Topical Formulations of Novel Angiostatic Steroids Inhibit Rabbit Corneal Neovascularization

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Purpose. To evaluate the antiangiogenic potential of topical ophthalmic formulations of the novel angiostatic steroids AL-3789 and AL-4940, using a rabbit model of corneal neovascularization.

Methods. Neovascularization was induced in the rabbit cornea by surgical implantation of a standard ethylene vinyl acetate copolymer (Elvax-40) pellet containing 1 μg lipopolysaccharide. Coded formulations of the control vehicle or the following test agents were administered in prevention and intervention treatment protocols: 1% formulations of AL-3789, AL-4940, and cortisol acetate as a positive drug control. Three doses of AL-3789 (0.01%, 0.1%, and 1%) were also evaluated in a prevention treatment protocol. Corneal responses were monitored throughout a 2-week treatment period, and 1 week after the last treatment dose. Observations included quantitative measurement of the area of new blood vessel growth and qualitative assessment of cellular infiltrate and edema. All treatments and observations were performed in a double-masked manner.

Results. All tested formulations, except the vehicle and the 0.01% AL-3789 preparation, significantly inhibited corneal neovascularization and other lipopolysaccharide-induced responses in the various treatment protocols employed. AL-4940, the free alcohol form of AL-3789, was slightly less effective than cortisol acetate or AL-3789. The extent of inhibition of the angiogenic response by the 1% and 0.1% AL-3789 suspensions ranged from 76% to 100% 1 week after the last treatment.


Angiogenesis, or neovascularization, the formation and growth of new blood capillaries from preexisting vessels, is associated with embryonic development, tumor growth, the reproductive cycle in women, and wound repair.1–3 Angiogenesis is a necessary stage of the wound-healing process and is regulated by the opposing actions of physiologic angiogenic and antiangiogenic factors.4–12 However, chronic and persistent activation of the angiogenic response after the constant release–production of stimulating factors and inappropriate local tissue responses to the primary insult lead to pathologic neovascularization.3,7

Many chronic ocular diseases are associated with the development of a persistent angiogenic stimulus. The development of new blood vessels compromises the structural and functional integrity of the eye and contributes to visual impairment. Ocular diseases and insults with an underlying angiogenic process are major causes of blindness worldwide and include proliferative diabetic retinopathy, retinopathy of prematurity, age-related macular degeneration, central retinal vein occlusion, neovascular glaucoma, trauma, corneal graft rejection, and chronic inflammation.13 Therefore, effective drugs that could specifically delay, arrest, or prevent the proliferation of new capillaries in the “adult” ocular tissues, with no or minimal side effects, are needed. An antiangiogenic drug could also
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be employed as adjunctive or second-line therapy to surgical procedures for various types of ocular neovascularization. Several distinct classes of inhibitors of neovascularization in various models have been described. However, results of controlled clinical trials with these agents are still awaited. The diversity of biologic activities involved in angiogenesis reinforces the earlier postulation that this process is a very complex cascade of events and also emphasizes the potential for effective inhibition of this process at multiple points of the cascade, once it is in progress.

The purposes of the current investigation were to evaluate in well-controlled studies the efficacy and potency of two novel angiostatic steroid structures, AL-3789 and AL-4940, as inhibitors of lipopolysaccharide (LPS)-induced angiogenesis in the rabbit cornea. Our data demonstrate that a 1% AL-3789 topical ophthalmic formulation, administered to the eye 2 to 4 times daily prevented corneal neovascularization in this model and also intervened effectively in the early phases of the response after the angiogenic process had been initiated.

MATERIALS AND METHODS

Angiogenic Stimulant

A standard angiogenic stimulus for the entire study was achieved using lipopolysaccharide-LPS–WS typhimurium (Difco Laboratories, Detroit, MI) and ethylene vinyl acetate copolymer (Elvax-40, Alza, Boston, MA) as a slow-release device. Five milligrams of lyophilized LPS were sequestered into 0.5 ml of Elvax-40 casting solution, thoroughly mixed, and allowed to dry under a laminar-flow hood to form a film of polymer. Individual implants of Elvax-40 were prepared using a grid under the magnification of a microscope; the implants were cut to a uniform size, corresponding to 1 μg LPS sequestered in each implant. From a group of implants prepared at the same time, implants were randomly selected for insertion into the rabbit corneas.

