Eyedrops Containing SA9000 Prodrugs Result in Sustained Reductions in Intraocular Pressure in Rabbits

John J. Arnold,^{1,*} Yash Choksi,¹ Xin Chen,² Atsushi Shimazaki,³ John Hatten,² Eric J. Toone,²
David L. Epstein,¹ and Pratap Challa¹

Abstract

Aim: Poor topical bioavailability and ocular irritation have impeded the development of the diuretic, ethacrynic acid (ECA) as a clinically useful ocular hypotensive for the treatment of glaucoma. Thus, the development of analogs and prodrugs of analogs with improved ocular penetration, potency, and tolerability is required. The aim of this work is to evaluate the corneal penetration and ocular distribution of SA9000, an ECA analog. Novel SA9000 prodrugs intended to further improve ocular pharmacodynamic effect were also evaluated.

Results: SA9000 penetrated porcine corneas more effectively than ECA in corneal diffusion studies. *In vivo* studies in Dutch-belted (DB) rabbits indicated that topical application of a single dose (0.3%) of SA9000 could significantly reduce intraocular pressure (IOP) (~25% vs. fellow untreated eye) but caused significant conjunctival hyperemia. Since this hyperemia was likely the result of its inherent thiol reactivity, SA9000 was formulated with equimolar cysteine, an exogenous thiol donor. The administration of increasing SA9000–cysteine adduct concentrations (0.3%, 0.6%, 0.9%) demonstrated that they cause less ocular irritation than unadducted SA9000 but could still significantly reduce IOP (0.3%: $8.7 \pm 2\%$; 0.6%: $14.4 \pm 5\%$; 0.9%: $23.3 \pm 4.4\%$) versus untreated contralateral control eyes.

Conclusions: These data suggest that novel thiol donor adduction can improve the ocular bioavailability and tolerability of SA9000. SA9000–cysteine prodrugs may represent a new option for the topical treatment of glaucoma.

Introduction

GLAUCOMA IS A MAJOR cause of visual morbidity for which topical ocular hypotensive medication therapy is a major treatment. An ideal therapeutic agent for the treatment of glaucoma would be a medication that displays meaningful and targeted penetration into the anterior chamber, causes minimal to no ocular irritation, and targets the diseased tissue (ie, a dysfunctional trabecular meshwork [TM]). However, few of the currently marketed topical therapies for the treatment of glaucoma elicit their main pharmacological action at the level of the TM. Timolol reduces intraocular pressure (IOP) secondarily to decreased aqueous humor production and, thus, does not treat the underlying disease mechanism.^{1–3} Furthermore, beta-blockers can often selectively diffuse into systemic circulation where they can

cause unwanted respiratory and cardiac effects. The prostaglandin analogs (ie, latanoprost) increase outflow facility through the uveoscleral (or unconventional) pathway, which can account for approximately 3–35% of total aqueous humor outflow.^{4–5} Approximately 80–90% of aqueous humor outflow occurs through the conventional pathway, which is composed of the TM and Schlemms canal (SC).⁶ Currently, no frequently used ocular hypotensive medications exclusively target this important site of aqueous humor outflow.

Ethacrynic acid (ECA) (Fig. 1), a commercially marketed diuretic, has previously been demonstrated to increase anterior chamber outflow facility *in vitro* and reduce IOP *in vivo*.^{7–13} The mechanism of action of ECA appears to involve cytoskeletal modulation and relaxation of TM/SC cells.^{14–17} Thus, ECA treats a critical target tissue through a novel

¹Department of Ophthalmology, Duke University School of Medicine, and ²Department of Chemistry, Duke University, Durham, North Carolina.

³Santen Pharmaceutical Co., Ltd., Tokyo, Japan.

*Current affiliation: Department of Pharmaceutical, Social, and Administrative Sciences, Samford University McWhorter School of Pharmacy, Birmingham, Alabama.

mechanism in the pathophysiology of glaucoma. However, the development of ECA into a useful topical hypotensive has been slow due to its poor ocular penetration, poor ocular distribution, and external side effects.¹⁴ Consequently, excessive concentrations (2–3% w/v) have been required for the topical administration of ECA in order to elicit an effect on IOP. Unfortunately, while effective in reducing IOP, these higher concentrations of ECA have also resulted in ocular toxicities, presumably due to its binding to free thiol groups in nontarget tissues. These inherent limitations experienced with ECA have spurred the search for more potent analogs that can be applied topically at lower concentrations and result in less toxicity.

