cells; NOS: endogenous arginine in serum free DMEM for 18 h with RAW 264-7 cells; Ca2+ pump: ATP in 10 mM Hepes–KOH (pH 7.0) at 30 °C for 6 min with Ca2+-ATPase; Na+/K+ pump: 86Rb and endogenous ATP in Earle's salt solution (pH 7.4) at 37 °C for 10 min with A7r5 cells; L-type Ca2+ channel: KCl in Hepes-buffered salt solution (pH 7.4) at 37 °C for 6 min with A7r5 cells; Na+/Ca2+ antiport: 45Ca in 160 mM NaCl and 20 mM Mops–Tris buffer (pH 7.4) at 25 °C for 0.5 min with bovine heart membrane vesicles; Na+/H+ antiport: 2Na in Earle's salt solution, 25 mM Hepes–Tris (pH 7.4) at 37 °C for 3 min with A7r5 cells; adenylyl cyclase: ATP and forskolin in 25 mM Tris–HCl buffer (pH 7.5) at 30 °C for 30 min with rat brain; guanylate cyclase: GTP and SNP in 40 mM triethanolamine (TEA)–HCl buffer (pH 7.4) at 30 °C for 15 min with bovine lung; β-adrenoceptor-G protein coupling (agonist effect): rat heart cells in 25 mM Tris–HCl buffer (pH 7.5) at 30 °C for 30 min; acetylcholinesterase: AMTCh in 0.1 mM phosphate buffer (pH 8.0) at 37 °C for 30 min with acetylcholinesterase; MAP kinase (ERK 42): [γ-32P]ATP and MBP in 50 mM phosphate buffer (pH 8) at 37 °C for 30 min with MAP kinase; protein kinase A: [γ-32P]ATP, cAMP and histone H1 in 0.2 mM EDTA (pH 7.0) at 30 °C for 20 min with protein kinase A.
kinase: $[\gamma^{33}\text{P}]\text{ATP} \text{ and histone H1 in 5} \text{mM Tris-HCl}$ buffer (pH 7.5) at 30°C for 20 min with protein kinase C$^{49}$; protein kinase p56$^{\text{ck}}$: $[\gamma^{33}\text{P}]\text{ATP} \text{ and poly GT in 50 mM}$ Tris–HCl buffer (pH 7.0) at 25°C for 30 min with protein kinase p56$^{\text{ck}}$; $^{50}$; protein kinase p55$^{\text{ck}}$: $[\gamma^{33}\text{P}]\text{ATP} \text{ and poly GT in 50 mM}$ Tris–HCl buffer (pH 7.0) at 25°C for 30 min with protein kinase p55$^{\text{ck}}$; $^{50}$; phospholipase C: $[\text{3H}]\text{PI, GT in 20 mM}$ acetic acid buffer (pH 5.5) at 37°C for 10 min with phospholipase C$^{51}$; $^{52}$; $\text{V}_{\text{i}}$-inositol phosphate (stimulated): $[\text{3H}]\text{inositol}$ and vasopressin in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C for 15 min with A7r5 cells$^{52}$; carbonic anhydrase: carbonic anhydrase in H$_2$O saturated with CO$_2$ at 4°C$^{53}$; neutral endopeptidase: DAGNP in 50 mM Tris–HCl buffer (pH 7.4) at 37°C for 30 min with neutral endopeptidase$^{54}$; MMP-3: NFF-2 in 50 mM Tris–HCl buffer (pH 7.5) at 37°C for 90 min with MMP-3$^{55}$; MMP-7: MMP-2/MMP-7 substrate in 50 mM Tris–HCl buffer (pH 7.5) at 37°C for 90 min with MMP-9$^{55}$; cell proliferation (serum-stimulated A7r5): A7r5 cells in DMEM at 37°C for 90 min with MMP-9$^{55}$; cell proliferation (TGF-$\beta$-inhibited Mv 1 Lu): Mv 1 Lu cells in DMEM at 37°C for 90 min with TGF-$\beta$1; cell proliferation (PGD-stimulated Balb/c/3T3): Balb/c/3T3 cells in DMEM at 25°C for 30 min with protein kinase p56$^{\text{ck}}$; phospholipase C: $[\text{3H}]\text{PIP2}$ GT in 50 mM Tris–HCl buffer (pH 7.0) at 25°C for 30 min with protein kinase p55$^{\text{ck}}$; $^{50}$; protein kinase p56 lck: $[\text{3H}]\text{ATP}$ and histone H1 in 5 mM Tris–HCl buffer (pH 7.5) at 30°C for 20 min with protein kinase C$^{49}$; protein kinase p56$^{\text{ck}}$: $[\gamma^{33}\text{P}]\text{ATP} \text{ and poly GT in 50 mM}$ Tris–HCl buffer (pH 7.0) at 25°C for 30 min with protein kinase p56$^{\text{ck}}$; $^{50}$; phospholipase C: $[\text{3H}]\text{PI, GT in 20 mM}$ acetic acid buffer (pH 5.5) at 37°C for 10 min with phospholipase C$^{51}$; $^{52}$; $\text{V}_{\text{i}}$-inositol phosphate (stimulated): $[\text{3H}]\text{inositol}$ and vasopressin in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C for 15 min with A7r5 cells$^{52}$; carbonic anhydrase: carbonic anhydrase in H$_2$O saturated with CO$_2$ at 4°C$^{53}$; neutral endopeptidase: DAGNP in 50 mM Tris–HCl buffer (pH 7.4) at 37°C for 30 min with neutral endopeptidase$^{54}$; MMP-3: NFF-2 in 50 mM Tris–HCl buffer (pH 7.5) at 37°C for 90 min with MMP-3$^{55}$; MMP-7: MMP-2/MMP-7 substrate in 50 mM Tris–HCl buffer (pH 7.5) at 37°C for 90 min with MMP-9$^{55}$; cell proliferation (serum-stimulated A7r5): A7r5 cells in DMEM at 37°C for 24 h$^{57}$; cell proliferation (PDGF-stimulated Balb/c/3T3): Balb/c/3T3 cells in DMEM at 37°C for 24 h$^{57}$; cell proliferation (TGF-$\beta$1-inhibited Mv 1 Lu): Mv 1 Lu cells in DMEM at 37°C for 24 h$^{58}$.

