Effects of Novel Ethacrynic Acid Derivatives on Human Trabecular Meshwork Cell Shape, Actin Cytoskeletal Organization, and Transcellular Fluid Flow

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To determine efficacy and therapeutic index in the context of ocular hypotensive activity of the new ethacrynic acid (ECA) derivatives of the 8000 series (SA8248 and SA8389), 9000 series (SA9000, SA9622 and SA9995) and ticrynafen, we undertook a comparative evaluation of the dose-dependent effects of these compounds on human trabecular meshwork (HTM) cell shape, actin cytoskeletal organization, focal adhesions and transcellular fluid flow. Responses were either scored using an arbitrary scale of 1—5 or quantified. Compounds of the 9000 series (SA9995>SA9000>SA9622) were found to be 14— to 20-fold more potent than ECA, ticrynafen or analogs from the 8000 series (SA8389>SA8248) in terms of ability to induce cell shape alterations in HTM cells. Similarly, compounds of the 9000 series (SA9995>SA9622>SA9000) were found to be much stronger (2 to 20 fold) than ECA, ticrynafen or analogs of the 8000 series in terms of affecting decreases in actin stress fiber content in HTM cells. Analogs of the 9000 series (SA9622>SA9995>SA9000) were also observed to be 8 to 10 fold more potent than ECA (SA8389>ECA>SA8248) at eliciting decreases in cellular focal adhesions. Interestingly, analogs of the 9000 series (SA9000>S9622>SA9995) and SA8248 demonstrated a huge increase (by many folds) in transcellular fluid flow of HTM cell monolayers as compared to ECA and ticrynafen. Collectively, these analyses revealed that the structural modification of ECA improves its ocular hypotensive efficacy, indicating that the SA9000 series compounds might be promising novel ocular hypotensive drugs.

Key words ethacrynic acid; SA9000; trabecular meshwork cell; actin cytoskeletal organization; ocular pressure

Increased intraocular pressure (IOP) is a major risk factor for primary open angle glaucoma (POAG), and lowering IOP is currently the only treatment available for the management of POAG. The conventional route of aqueous humor outflow through trabecular meshwork (TM) and Schlemm’s canal (SC) is generally thought to be the major pathway for the drainage of aqueous humor from the eye. Impaired drainage through the conventional outflow pathway is believed to be responsible for the increased IOP in POAG. A balance between aqueous humor inflow by the ciliary body and its outflow through the TM determines IOP. Both TM and SC cells are thought to have important roles in the regulation of aqueous humor outflow through the conventional pathway. Since there is no single drug currently available for primary open angle glaucoma (POAG), and lowering IOP is the only available treatment for the management of POAG, the need for the development of novel ocular hypotensive drugs is evident. The contractile function, cell shape, and cell adhesion properties of TM and SC cells have been implicated extensively in modulation of aqueous humor outflow through the conventional pathway, and various pharmacological agents that induce changes in cell shape, increase actin depolymerization, and decrease focal adhesions (cell–ECM interaction) have been demonstrated to consistently increase aqueous humor outflow facility in vitro perfusion models of human and porcine enucleated eyes and in living primates. Ethacrynic acid (ECA), a sulphydryl reactive diuretic and a Na+/K+/Cl− co-transporter inhibitor, has been demonstrated to lower IOP in living animals, including humans, and perfusion of ECA in enucleated eyes has been reported to increase aqueous humor outflow facility through the conventional outflow pathway.

In cultured TM cells, ECA induces changes in cell shape and decreases actin stress fibers and focal adhesions. Such changes are thought to influence the permeability barrier function of SC cells, the geometry of TM tissue, and ultimately, increase aqueous outflow facility. Interestingly, the non-sulphydryl reactive phenoxyacetic acid ticrynafen, has also been shown to increase aqueous humor outflow facility by affecting cell morphology and actin cytoskeletal organization. Although a pilot study on intracameral injection of ECA in human eyes with chronic open-angle glaucoma demonstrated both efficacy and safety, higher concentrations of the drug in nonhuman primates were found to potentially produce a reversible focal corneal edema. Therefore, there is a need for new derivatives of ECA with greater ocular safety and greater corneal penetration potential.

With this goal in mind, we have produced various derivatives of ECA by structural modification of phenoxyacetic acid and acryloyl moieties. In this study we evaluated the effects of such ECA derivatives on human trabecular meshwork (HTM) cell shape, actin cytoskeletal integrity and transcellular fluid flow as morphological correlates of ocular hypotensive efficacy and therapeutic index.