SA9000 (Fig. 1), an analog of ECA, has previously been shown to display striking *in vitro* potency for TM cell shape alteration.¹⁸ As compared to ECA, SA9000 has demonstrated superior efficacy for the reduction of IOP in both cats and monkeys following intracameral injection with minimal ocular toxicity.^{19,20} These findings have suggested that SA9000 may represent a potentially useful medication for the treatment of glaucoma. Notwithstanding, several important pharmacokinetic and pharmacodynamic questions remain about the utility of SA9000 as a chronic topical treatment for glaucoma. The purpose of this study was to characterize the ocular penetration, efficacy, and tolerability of SA9000, especially as compared to its parent compound, ECA. Furthermore, we describe new strategies including thiol adduction (Fig. 1) in order to enhance the ocular penetration and tolerability of SA9000.

Experimental Procedures

Materials and animals

SA9000 was obtained from the Santen Corporation (Tokyo, Japan). ECA and cysteine were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All chemicals used for High performance liquid chromatography (HPLC) were of reagent grade. Male Dutch-belted (DB) rabbits (2.5–4.0 kg) were obtained from Covance (Princeton, NJ). Male New Zealand White (NZW) rabbits (3.0–4.0 kg) were obtained

from Charles River Laboratories, Inc. (Boston, MA). Animals were fed *ad libitum* with a normal light/dark cycle. Animal care and treatment were in accordance with the guidelines established by the Association for Research in Vision and Ophthalmology (ARVO).

Formulation of SA9000 and SA9000–cysteine adduct

Seventy milligrams of SA9000 was suspended in 1.5 mL of 0.2 M NaOH aqueous solution. Sonication and vortexing was performed until a clear solution was obtained. Next, 10.1 mL of pH 7.0 phosphate-buffered saline (PBS) was added in portions. Sonication and vortexing were then repeated. This resulted in a 0.6% solution of SA9000 in a pH 7.0 PBS. For the cysteine adduct, 44 mg of cysteine HCl salt was dissolved in 2.0 mL of 0.2 M NaOH aqueous solution. Seventy milligrams of SA9000 was then added to the cysteine solution. Sonication and vortexing were performed until a homogeneous suspension was obtained. Next, 1 mL of 0.2 M NaOH aqueous solution was added, and sonication and vortexing were performed until a clear solution was generated. Finally, the solution was diluted with 8.6 mL of pH 7.0 PBS. The resulting solution was 0.6% of SA9000–cysteine in pH 7.0 PBS.

Corneal penetration experiments

Experiments were conducted in two-sided diffusion cells. These chambers allow drug solutions to be incubated in the donor (ie, epithelial) chamber (20 mL) and samples to be collected from the receiver (ie, endothelial) chamber (6 mL). Fresh enucleated porcine eyes were collected at a local abattoir (Burlington, NC) and used within 10 h of animal death. Isolated porcine corneas have previously demonstrated utility as surrogates of human corneas in both penetration and ocular enzymatic studies.²¹ Corneal and conjunctival–scleral rings, ~3 mm in diameter, were collected, suspended, and anchored with an O-ring between the donor and receiver compartments. The ocular tissues were bathed on either side with Dulbecco's phosphate-buffered saline (dPBS) with glucose (1 mg/mL) added.

FIG. 1. Novel topical ocular hypotensives.

Comparative diffusion experiments across cornea or conjunctiva-sclera were conducted with ECA and SA9000. Solutions containing either drug at a concentration of 1 mM were incubated in the donor compartment for 3 h at 37°C. Concurrently, samples (600 μ L) were collected from the receiver compartment at ~15-min intervals. In order to assess drug movement, absorbance was measured in a UV-light spectrophotometer. Samples were quantitated with previously determined standard curves of known concentrations of either ECA or SA9000. Following measurement, samples were immediately returned to the receiver chamber of the diffusion cell.