Statistical Analysis Statistical comparisons of vehicle- and drug-induced changes were made using an analysis of variance followed either by Student’s t-test or by Dunnett’s multiple comparison test (Yukms STAT LIGHT; Yukms, Tokyo, Japan). Student’s t-test was performed after F analysis in the cat study, while Dunnett’s multiple comparison test was performed after Bartlett analysis and ANOVA analysis in the monkey study. The level of significance was set at $p<0.05$.

RESULTS

Intraocular Pressure Measurements Ocular normotensive cats were intracamerally injected with 20 $\mu$L of 0.1 mM SA9000. The initial IOPs (mean±standard error) of 0.1 mM SA9000 and vehicle group were 17.1±1.2 mmHg (n=4) and 17.4±0.9 mmHg (n=4), respectively. By comparison to that of the vehicle-treated group, the IOP in the SA9000-treated group was significantly lower at 4 and 6 h after the injection (Fig. 2A). The maximal IOP reductions in the vehicle- and SA9000-treated groups were 1.3 and 3.8 mmHg, respectively (Fig. 2B).

Cynomolgus monkeys were intracamerally injected with 10 $\mu$L of either ECA or SA9000 at dosing concentrations from 0.3 to 3 mM. The initial IOPs (mean±standard error) of 0.3 mM ECA, 1 mM ECA, 3 mM ECA, 0.3 mM SA9000, 1 mM SA9000, 3 mM SA9000 and vehicle group were 17.3±2.0 mmHg (n=3), 16.5±0.2 mmHg (n=4), 17.0±0.7 mmHg (n=4), 16.5±1.2 mmHg (n=3), 16.5±0.8 mmHg (n=4), 17.5 mmHg (n=2) and 17.8±1.3 mmHg (n=4), respectively. ECA at 3 mM reduced IOP by 4.5 mmHg at 4 h after injection (Figs. 3A, C). Intracamerical injection of SA9000 reduced IOP in a dose-dependent manner (Figs. 3B, C). The maximal IOP reduction in the 1 mM SA9000-treated group was 7.0 mmHg (Figs. 3B, C) and it was statistically significant ($p<0.01$). The maximal IOP reduction in the 3 mM SA9000-treated group was 15.5 mmHg (Figs. 3B, C) and statistical analysis could not be applied to the 3 mM SA9000-treated group due to the small number of animals (n=2); however, it may indicate the tendency of ocular hypotensive efficacy at 3 mM that 3 mM SA9000 dramatically reduced the IOP (by more than 10 mmHg) from 4—24 h after the injection.

Electron Microphotography Corneal endothelial and epithelial cell morphology was observed by electron microscopy. With SA9000 at 2 mM, there was no evidence of corneal toxicity (such as damage to microvilli, focal breaks in intercellular junctions, or cellular edema) in the rabbit corneal epithelium or endothelium (Fig. 4).

Receptor Binding and Biological Assays To try to clarify the mechanism underlying the effect of SA9000, receptor binding and biological assays were performed. The concentration of SA9000 used was 0.01 mM (assumed to be approximately the final concentration at the level of the trabecular meshwork after intracameral injection of 0.1 mM SA9000 in cats). SA9000 at 0.01 mM exhibited negligible binding affinities or inhibition in our study of forty-three receptors, second messenger systems, and ion-channel proteins (data is not shown). SA9000 did not show a clear inhibition of Na$^+$/K$^+$/Cl$^-$ cotransport in our study (Table 1).