MATERIALS AND METHODS

Cell Cultures Primary cultures of HTM cells were isolated from cadaver donor eyes (obtained from the National Disease Research Interchange, Philadelphia, PA, U.S.A.) by collagenase IV digestion, as described previously by us. Cells were cultured at 37 °C under 5% CO2, in Dulbecco’s...
modified Eagle medium (DMEM, Gibco BRL, Gaithersburg, MD, U.S.A.) containing 10% fetal bovine serum, penicillin (100 Units/ml) and streptomycin (100 μg/ml). All experiments were conducted using several independent replicates with minimum of three.

**Drug Treatments** Stock solutions of different drugs were freshly prepared in dimethyl sulfoxide (DMSO). ECA was obtained from Sigma/Aldrich Chemical Co (St. Louis, MO, U.S.A.). Drug effects were evaluated in the absence of serum. Final concentration of DMSO in cell culture media was less than 1%.

**Cell Shape Changes** HTM cells were grown to confluence on gelatin (2%)-coated glass coverslips in complete medium and washed twice with serum-free media prior to initiating drug treatment. Cells were evaluated periodically for the first hour following addition of a drug to score for morphological changes using a Zeiss phase contrast microscope. Results were recorded photographically, using Kodak black and white film. Cell shape changes and all other analyses were carried out using concentrations of a drug ranging between 0.05 to 500 μM.

**Changes in Actin Cytoskeletal Organization and Focal Adhesions** For evaluating changes in actin cytoskeletal staining, cells grown to confluence on gelatin-coated glass coverslips were washed twice with serum-free media before treatment with the different ECA analogs for 1 h. Cells were fixed with 3.7% paraformaldehyde in PBS buffer at room temperature and permeabilized with 0.1% Triton X-100 as we described earlier.11) Cells were stained for actin with rhodamine-phalloidin, and focal adhesions were immunostained with a monoclonal primary antibody raised against paxillin (Sigma-Aldrich, St. Louis, MO, U.S.A.), followed by a TRITC (tetramethylrhodamine isothiocyanate)-conjugated secondary antibody. Micrographs were recorded using a Zeiss Axioplan-II fluorescence microscope. Images were recorded from five different positions (with respect to the coverslips) in all cases and stored on a Macintosh computer using Scion Imaging software.

**Hydraulic Conductivity Measurement** HTM cells (5.0×10⁶ cells/cm²) were seeded onto permeable methylcellulose filters (0.45-mm pores; catalog no. PIHA01250, Millipore, Bedford, MA, U.S.A.) for hydraulic conductivity (HC) measurements. When cells formed confluent monolayers (~day 14), the fetal bovine serum concentration was decreased to 10%, and the cells were fed every second day with fresh media. When the cultured cells reach their most resistive state, generally two weeks after becoming confluent, HC measures 1.0 to 3.0-μl/min/mmHg/cm². At this time, perfusion experiments using serum-free conditions were carried out according to protocols described in our earlier studies.21,22) Non-leaky preparations (i.e., HC 1 μl/min/mmHg/cm²) were chosen based on their baseline HC measurements made during the first 30 min. Multiple sets of HTM preparations were either treated in media without serum, with ECA or different derivatives of ECA, or with ticrynafen at different concentrations ranging from 0.01 μM to 500 μM. The threshold concentration for different drugs was determined. Two perfusions were carried out in sequence. During the first perfusion, which lasted for 30 min, the HTM cell preparations were perfused with media without serum. At the end of the first perfusion, the preparations were disconnected from the flow apparatus, the media were exchanged with new media without serum, with or without ECA or ECA derivatives, and ticrynafen at different concentrations. The second perfusion lasted for 90 min, and the resultant peak HC was plotted as a function of the concentration of a given agent and compared with controls that received no ECA derivatives in media without serum. Flow (Q) was calculated on the basis of measurements obtained from two pressure gauges (P1 and P2) in series with a piece of tubing of known resistance (“resistor”) and cell monolayers. HC was derived from the following equation: HC=Q/P2/A, where A is surface area of the monolayer.