Eyedrop administration

Male DB rabbits were treated via eyedrop administration to one eye with the contralateral eye not receiving drug formulation in order to serve as an untreated control. For the initial study, to assess the IOP-reducing potential of SA9000 (0.3%), single-dose treatment involved the administration of four drops (containing 40 μ L in each drop) separated by 1-min intervals. Multiple dose studies with formulations of SA9000 (0.3, 0.6, 0.9%) plus cysteine necessitated initial treatment with four drops (~160 μ L) separated by 1-min intervals of drug. Subsequently, animals were treated twice daily (1,000 and 1,800) with two drops (~80 μ L) separated by 1-min intervals for 5 days. Prior to drug treatments, eyes were anesthetized with 0.5% proparacaine topical drops to facilitate IOP measurements.

Subsequent ocular disposition studies were conducted in less expensive male NZW rabbits. The animals were treated with SA9000 (0.6%) and SA9000 (0.6%) plus cysteine in which eight drops separated by 1-min intervals were administered. The animals were sacrificed and tissues collected as outlined below.

IOP measurements

IOP measurements (mmHg) were made in the DB rabbits using a calibrated pneumatonometer (Medtronic Solan, Jacksonville, FL). Proparacaine (0.5%) (Bausch and Lomb Inc., Tampa, FL) was applied to the cornea of each eye before measurements in order to minimize the discomfort to the animal. Animals were measured several days prior to the start of an experiment in order to establish baselines and minimize fluctuations. IOP measurements were made serially, and three values were obtained from each eye that was averaged. Statistical assessments of compiled data were assessed via Graphpad Prism, Version 4 computer software (San Diego, CA).

Ocular irritation

Prior to the IOP measurements and drug administration in the DB rabbits, ocular irritation was assessed by biomicroscopic examination. DB rabbits were assigned relative irritation scores (0–4+; 0 no irritation, 1+ slight redness, 2+ more uniform, diffuse redness, 3+ severe diffuse redness, moderate inflammation, 4+ diffuse highly inflamed red eye) based on the level of hyperemia observed at that particular time point. DB rabbits were also examined via slit lamp in

order to assess whether anterior chamber inflammation was present and recorded as the number of visible cells per 1 mm cross-sectional area of the slit-lamp light beam.

Ocular disposition

NZW rabbits were sacrificed 2 h following topical administration of either SA9000 or SA9000–cysteine. Subsequently, samples of aqueous humor, cornea, conjunctiva-sclera, and iris-ciliary body were carefully isolated and immediately stored at –25°C. Aqueous humor was mixed with equal volumes of acetonitrile and injected directly in the HPLC for determination of SA9000 and SA9000–cysteine content. The other frozen samples were minced into a fine tissue and incubated with 2 mL of acetonitrile:methanol (50:50) on a shaker overnight at 20°C. Supernatant (1.5 mL) was removed from each sample twice following centrifugation (10,000 rpm for 10 min). The supernatant was subsequently removed, utilizing a speed vacuum pump. The samples were resuspended with acetonitrile and injected directly into the HPLC for determination of drug content.

Sample analysis was performed on a 4.6 $\times$ 250 mm reversed-phase C-18 column (Supelco/Sigma-Aldrich Company, St. Louis, MO) at a flow rate of 0.8 mL/min at 25°C. SA9000 and SA9000–cysteine were separated via gradient elution. Gradient was achieved by initially perfusing 80% water (plus 10 mM acetic acid)/20% acetonitrile for 5 min, then linearly increasing to 90% acetonitrile/10% water (plus 10 mM acetic acid) over 45 min. Sample analysis was carried out at a wavelength of 25 nm.

