Effects of the New Ethacrynic Acid Oxime Derivative SA12590 on Intraocular Pressure in Cats and Monkeys

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In glaucomatous eyes, an elevated intraocular pressure (IOP) is one of the risk factors for axonal damage in the optic nerve and subsequent retinal ganglion cell death, potentially leading to blindness.1−3)

Currently, the use of ocular hypotensive drugs is the only available approach to glaucoma medication, and extensive efforts have been made to develop new anti-glaucoma drugs that lower IOP. In the efforts to manage and control IOP in glaucoma patients, use has been made of a number of drugs (namely, pilocarpine, β-adrenergic receptor antagonists, epinephrine and its derivatives, prostaglandin-related compounds, and carbonic anhydrase inhibitors).4,5) Such ocular hypotensive drugs operate either by altering aqueous humor or outflow by acting at sites in the trabecular meshwork (TM) or ciliary muscle, or by inhibiting the production of aqueous humor by the ciliary body.5−10) Aqueous humor outflow consists of conventional TM outflow and unconventional uveoscleral outflow. Although prostaglandin derivatives that modulate uveoscleral outflow are in use as major anti-glaucoma drugs, there are currently no drugs that act directly on TM to increase conventional outflow, even though this constitutes approximately 90% of the normal eye’s total aqueous outflow.11) It has been proposed that in human and primates, TM plays the major role in regulating normal aqueous humor outflow-resistance so as to maintain a normal range of IOP.12−15) If this is so, an effective modulator of conventional outflow might exert a powerful ocular hypotensive effect, and thus be of great value as a next-generation anti-glaucoma drug.

Ethacrynic acid (ECA), a sulfhydryl (SH)-reactive diuretic, has been observed to increase the conventional outflow facility in both enucleated calf eyes and human eyes, as well as in monkeys following anterior-chamber perfusion.16−20) Intracameral injection of up to 3 mM ECA has been found to lower IOP in monkeys, although at concentrations higher than 3 mM it produced some focal reversible corneal edema.13) However, a pilot study of intracameral ECA injection in humans with chronic open-angle glaucoma demonstrated remarkable degrees of both efficacy and safety.21) ECA thus seemed to have potential as an ocular hypotensive agent. Nevertheless, because of its possible ocular side effects, there is a need for derivatives of ECA with even greater ocular safety,19,20) and corneal penetration.22) In fact, a number of attempts have been made to improve its profile, with respect to corneal penetration and corneal toxicity, by modifying the ECA molecule.23−25) A broader therapeutic index, which may be defined as the dose-ratio between its IOP-lowering effects and its potential side effects, would represent an advance toward the development of a drug that enhances conventional outflow.

We made structural modifications to ECA involving both the phenoxyacetic acid and acryloyl moieties. This led us to a new ECA derivative, SA9000, which appeared to have a broader therapeutic index and which, upon intracameral injection at 1 mM, lowered IOP in cats and monkeys without corneal toxicity.27)

In the present study, we evaluated SA12590 (a drug we developed from SA9000 during our attempts to improve its therapeutic index) for its effects on IOP in cats and monkeys following topical administration, its corneal toxicity in rats, and its SH reactivity in vitro.

MATERIALS AND METHODS

Animals European cats (IFFACREDO, Lyon, France), cynomolgus monkeys (KEAR Co., Ltd., Osaka, Japan), and Sprague-Dawley rats (Japan SLC, Osaka, Japan) weighing approximately 3.5−5.0 kg, 5.0−7.0 kg, and 200−400 g, re-

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spectively, were used in the studies. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Chemicals, Drug Preparation, and Drug Administration** SA9000 (4-(2-phenyl acryloyl) cinnamic acid) and SA12590 (4-(1-methoxy imino-2-phenyl allyl) cinnamic acid) were synthesized by Santen Pharmaceutical Co., Ltd. Their structures are shown in Fig. 1. SA9000 and SA12590 were each dissolved in sodium hydroxide solution, and this solution was then neutralized to pH 6.0—7.0 using hydrochloric acid. Drug solutions were topically administered.

**IOP Study** A calibrated pneumatic tonometer (Model 30 Classic; Mentor Co., Norwell, MA, U.S.A.) was used to monitor IOP in all IOP studies. For these measurements, all animals' eyes were anesthetized by topical administration of 0.4% oxybuprocaine hydrochloride solution (Benoxil® 0.4% solution; Santen Pharmaceutical Co., Ltd., Osaka, Japan).