**Cell Viability and Cytotoxicity** To evaluate the effects of ECA analogs on viability, HTM cells were grown to confluence on gelatin coated glass coverslips. Following drug treatment, cells were rinsed twice with PBS and treated with fluorescein diacetate and propidium iodide for 10 min. Viable cells and dead/damaged cells, which stain green and red respectively, were examined under a fluorescence microscope and photographed using Kodak color film.

**Reversibility of Drug-Induced Effects on Cell Shape and Actin Stress Fibers** To determine the reversibility of ECA analog-induced effects on cell shape and actin cytoskeletal changes upon drug withdrawal from the media, initially confluent HTM cultures grown on coverslips were treated with desired drugs for one hour in the absence of serum. After 1 h, phase contrast pictures were recorded and one set of coverslips fixed for actin staining. The second set of samples was washed thrice with media and then cultured for a further 24 h in 10% serum containing culture medium minus the drug. Cells were first photographed with a phase contrast microscope and fixed and stained for actin stress fibers with rhodamine-phalloidin. These experiments were carried out only with the 9000 series compounds along with ECA. The results of drug withdrawal were compared with drug-induced changes as well as with control untreated samples.

**Arbitrary Scales for Grading the Relative Effects of Individual Drugs** To determine the relative effects of different ECA analogs on HTM cell shape, actin cytoskeletal organization, focal adhesions and cell viability, we treated the cells with different compounds in a dose dependent manner and recorded their effects photographically by two independent persons. After examining photographically recorded data on cell shape changes, actin staining, focal adhesions, and cytotoxicity profiles of the different drugs under study, we developed the templates for each parameter with an arbitrary scale of 0—5 to compare the relative effects of individual drugs. The scale of 0 to 5 corresponds with a “no effect” to “maximum effect” for any given parameter in this study.

The ratio of the drug concentration required to induce scale 1 cytotoxicity to that required to induce a scale 1 morphological response (cell shape change) or actin cytoskeletal response (decrease in actin stress fibers or focal adhesions), was used to assess the compound(s) safety margin and identify the candidate possessing the “best fit” profile—i.e., a profile characterized by low toxicity and high efficacy as assessed by cell morphological and cytoskeletal responses.
RESULTS AND DISCUSSION

In this study we screened ECA and new derivatives of ECA for efficacy and safety margin of their ocular hypotensive activity. ECA, a clinically used diuretic drug, exhibits ocular hypotensive activity both in in vitro perfusion models and in living primates including humans.\(^{15—17}\) However, at higher concentration, it has been found to cause corneal edema.\(^{18}\) Therefore, a number of derivative compounds produced by modification of phenoxyacetic acid and acrylyol moieties (Table 1) were evaluated for efficacy based on their effects on TM cell morphology, actin cytoskeletal organization, focal adhesions and transcellular fluid flow.

It has been extensively documented that the cell morphology and actomyosin organization of TM cells influence aqueous humor outflow facility.\(^{5—14}\) ECA and inhibitors of Rho kinase and Myosin Light Chain Kinase, and cytochalasin D and latrunculin-induced increases in aqueous outflow facility have been shown to correlate well with alterations in cell shape and decreased actin stress fibers and focal adhesions.\(^{5—14}\) Therefore, we evaluated the effects of ECA derivatives on TM cell shape, actin cytoskeletal organization, focal adhesions, transcellular fluid flow, and cell viability on a comparative basis with the intention of evaluating their low toxicity and high efficacy as assessed by cell morphological and cytoskeletal responses.

Arbitrary grading scales (with no effect to maximum effect) were generated to score the relative effects of ECA derivatives on HTM cell shape (Fig. 1), actin cytoskeletal organization (Fig. 2), focal adhesions (Fig. 3), and cytotoxicity (Fig. 4), respectively. These templates were generated based on ECA derivative-induced changes in HTM cells. The 9000 series compounds (SA9622, SA9995, and SA9000) induced a +1 change in HTM cell shape change at a concentration of 3—4 \(\mu M\), while analogs of the 8000 series, ECA, and ticrynafen required concentrations 25—100 \(\mu M\) to achieve a similar degree of effect. Concentrations of 5—10 \(\mu M\) for the 9000 series analogs, and 100—200 \(\mu M\) for SA8248, SA8389, ECA, or ticrynafen, were required to induce a +2 change in HTM cell shape. Table 2 shows the individual grading values for the different ECA analogs.