Preparation of the Slow-Release Polymer

Fifty grams of polyvinyl acetate copolymer, purchased as dry beads, was extensively washed in 3 l of absolute alcohol for 20 days, with a change in the washing solution every day. On day 21, 1 g of the washed beads was dissolved in 10 ml of methylene chloride to obtain a 10% solution of Elvax-40. This preparation, to be used as a casting solution for sequestering the lyophilized LPS, was tested first for its biocompatibility in the absence of LPS as follows: Ten implants of the Elvax-40 preparation were inserted intrastromally into the cornea in 10 rabbit eyes. In each eye, the implant was positioned 2.5 mm from the limbus and the corneal reaction monitored daily for 14 days after implantation. If any of the 10 implants induced the slightest clinical or histologic reaction in the rabbit cornea, the casting-stock solution of Elvax-40 was discarded. In this case, further washing of the polyvinyl acetate beads was carried out for 10 additional days, and a new casting solution was prepared and retested, as described earlier. A casting-stock solution was eligible for routine use in experiments only when all 10 test implants remained absolutely inert during the 14-day examination period. The same casting solution was then used throughout the study.

Animals

Albino male and female rabbits weighing 3 to 4 kg were obtained from a local breeder. The ARVO Statement for the Use of Animals in Ophthalmic and Vision Research was followed. The rabbits were anesthetized by intravenous injection of 0.5 ml/kg 1.5% thiopental sodium into the marginal ear vein. Before surgery, two drops of Benoxinate 0.2% (Oxybuprocaine HCl, Novesine, Hadassah, Jerusalem) were instilled for additional (local) anesthesia of the cornea.

The In Vivo Model

The preparation of the implants, the surgical technique, testing, and recording of the corneal reactions (edema, infiltrate, and neovascularization) were carried out as published previously. Each rabbit in the study had a single implant in one eye. For precision, the surgical implantation of the LPS-sequestering pellets was carried out under the high magnification of a surgical microscope. During the surgery, the cornea was irrigated with 0.5% chloramphenicol eye drops. The animals were assigned at random to different groups (four to six rabbits per group) for treatment with coded formulations, as described later. After instillation of the first drop of the assigned treatment, the surgical eyes received a prophylactic treatment consisting of 1 drop 1% atropine sulfate and 5% chloramphenicol ophthalmic ointment both applied daily for the duration of the study.

Tested Compounds

The formulations included 0.01%, 0.1%, and 1% AL-3789; 1% AL-4940 (the free alcohol form of AL-3789); 1% cortisol acetate; and the common proprietary topical ophthalmic vehicle. All formulations and the vehicle were assigned unique codes. The study was performed in a masked manner: The investigators involved in the surgery and evaluation of the corneal reaction did not prepare, or know the identities of, the formulations until after the study was completed and the data were analyzed. The chemical structures of the tested compounds are shown in Figure 1. AL-3789 and AL-4940 were synthesized at Alcon Labora-
Cortisol Acetate

AL-3789

AL-4940

FIGURE 1. Chemical structures of cortisol acetate, AL-3789, and AL-4940.