In the ocular normotensive cat study, the cats were anesthetized throughout the experiment with a continuous infusion of pentobarbital in Ringer's solution (approximately 12.5 μg/kg/min, i.v.) after an intramuscular injection of ketamine (Ketaral; Sakyo Co., Tokyo, Japan; 10 mg/kg). Fifty microliters of 3% SA9000, 3% SA12590, or vehicle was topically administered once, with the fellow eye remaining untreated. IOP was measured 1 h before, immediately before, and at 4, 6, and 8 h after the topical administration. The IOP value was corrected by subtracting the IOP of the contralateral non-treated eye from that of the treated eye (in order to minimize the influence of anesthesia).

In ocular normotensive monkeys, IOP measurement was performed without systemic anesthesia. In the first study, 20 μl of 3% SA12590 or vehicle was topically administered 3 times with 5-min intervals. IOP was measured 1 h before, immediately before, and at 2, 4, 6, 8, and 24 h after such topical administration. In the second study, 20 μl of 1% or 3% SA12590 or vehicle was topically administered once a day for 3 d, with IOP being measured 1 h before, immediately before, and at 12, 24, 36, 48, 60, and 72 h after the first topical administration. The change in the IOP value from the predosing IOP value obtained at 0 h was calculated for each animal.

**SH Reactivity** For this assay, glutathione was used to make a conjugate with either SA9000 or SA12590 as a model molecule with a sulfhydryl group. One hundred microliters of either 2 mM SA9000 or 2 mM SA12590 were added to 20 mM glutathione solution in 80% methanol (900 μl) at room temperature. Sampling from the mixture was performed at 3, 15, 30, and 60 min, and 24 h after addition of SA9000 or SA12590. The sampled solution was spotted on a thin layer chromatography (TLC) plate (50 TLC aluminium sheets silica gel 60 F254; Merck KGaA, Darmstadt, Germany) using capillary glass (250 precision disposable micropipettes; Drummond Scientific Company, Broomall, Pennsylvania, U.S.A.). TLC was performed using a developing solvent containing ethyl acetate: hexane=20:1. Spot detection was conducted by exposure to UV (254 nm) for aromatic rings or by spraying 0.2% ninhydrin ethanol solution for amino groups. Binding of SA9000 or SA12590 to glutathione was determined by the disappearance of a given spot on the TLC plate under UV. Glutathione-bound SA9000 or SA12590 was determined as a shifted spot given a color by the ninhydrin solution. Spots were evaluated by comparison with the spot size produced by 0.2 mM SA9000 or SA12590 ethanol solution (the same concentration of SA9000 or SA12590 as in the above mixture solution). For this, the following scale was used: + + = no change; + = slight decrease in size; and − = spot not detected.

**Corneal Toxicity** Five microliters of 3% SA9000, 3% SA12590, or vehicle was topically administered to rats 3 times with 5-min intervals. Twenty-four hours later, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (35 mg/kg). One percent fluorescent (acid yellow 73; SIGMA, St. Louis, MO, U.S.A.) solution was topically applied to both eyes in each rat. Five minutes later, the surface of the cornea was rinsed with saline. Corneal toxicity was assessed as the superficial punctuate keratitis (SPK) or corneal epithelium defect detected on photos taken using a photo-slit lamp (PHOTOSLITLAMP SC-1200; KOWA, Tokyo, Japan). For this, the following scale was employed: 0 = no damage; 1 = slight SPK (staining area, 0—30%); 2 = moderate SPK (30—50%); 3 = severe SPK (50—100%); or 4 = corneal epithelium defect.

**Statistical Analysis** In the IOP studies, statistical comparisons of vehicle- and drug-induced IOP changes were made using an analysis of variance, with F analysis, followed by either a Student’s t-test or Aspin–Welch t-test (Exsas; Arm Systex, Osaka, Japan). In the multigroup IOP study with SA12590, statistical comparisons were made using an analysis of variance, Bartlett analysis, followed by either a Tukey-type or Kruskal–Wallis test. The level of significance was set at p<0.05. In the corneal toxicity study, statistical comparisons of vehicle- and drug-induced changes were made using an analysis of variance, employing the Kruskal–Wallis test, followed by the Steel–Dwass test.

**RESULTS**

**Effect of SA9000 Topically Administered in Cats** Ocular normotensive cats received 50 μl topically administered 3% SA9000 or vehicle (once). The initial IOP values (mean±standard error) for the 3% SA9000 and vehicle groups were 18.6±1.8 mmHg and 15.3±1.7 mmHg (n=6), respectively, which were not statistically different. SA9000 showed only a non-significant tendency to lower IOP in the
8 h after topical administration (Fig. 2A). The maximal IOP reductions in the vehicle- and SA9000-treated groups were 1.8 ± 0.6 and 2.8 ± 0.9 mmHg, respectively (not statistically different; Fig. 2B).