DISCUSSION

We observed that SA9000 (at 0.1 and 1 mM in cats and monkeys, respectively) demonstrates potent ocular hypotensive effects without corneal cytotoxicity. In the monkey study, the maximal IOP reductions in the 1 and 3 mM SA9000-treated groups were 7.0 and 15.5 mmHg, respectively. We calculate that SA9000 at 0.1 and 1 mM injection concentration would have been diluted approximately ten-fold, equivalent to levels of 0.01 and 0.1 mM, respectively, in the aqueous humor after intracameral injection. Although we sought to obtain evidence of the mechanism underlying this potent ocular hypotensive effect using forty-three receptors, enzymes, second messenger systems, and ion channel proteins including the Na$^+$/K$^+$/Cl$^-$ cotransport system, SA9000 at 0.01 mM exhibited negligible binding affinity and inhibitory effects. Thus, SA9000 may reduce IOP and modulate outflow facility via effects on other second messenger systems related perhaps to the cytoskeleton of the trabecular...
meshwork cell, although this remains to be clarified.

At a concentration of 1 mM, SA9000 reduced IOP 7 mmHg in living monkeys without evidence of *in vivo* or *in vitro* toxicity. At a concentration of 3 mM, SA9000 did show evidence of moderate corneal cytotoxicity (data not shown), but our results point to the possibility of separating efficacy from cytotoxicity by use of such ECA derivatives.

In the present study, 3 mM ECA also reduced IOP in monkeys, although more weakly than SA9000, and at this concentration produced some corneal cytotoxicity. Similarly, ECA at concentrations up to 3 mM has been reported to reduce IOP, but at 3 mM was observed to cause side effects such as corneal edema. Therefore, there would appear to be a narrower therapeutic index with ECA compared to SA9000. Also, the maximal ocular hypotensive effect of SA9000 was more than 3 times greater than that of ECA, and SA9000 did not demonstrate corneal toxicity at its lowest effective dose.

In addition, intracameral injection of 2 mM SA9000, which is twice the lowest effective dose in the monkey, did not affect corneal endothelial and epithelial morphology in rabbits. Calculated final concentrations of SA9000 in aqueous humor were 0.2 and 0.1 mM after 20 μl injection of 2 mM SA9000 in rabbits and 10 μl injection of 1 mM SA9000 in monkeys, respectively. Thus, at 1 mM the ocular hypotensive efficacy of SA9000 may be separated from any corneal cytotoxicity.

Therefore, the purpose of the present study, which was to determine if a wider safety margin than that of ECA might be observed with the new ECA derivative SA9000 in vivo, was, in fact, substantially achieved. These findings indicate that the separation of efficacy from cytotoxicity may validate the direction of chemical modification of ECA derivatives and

![Fig. 3. Effects of Ethacrynic Acid and SA9000 on Intraocular Pressure (IOP) in Anesthetized Ocular Normotensive Monkeys](image-url)
SA9000 may be clinically a potent ocular hypotensive drug.

In the present study, we observed a difference in the effective IOP lowering dose of SA9000 between monkey and cat (1 and 0.1 mM, respectively). Thus, there may be a species difference in the drug response (cf. the prostaglandin FP receptor agonist latanoprost, which lowers IOP in monkeys and humans but not in rabbits), although further experiments will be needed to clarify the exact dose response characteristics.

ECA is well known to be a potent Na\(^+/\)H\(^+\)/K\(^+/\)H\(^+\)/Cl\(^-/\)H\(^+\) co-transporter inhibitor, whereas the new ECA derivative, SA9000, had only a very weak effect on this co-transporter (Table 1), despite the ocular hypotensive efficacy of SA9000 being more potent than that of ECA in the monkey (Fig. 3). These results suggest that the effective IOP lowering action of SA9000 in cats and monkeys may not be mediated via an inhibition of the Na\(^+/\)K\(^+/\)Cl\(^-\) co-transport system, and may instead be mediated by an alteration in conventional outflow facility achieved via another mechanism, such as an inhibition of a second messenger related to the cell cytoskeleton.\(^{18}\)

The Na\(^+/\)K\(^+/\)Cl\(^-\) co-transporter plays a prominent role in, for example, cell volume regulation in the corneal endothelium and trabecular meshwork in rabbits, calves, and humans.\(^{59,60}\) If inhibition of the Na\(^+/\)K\(^+/\)Cl\(^-\) co-transporter is one of the causes of the corneal edema that develops after instillation of ECA, the improvement in the selectivity of pharmaco logical properties shown by SA9000 may be due partly to a decrease in corneal cytotoxicity secondary to its weak effect on this co-transporter.

In efforts to discover a high potent ocular hypotensive drug, many research groups have been engaged on research related to cytoskeletal modulator of trabecular meshwork cells. They reported that the candidates show cytoskeletal modulating efficacy in vitro and reduce IOP in vivo.\(^{61,62}\) Our findings also suggested the possibility of cytoskeletal modulator as an ocular hypotensive drug because SA9000, which has potent cytoskeletal modulating efficacy of trabecular meshwork cells, showed ocular hypotensive effect in cats and monkeys.

Further modulations of the chemical structure of SA9000 and additional investigations of the pharmacological properties of SA9000 and its derivatives may lead to the development of a new category of ocular hypotensive drugs for the treatment of glaucoma.

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