Results

In vitro ocular penetration and IOP-reducing effects of SA9000

Initially, the ability of ECA and SA9000 to pass through the cornea and conjunctiva-sclera was assessed in two-sided diffusion chambers as an *in vitro* model of ocular penetration. Buffer solutions containing ECA (1 mM) were incubated in the donor (ie, epithelial) compartment for 3 h at 37°C. The receiver chamber was sampled every 15–30 min and drug content was determined spectrophotometrically as described (Fig. 2). Following the 3-h experiment, only about 1.2% of the total ECA available for corneal penetration in the donor chamber was found in the receiver chamber (Fig. 2A). Conversely, a similar experiment conducted with SA9000 demonstrated that the analog demonstrated better penetration across the cornea (~4% of the total SA9000 at the end of the 3-h experiment) (Fig. 2A). A similar result was noted for the penetration of each compound when conjunctiva-scleral tissue replaced the cornea in the diffusion chambers (Fig. 2B).

Subsequently, the *in vivo* efficacy of SA9000 was tested in normotensive DB rabbits. Previous data have suggested that SA9000 can effectively modulate TM cell shape and reduce IOP at concentrations approximately a tenth of those used with ECA. For this reason, a topical concentration of 0.3% (vs. 3% commonly used for efficacy with ECA) was selected to study with SA9000 *in vivo*. A single four-drop dose of SA9000 (0.3%) was applied topically at time zero to a single eye of the DB rabbits, while the fellow eye was left as an untreated

FIG. 2. *In vitro* corneal (A) and conjunctival-scleral (B) penetration of ethacrynic acid (open circles) and SA9000 (closed circles). Data represents mean $\pm$ SD ($n = 3$).

FIG. 3. Pressure-lowering effect of SA9000 following topical administration. IOP of treated (open circle) and untreated (closed circle) eyes following a single dose of SA9000 (0.3%). Data represents mean $\pm$ SEM, ($n = 4$). Asterisks indicate significantly different values via paired *t*-test ($P < 0.05$).

control. The results of the experiment are presented in Figure 3. As compared to the untreated eyes, those treated with SA9000 responded with significant reductions in IOP. The IOP of SA9000-treated eyes were reduced ~ 5 mmHg (25%) versus fellow untreated control eyes at 36 h postdose (Fig. 3). As compared to untreated eyes, however, those treated with SA9000 displayed significant ocular irritation (primarily hyperemia/injection and conjunctival chemosis). Ocular irritation/inflammation can itself produce lower IOP levels, irrespective of any specific therapeutic action by the ocular hypotensive. Indeed, time-dependent comparison of the change in IOP versus relative hyperemia scores suggested that the pressure reductions might partially be due to ocular irritation (data not shown).

Ocular distribution of SA9000 and SA9000-cysteine adducts

In order to assess the ocular distribution of SA9000 and its cysteine adduct, these drug formulations were administered topically to NZW rabbits. The animals were subsequently sacrificed after 1 h, and various ocular tissues were collected for determination of drug content via reversed-phase HPLC. When standards of SA9000-cysteine adduct and SA9000 were injected, retention times of approximately 18 and 25 min were noted, respectively (data not shown). Control aqueous humor collected from animals treated with sham formulations containing saline demonstrated spectra devoid of the characteristic peaks of SA9000 or SA9000-cysteine

adduct (Fig. 4A). However, following administration of SA9000 or SA9000–cysteine adduct at a concentration of 0.6%, both of the drug species could be detected in all ocular tissues sampled (ie, aqueous humor, cornea, conjunctiva–sclera, iris–ciliary body). Representative spectra derived from conjunctival–scleral tissue and aqueous humor following SA9000–cysteine adduct administration demonstrated peaks corresponding to both the cysteine prodrug and free SA9000 (Fig. 4B, C). Subsequently, the integration derived from spectra of the various ocular tissues was compared to those derived from known concentrations and quantitated for SA9000 and SA9000–cysteine contents. Compared to animals treated with the SA9000–cysteine adduct, SA9000-treated rabbits displayed higher concentrations of the drug in extraocular tissues such as cornea (3.0-fold) and conjunctiva–sclera (4.1-fold) (Table 1). Conversely, the difference in disposition between the SA9000 and SA9000–cysteine adduct was less in intraocular tissues such as the iris–ciliary body (2.1-fold) and aqueous humor (1.9-fold).