The laboratories (Ft. Worth, TX) and their chemical structures verified by elemental analysis, nuclear magnetic resonance (NMR) and mass spectroscopy. Cortisol acetate was purchased from Sigma (St. Louis, MO). In this set of experiments, the cortisol acetate formulation was included as a positive inhibitory control on the basis of angiostatic activity previously demonstrated for the Na+ hemisuccinate ester of cortisol (hydrocortisone) evaluated in an Elvax-40 implant in this model.21 A phosphate buffered saline solution was also coded and included as a negative treatment control for direct comparison with the vehicle control suspension. The experiment was designed to evaluate the relative efficacy of the 1% coded formulations at dose frequencies of 2 or 4 drops daily, employing prevention treatment and intervention treatment strategies; and to determine whether 0.01%, 0.1%, and 1% AL-3789 formulations produced dose-dependent inhibition of the neovascularization response in a prevention Treatment protocol with a dose frequency of 2 drops daily. Treatment with the test formulations was initiated on the day of LPS implantation (prevention treatment protocol) or 2 days after LPS implantation (intervention treatment protocol). To replicate the treatment of human patients as closely as possible, the formulations were prepared in a standard droptainer (drop volume approximately 40 µl), and each treatment consisted of 1 drop administered at two or four approximately equal time intervals during a 24-hour period.

In all studies, the coded compounds were adminis-

TABLE I. Summary of Studies Conducted to Evaluate the Effects of 1% Topical Ophthalmic Formulations of AL-3789, AL-4940, and Cortisol Acetate on Lipopolysaccharide-Induced Rabbit Corneal Neovascularization

<table>
<thead>
<tr>
<th>Study Number</th>
<th>Treatment Protocol</th>
<th>Drops (per day)</th>
<th>Formulation*</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prevention</td>
<td>4</td>
<td>1% AL-3789</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1% AL-4940</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1% Cortisol acetate</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vehicle</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Prevention</td>
<td>2</td>
<td>1% AL-3789</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1% AL-4940</td>
<td>6</td>
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<tr>
<td></td>
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<td></td>
<td>1% Cortisol acetate</td>
<td>6</td>
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<td></td>
<td></td>
<td></td>
<td>Vehicle</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Intervention</td>
<td>4</td>
<td>1% AL-3789</td>
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<td>1% AL-4940</td>
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<td></td>
<td></td>
<td></td>
<td>1% Cortisol acetate</td>
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<td>Vehicle</td>
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<tr>
<td>4</td>
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<td>1% Cortisol acetate</td>
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<td></td>
<td></td>
<td></td>
<td>Vehicle</td>
<td>6</td>
</tr>
</tbody>
</table>

* All formulations were coded. Each study also included a separate control group treated with buffered saline solution (also coded) according to the respective dosing schedule.
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tered for 12 to 15 days. Monitoring of the corneal reactions, however, was extended for another week, for a total study duration of 3 weeks. All procedures and observations were conducted in a double-masked manner. The identities of the coded formulations were revealed only after the entire series of studies was completed and the data tabulated. The designs of the four experiments conducted with the set of 1% suspensions are summarized in Table 1.

Monitoring of Corneal Responses and Reactions In Vivo

After the implantation of the LPS-sequestering implants, the corneal responses were monitored under an operating microscope once every 24 hours, with random selection of animals for observation. The corneal responses evaluated included: corneal edema; intensity of cellular infiltrate into the cornea, observed clinically; and total area of new blood capillaries. Corneal edema and cellular infiltrate were graded, using an arbitrary scoring system that ranged from 0 (none) to 5 (severe). The surface of the neovascular response was obtained by multiplying the length of the leading vessel growing from the limbus toward the LPS implant by the length of the active base of new capillaries sprouting at the limbus and dividing the result by 2. Statistical significance of differences between group mean values was determined by use of the Student’s t-test comparison (SigmaStat Statistical Software, Jandel Scientific, San Rafael, CA).