SH-Reactivity of SA9000 and SA12590

The staining spot obtained using SA9000 decreased with time in the glutathione assay (Table 1), whereas the SA12590 spot did not change under the same conditions.

Effect of SA12590 Topically Administered in Cats

Ocular normotensive cats received 50 μl topically administered 3% SA12590 or vehicle (once). The initial IOP values in the SA12590 and vehicle groups were not different [21.7 ± 1.3 mmHg and 21.3 ± 1.2 mmHg (n = 5), respectively]. SA12590 significantly lowered IOP at 6 h after its administration compared to vehicle (Fig. 2C). The maximal IOP reductions in the vehicle- and SA12590-treated groups were 0.3 ± 1.0 and 3.8 ± 0.7 mmHg, respectively (Fig. 2D), values that were significantly different according to the Student's t-test (p < 0.05).

Effect of SA12590 Topically Administered in Monkeys

Cynomolgus monkeys received 20 μl topically administered 3% SA12590 3 times with 5-min intervals to examine its potency at a dosage that was confirmed to show good tolerability in the corneal toxicity study (see below). The initial IOP values in the 3% SA12590 and vehicle groups were not different [20.5 ± 0.9 mmHg and 20.3 ± 0.6 mmHg (n = 4), respectively]. The SA12590-treated group first tended to exhibit signs of IOP lowering at 6 h after drug administration (Fig. 3A). The maximal IOP reductions in the vehicle- and SA12590-treated groups were 0.6 ± 0.3 and 8.6 ± 2.5 mmHg, respectively (Fig. 3B) (p < 0.05).

Next, cynomolgus monkeys received 20 μl topically administered 1% or 3% SA12590 once a day for 3 d. The initial IOP values in the 1% SA12590, 3% SA12590, and vehicle
We previously observed that upon intracameral injection, SA9000 (a derivative of ECA) has a potent ocular hypotensive effect at 0.1 mmHg in monkeys.27 Further, topical SA9000 lowers IOP in the laser-induced ocular hypertensive monkey.28 In addition to its greater efficacy, SA9000 has a wider safety margin than ECA (between its ocular hypotensive effect and corneal toxicity).26 However, topical administration of 3% SA9000 only tended (non-significantly) to lower IOP in our cats (Fig. 2). This concentration, 3%, of SA9000 was expected to be enough to induce an IOP-lowering effect, because it is 2500 times that used for intracameral injection (0.1 mm). Reportedly, a one hundred to one thousand times higher concentration is needed for topical administration to achieve a given intracameral drug level, due to the corneal barrier properties.29,30 If SA9000 has a fairly average corneal penetration rate, the concentration achieved in the aqueous humor following its topical administration at 3% would be more than 0.1 mm. Since the latter concentration, when injected intracameral, has an IOP-lowering effect, topical 3% SA9000 might be expected to induce an IOP-lowering effect as big as, or greater than that.27 However, 3% SA9000 did not show significant efficacy upon topical administration, and so we hypothesized that it has a low corneal penetration rate in the current setting. An additional point is that a single drop of 3% SA9000 might be the maximum possible dosage if tolerability to corneal toxicity is to be maintained. In fact, this dosage of SA9000 caused slight corneal epithelia edema at 24 h after topical administration (data not shown).

We therefore sought to develop a new compound that might have a greater therapeutic index than SA9000. SA9000 contains aromatic rings, which increases the ocular hypotensive effect, situated adjacent to an \( \alpha, \beta \)-unsaturated carbonyl group. This \( \alpha, \beta \)-unsaturated carbonyl is assumed also to increase SH reactivity because of the electron-withdrawing property of the aromatic group. SH reactivity is believed to be involved in corneal toxicity,23 and it may also lead to protein binding at the cornea, which could further increase corneal toxicity. We modified SA9000 by maintaining the necessary chemical skeleton while making changes designed to lower its SH reactivity. SA12590 was discovered during this process (Fig. 6).

The SH reactivity of SA12590 was lower than that of SA9000 (Table 1) and no corneal toxicity was observed (Fig. 5). Moreover, topical 3% SA12590 exhibited a substantial, statistically significant ocular hypotensive effect in monkeys (Figs. 3, 4). In corneal-chamber studies, we have confirmed an enhanced corneal penetration rate of SA12590 compared to SA9000 (data not shown). Hence, the oxime-derivative SA12590, with its minimal SH reactivity, resolves some important pharmacokinetic issues surrounding SA9000, and represents a promising new lead in the search for an ECA derivative for glaucoma therapy.

We conclude that SA12590, and possibly other analogues, has important potential for use as novel ocular hypotensive eye drops.

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REFERENCES AND NOTES

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