The 9000 series compounds (SA9622, SA9995 and SA9000) induced loss of actin stress fibers at a concentration of 2—5 \(\mu M\) (scale =−2, with the minus sign denoting a decrease in the parameter under study), while concentrations of 10 \(\mu M\) and 85—110 \(\mu M\) were required for ECA and analogs of the 8000 series and ticrynafen, respectively to affect the same change. SA9622, SA9995 and SA9000 were found to be more potent than ECA, SA8389, SA8248, and ticrynafen (SA9622>SA9995>SA9000>ECA), at decreasing the actin stress fiber content of HTM cells (Fig. 5). Interestingly, compound SA9622 exhibited a biphasic effect on actin staining, with 0—5 \(\mu M\) inducing a dose-dependent decrease in actin stress fibers and higher concentrations being less effective (Fig. 5). At a concentration of 2—3.5 \(\mu M\), compounds of the 9000 series induced a loss of focal adhesions to a scale of +2 (SA9622>SA9995 and SA9000). On the other hand, SA8389, SA8248, ECA, and ticrynafen required a wide (7—50 \(\mu M\)) but much higher range of concentration than analogs of the 9000 series (order of potency: SA8389>ECA>SA8248 and ticrynafen; Fig. 6). Based on the changes in cell

### Table 1. Chemical Structures of the ECA Derivatives Used in This Study

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Structure</th>
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<tbody>
<tr>
<td>ECA (sulfhydryl reactive)</td>
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<tr>
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<td><img src="image2" alt="SA9000" /></td>
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<tr>
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<tr>
<td>SA8389</td>
<td><img src="image5" alt="SA8389" /></td>
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<tr>
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<td><img src="image6" alt="SA8248" /></td>
</tr>
<tr>
<td>Ticrynafen (non-sulfhydryl reactive)</td>
<td><img src="image7" alt="Ticrynafen" /></td>
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</tbody>
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Fig. 1. ECA Analogs Induced Morphological Changes in HTM Cells

Confluent cultures of HTM cells grown on gelatin coated glass coverslips were treated with different ECA analogs at 37° C for 1 h in the absence of serum. After 1-h drug treatment, cells were photographed for morphological changes using a Zeiss phase contrast microscope. To enable comparative analysis of ECA analogs-induced changes in HTM cell shape, we have selected examples of photographs representing different degree of morphological changes as shown in this figure. The scale of +1 to +3 indicates increasing degree of changes in cell shape. Control cells were shown with (−). This is a standard template used to score the effects induced by all ECA analogs.
HTM cells were treated with different ECA analogs for 1 h at 37 °C under serum free condition, and subsequently fixed and stained for F-actin with rhodamine-conjugated phalloidin. Changes in actin stress fibers were recorded using a Zeiss Axioplan-II fluorescence microscope. Representative templates have been shown to depict the loss of actin stress fibers caused by different ECA analogs. Compared to untreated control cells (0), the scale of 1 to 4 depicts increasing degree of loss of actin stress fibers in drug treated samples. This is a standard template used to score the effects induced by all ECA analogs.

Table 2. Dose-Dependent Effects of ECA Derivatives on HTM Cell Shape

<table>
<thead>
<tr>
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<th>0.5</th>
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Grading was done using the templates described in Materials and Methods based on drug-induced changes in cell morphology. Values are the mean of three independent experiments.
shape (Table 2), actin cytoskeletal organization (Fig. 5) and focal adhesions (Fig. 6) induced by different ECA derivatives tested in this study, the 9000 series compounds, including SA9622, SA9995, and SA9000 consistently demonstrated a much better or higher efficacy than ECA, SA8339, SA8248, and ticrynafen.