RESULTS

Prevention Treatment Protocol With 1% Drug Formulations and Drug Vehicle

Figure 2 shows the time course of the corneal neovascularization response for each of the tested formulations (1% AL-3789, 1% AL-4940, 1% cortisol acetate, and drug vehicle), administered as 4 drops daily in the prevention protocol (study 1). In the control group, which received buffered saline eye drops, florid corneal neovascularization developed, achieving a maximum area of new blood vessels within 10 days after LPS implantation. In this experiment the surface of neovascular response remained near this maximum level (45 to 50 mm²) for the remainder of the 22-day study. The angiogenic response of eyes treated with vehicle or of eyes treated with normal saline was identical until day 10; thereafter, the extent of corneal blood vessels of the vehicle-treated group decreased slowly until the end of the study. During the 2-week treatment period, AL-3789 and cortisol acetate completely inhibited the LPS-induced neovascularization (P < 0.0005 for the AL-3789 and cortisol acetate-treated groups compared with the vehicle-treated group at day 15). The mean area of neovascularization of the

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933196/ on 04/27/2017)
FIGURE 3. Representative photographs of corneas 8 days after lipopolysaccharide implantation for each experimental group in the prevention treatment protocol, 4 × 1 drop daily. (A) demonstrates the progression of new blood vessels toward the implant in corneas treated with saline solution only. (B) demonstrates the extensive neovascular process in the corneas treated with vehicle suspension. Note also the marked leukocyte infiltrate and the corneal edema observed in these two photographs. (C) A mild angiogenic process with a localized edema of the cornea was observed when the corneas were treated with 1% AL-4940; a greater variability of response was observed among the individual experiments in this treatment group. No angiogenesis, edema, or cellular infiltrates were observed in the corneas treated with 1% cortisol acetate (D) or with 1% AL-3789 (E). An arrow shows the site of the lipopolysaccharide implant in each cornea.

Intervention Treatment Protocol With 1% Drug Formulations and Drug Vehicle

The 1% drug formulations and drug vehicle were subsequently evaluated in intervention treatment protocols according to both dose schedules used in the prevention protocols. The data in Figure 5 depict the results of the study (study 4) in which 2 drops of each coded formulation were administered daily to the eye, group). Four days after the last dose, a very small response of new capillaries appeared in the corneas of the AL-3789-treated group; however, the area of these vessels did not increase by the end of the study, 1 week after the final treatment. The group treated with 2 drops daily of 1% AL-4940 showed an initial delay in progression of neovascularization, relative to progression in the vehicle-treated control group, and eventual stabilization of the response 6 days after LPS implantation at a value approximately 15% of the area of the saline-treated control group. The corneal cellular infiltrate and edema scores of each treatment group (Figs. 4B, 4C) also responded in a manner qualitatively similar to the neovascularization response (Fig. 4A). Indeed, in all findings, the qualitative corneal responses consistently corroborated the quantitative neovascularization responses in all treatment groups (data not shown), similar to those shown in the data in Figure 4.
beginning 2 days after initiating the neovascularization response by LPS implantation. Very similar results (data not shown) were obtained in an intervention study with a treatment of 4 drops daily (study 3). At the time of drug intervention in both studies, the corneas displayed small but detectable neovascularization, with measurable edema and cellular infiltrate typical of this stage of the response. As shown in Figure 5, all three drug formulations could intervene very effectively in the neovascularization process ($P < 0.0001$ for each of the three drug-treated groups compared with the vehicle-treated group at day 13). At various times during this study, each of the three groups treated with a study drug exhibited minimally detectable neovascularization, which showed no consistent tendency to progress for up to 1 week after the last dose.

**Prevention Treatment Protocol With 0.01%, 0.1%, and 1% AL-3789 Formulations and Drug Vehicle**

On the basis of the results with the 1% formulation of AL-3789, a prevention treatment protocol was undertaken with coded 0.01%, 0.1% and 1% AL-3789 formulations. Two drops of the drug formulation, drug vehicle, or buffered saline solution were administered daily to eyes with the LPS corneal implant for a
AL-3789, administered topically to eyes of rabbits with