In order to assess the effect of the SA9000–cysteine adduct on IOP following topical administration, ocular

formulations (0.3, 0.6, and 0.9%) were administered as eye-drops twice daily for 1 week in normotensive DB rabbits (Fig. 5). IOP and ocular irritation were assessed prior to each drug administration. A separate cohort of animals received vehicle without drug. No effect of vehicle treatment on IOP was noted (data not shown). As compared to untreated eyes, SA9000–cysteine adducts (0.3%) significantly reduced IOP with no apparent ocular irritation (Table 2). Since no ocular irritation was noted with the 0.3% concentration, the amount of SA9000–cysteine adduct applied was increased (0.6, 0.9%) in order to determine if amount of IOP lowering could be maximized (Fig. 5B, C, Table 2). Application of increasing amounts of the SA9000–cysteine adduct resulted in dose-dependent reductions in IOP (Table 2). Concurrently, the amount of hyperemia increased with increasing concentrations of the SA9000–cysteine adduct dosed. Nevertheless, the hyperemia noted even with the highest concentration (0.9%) of the SA9000–cysteine adduct tested was significantly less than that seen following administration of the unadducted drug at 0.3% (0.75+ vs. 3.5+). Furthermore, consistent with previous *in vitro* and *in vivo* data, the amount of

FIG. 4. Ocular distribution of SA9000–cysteine adduct. High performance liquid chromatography (HPLC) spectrums derived from (A) aqueous humor following topical administration of saline or (B) conjunctiva–sclera and (C) aqueous humor following administration of SA9000–cysteine adduct (0.6%) in New Zealand White rabbits. Asterisks denote peaks representing SA9000–cysteine adduct. Double asterisks denote peaks representing SA9000.

TABLE 1. SA9000 AND SA9000-CYSTEINE DRUG CONCENTRATIONS IN VARIOUS OCULAR TISSUES 1 h FOLLOWING ADMINISTRATION OF 0.6% OF EITHER SA9000 OR SA9000-CYSTEINE IN DUTCH-BELTED RABBITS

Compound	Drug concentration in different ocular tissues			
	Aqueous humor ($\mu\text{g/mL}$)	Cornea (ng/mg)	Conjunctiva/sclera (ng/mg)	Iris-ciliary body (ng/mg)
SA9000	5.4 ± 1	30.6 ± 24	19.8 ± 13	3.7 ± 2
SA9000-cysteine	2.8 ± 1	10.3 ± 4	4.8 ± 1	1.7 ± 0.1

the SA9000-cysteine adduct (0.6–0.9%) required to significantly lower IOP was less than that previously reported for ECA-cysteine adducts (3–4%).¹⁴

Discussion

Medications applied topically to the eye typically experience poor ocular bioavailability, with <1% of the dose applied penetrating the cornea into the anterior chamber.^{22,23} The majority of the applied topical dose is often lost to drainage into the nasolacrimal system or systemic absorption into the well-vascularized conjunctiva often necessitating the administration of high drug concentrations. For promising yet irritating drugs such as ECA, requirement of these large topical concentrations can lead to unacceptable toxicities. Consequently, efforts have been underway to produce new ECA analogs that may more potently reduce IOP with fewer ocular side effects. The ECA analog, SA9000, has demonstrated promise as a potentially potent and safe ocular hypotensive both *in vitro* and *in vivo*. Consistent with its ability to alter TM cells, intracameral injection of SA9000 in cats and monkeys has demonstrated superior potency with fewer deleterious effects on endothelial cells. The experiments described in this article were conducted in order to assess the feasibility of SA9000 or a prodrug equivalent as an eyedrop medication.

In vitro experiments conducted in two-diffusion chambers confirmed that ECA possesses relatively poor corneal penetration consistent with the large topical concentrations (3–4%) of the drug required to elicit *in vivo* effects on IOP following topical administration (Fig. 2). Conversely, similar experiments indicated that SA9000 possessed superior corneal penetration as compared to ECA. Consequently, in addition to the superior *in vitro* potency to ECA reported

with SA9000, we postulated that this enhanced ocular penetration would result in better IOP reductions at lower topical concentrations. Indeed, a single dose of SA9000 (0.3%) resulted in dramatic reductions in IOP that persisted for 3 days. Nevertheless, even at this lower concentration the SA9000 resulted in significant ocular irritations including hyperemia and conjunctival chemosis. This ocular irritation was most likely due to the ability of SA9000 to undergo retro-Michael additions to nontarget proteins. Interestingly, consistent with reported data detailing its lack of activity with the Na^+/K^+ cotransport inhibitor, SA9000 produced no corneal edema following topical administration.