The endothelial monolayer lining of the inner wall of SC is thought to play an important role in maintaining resistance to aqueous humor flow through the conventional route.3,4) Further, modulation of SC cell junctions, morphology, and contractile characteristics have been reported to influence HC of SC cells.21,22) Since ECA is known to influence cell morphology and actomyosin organization, we have quantitatively determined the effects of ECA derivatives on transcellular fluid flow of HTM monolayers maintained on permeable methylcellulose membranes. Figure 7 depicts ECA and ECA derivative-induced changes in HTM cell transcellular fluid flow. The 9000 series of compounds and SA8248 exhibit a dose-dependent ability to enhance HC, with the 9000 series ECA derivatives increasing transcellular fluid flow (HC) at concentrations ranging $1 \times 10^{-13}$ to $5 \times 10^{-8}$ M. ECA showed a marginal change in HC at $5.7 \times 10^{-6}$ M, but at a higher concentration ($2 \times 10^{-4}$ M), it induced increased HC markedly (Fig. 7). ECA showed no effect on hydraulic conductivity of TM monolayers at the drug concentration of 1 nM; however, the 9000 series and SA8248 increased HC by several fold. Among various 9000 series of compounds tested, SA9995 demonstrated the strongest effect on HC, followed by SA9622 and SA9000 at $1 \times 10^{-11}$ M (Fig. 7). Interestingly, the SA8248 compound also increased transcellular fluid flow, and its response was found to be as good as (Fig. 7), or even better than, the 9000 series compounds, particularly at lower concentration ($1 \times 10^{-11}$ M) (Fig. 7). Ticrynafen effects on HC tested at different concentrations, ranging $5.7 \times 10^{-6}$, $5.7 \times 10^{-7}$ and $2 \times 10^{-4}$ M, were found to be slightly, but consistently lower, than the ECA-induced changes by 25 to 40%
However, collectively, ECA derivatives exhibited very strong effects on transcellular fluid flow, demonstrating that structural modification of the parent molecule, especially derivatives based on modification of the phenoxyacetate moiety, generates compounds with better efficacy than ECA per se. Among different parameters utilized in this study to evaluate the relative potency of ECA derivatives including cell shape, actin cytoskeletal organization, focal adhesions and HC, the HC assay appeared to be very sensitive and revealed a dramatic difference between ECA and ECA
derivative-induced response.

SA9962 (3.5 μM) and SA9995 (5 μM) induced cytotoxicity at the +1 scale, while SA8389 and SA9000 required 20 and 30 μM, respectively, to cause the same degree of cytotoxicity. On the other hand, SA8248 (50 μM), ECA (130 μM) and ticrynafen (175 μM) were found to be much less toxic to HTC cells (Fig. 8). Changes in cell shape and actin stress fibers induced at a 5 μM concentration of the 9000 series of compounds (SA9000, SA9995, and SA9962), were found to be reversible within 24 h of drug withdrawal. Reversibility of effects noted in response to higher concentration of drugs has not yet been analyzed.

Since we do not have robust quantitative methods to determine changes in cell morphology, actin stress fibers, and focal adhesions, we utilized a grading system in this study that scores drug-induced changes on a relative basis ranging from marginal to strong effects (Figs. 1—4). Based on the ratio of the drug concentration required to produce a scale 1 cytotoxic response to that required to induce a cell shape change at the scale 1 level, SA9000 and ECA (SA9000->ECA) were found to have a broader safety margin or therapeutic index when compared to all other compounds evaluated in this study (Table 3). The data obtained from morphological change reveals a higher efficacy and broader safety margin for SA9000, corroborating very well with our earlier independent work carried out in Santen Laboratories in Japan, in which similar results were documented using primary TM derived from bovine species. 23) Based on the ratio of cytotoxicity/loss of actin stress fibers, ECA, SA9000, and SA8248 were found to exhibit a better safety margin.
(ECA > SA9000 > SA8248) than the rest of the compounds. In contrast, the values of ratios of toxicity/focal adhesions suggest that ECA and SA8248 possess a better safety margin than SA9000 (Table 3). Interestingly, when we compared the safety margin data based on scale 2 values of cytotoxicity, cell morphology and actin cytoskeletal changes, the SA9000 compound revealed an even much higher safety margin than ECA (data not shown).

These analyses collectively indicate that the structural modification of ECA influences the efficacy of the compound. Compounds of the 9000 series in particular—SA9622, SA9995, and SA9000—exhibit better efficacy than the parent compound, ECA. The safety margin, based on the effects of the ECA analogs on cell shape, actin cytoskeletal organization, focal adhesions and cytotoxicity reveals that SA9000 and ECA exhibit a similar response. Interestingly, in accordance with these observations, SA9000 has been shown to maximally decrease IOP with minimal side effects in living cats and monkeys, compared to ECA, as determined by intracameral injection or by topical application.\(^2\) Therefore, SA9000 appears to be a promising novel ocular hypotensive drug worthy of further investigation per se. Modification of SA9000 should also afford enormous pharmacological potential in terms of generating derivatives that lend themselves to structure-function analysis in the context of aqueous outflow function.

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## REFERENCES