Previously, Tingey and colleagues¹⁴ have demonstrated that cysteine adduction of ECA can reduce ocular toxicity while maintaining IOP-reducing capacity following topical administration. The authors postulated that a cysteine adduct of ECA could penetrate the cornea without binding to secondary thiol groups. Furthermore, once in the anterior chamber, the ECA-cysteine adduct might dissociate to the free drug via a retro-Michael reaction. We theorized that such an approach might be feasible in order to enhance the ocular tolerability while maintaining the pressure-lowering capabilities of SA9000 (Fig. 1). Indeed, the administration of the SA9000-cysteine adduct (0.6%) to NZW rabbits resulted in significantly reduced ocular irritation compared to the administration of SA9000 alone (data not shown). Subsequently, the pharmacokinetics of the SA9000-cysteine adduct versus unadducted SA9000 were studied in order to determine what effect cysteine adduction might have on ocular distribution. Analysis of SA9000 distribution following ocular administration suggested that this lack of observed toxicity with the SA9000-cysteine adduct was the result of less extraocular distribution (Table 1). Consequently, we have

TABLE 2. PHARMACODYNAMICS OF SA9000-CYSTEINE ADDUCTS FOLLOWING TOPICAL ADMINISTRATION TO DUTCH-BELTED RABBITS FOR 7 DAYS

SA9000-cysteine	Pharmacodynamics of SA9000-cysteine			
	Maximum IOP change vs. untreated control	Change in IOP AUC_{0-152}	Time to maximum reduction in IOP vs. untreated control	Maximum ocular irritation scale 0–4+ (range)
0.3%	$1.8 \pm 0.4 \text{ mmHg}$ ($8.7 \pm 2\%$)	$160 \pm 50 \text{ mmHg.h}$	56 h	0+
0.6%	$3.0 \pm 1.1 \text{ mmHg}$ ($14.4 \pm 5\%$)	$360 \pm 53 \text{ mmHg.h}$	152 h	0.5+ (0–1.0)
0.9%	$4.8 \pm 0.9 \text{ mmHg}$ ($23.3 \pm 4.4\%$)	$560 \pm 83 \text{ mmHg.h}$	56 h	0.75+ (0–2.0)

FIG. 5. IOP reductions in Dutch-belted rabbits following the administration of SA9000-cysteine adducts. SA9000-cysteine was administered at concentrations of (A) 0.3%, (B) 0.6%, and (C) 0.9% to one eye (open circles) while the fellow eye was left untreated (closed circle). Data represent mean $\pm$ SEM (0.3% $n = 8$; 0.6% $n = 4$; 0.9% $n = 4$). Values were assessed via paired t -test at each time point. Single asterisks ($P < 0.05$), double asterisks ($P < 0.01$), and triple asterisks ($P < 0.001$) indicate significance.

postulated that the presence of an exogenous thiol donor such as cysteine may simply redirect the SA9000 from the extraocular tissue until the drug molecule can penetrate into the anterior chamber and its site of action. However, care must be applied to these data since single time points may not always be fully extrapolated to comprehensive pharmacokinetic profiles.

Conclusions

The ECA analog, SA9000, displays higher intrinsic corneal permeability than its parent drug. Furthermore, formulation and prodrug strategies such as thiol adduction are a viable option to improve ocular tolerability and bioavailability. Coupled with its observed increased IOP-reducing potency over ECA, SA9000 is a promising ocular hypotensive candidate for clinical use.

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Reprint Requests: Dr. Pratap Challa
Duke Eye Center
Box 3802 DUMC
Durham, NC 27710

E-mail: Chall001@mc.duke.edu