The data obtained from this series of studies have

DISCUSSION

The data obtained from this series of studies have
documented the efficacy of a new angiostatic agent,
AL-3789, administered topically to eyes of rabbits with
experimentally induced corneal neovascularization. The 1% AL-3789 topical ophthalmic formulation was
highly effective in prevention treatment and in inter-
vention treatment protocols. AL-3789 and its free alco-
hol form AL-4940 produced almost complete inhibi-
tion of corneal neovascularization in the most strin-
gent protocol of antiangiogenic activity investigated,
that is, the intervention study with a treatment fre-
cquency of only 2 drops of drug formulation adminis-
tered daily. In all treatment protocols, no significant
neovascularization developed in the corneas treated
with 1% AL-3789 by the end of the study—that is,
1 week after the last treatment, in contrast with the
somewhat variable posttreatment neovascularization
response observed with AL-4940. These data may indi-
cate a prolonged action of AL-3789 in the cornea.
Taken together, these results suggest that AL-3789 in
this ophthalmic formulation has properties that may
be advantageous for treatment of corneal neovascu-
larization.

Results of Michaelson’s earlier study,22 our own
observations,15,20 and those of others24–26 demonstrate
the angiostatic potential of various forms of cortisone,
cortisol (hydrocortisone), and tetrahydrocortisol (an
endogenous cortisol metabolite). In these experi-
ments, however, the steroid only partially inhibited
the neovascularization process. More recently, we re-
ported on the total inhibition of the LPS-induced cor-
neal angiogenic process using cortisol hemisuccinate
ester, Na+ salt (Solu-Cortef, Upjohn, Belgium) seques-
tered in Elvax-40 as a slow delivery system.21 In the
current study, 1% cortisol acetate in the proprietary
ophthalmic vehicle was efficacious as an antiangiog-
genic agent. These results and data from other studies
of corneal neovascularization in the rabbit model24,26
suggest that the chemical form of cortisol, together
with the specific route of delivery and ophthalmic for-
mulation, can significantly influence the ocular tissue
penetration and the therapeutic activity of this drug.
In addition, the cortisol structure may provide angios-
tatic activity that is additive to its glucocorticoid activ-
ity.

Glucocorticoids, with varying potency, in different
chemical forms (as exemplified by hydrocortisone-
cortisone), and in various ophthalmic preparations,
have been extensively used for treating a broad range
of ocular diseases.27 The beneficial effects of this class
of steroids are well-documented, but their clinical util-
ity in chronic ocular neovascularization conditions is
limited by unacceptable side effects, most notably ele-
vation of intraocular pressure (IOP).27–29 There is con-
siderable evidence that many glucocorticoids, even
those with low potency, can elevate IOP in susceptible
humans and in rabbits, if sufficient doses are adminis-
tered in a prolonged regimen.27–29 However, there is
preliminary experimental evidence that AL-3789 does
not elevate IOP in rabbits and can antagonize the
dexamethasone-induced increase in IOP in rabbits.30
Consistent with these results, AL-3789 is devoid of anti-
inflammatory activity when tested in three models of
inflammation: carrageenan-induced footpad edema
in rats, endotoxin-induced uveitis in rabbits, and inhi-
bition of IL-1 production in cultured human U937
cells (manuscript in preparation). Additional studies
of the ocular effects and molecular pharmacology of

![Figure 6. Effects of various doses of AL-3789 on lipopolysac-
charide-induced rabbit corneal neovascularization: prevention treatment protocol, 2 X 1 drop daily. Treatment was ini-
tiated at the time of lipopolysaccharide implantation on day 0, with the last dose on day 14. Final observations were
made on day 22. Representative statistically significant differences in effect in eyes treated with each AL-3789 dose, com-
pared with that in vehicle-treated eyes, are shown in parenthesis: •, vehicle control; △, 0.01% AL-3789; ○, 0.1% AL-
3789 (b, P < 0.001; c, P < 0.005); ■, 1% AL-3789 (a, P < 0.02; β, P < 0.04). Data shown are mean area of neovascu-
larization (mm²) ± SEM, n = 4.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933196/)
AL-3789 are in progress, with recent findings that this compound can inhibit ocular angiogenesis in other models—a rat model of retinopathy of prematurity and a mouse model of intraocular tumor growth and angiogenesis. The ability of AL-3789 to inhibit neovascularization of the intraocular structures may have potential clinical relevance for treatment of proliferative diabetic retinopathy and age-related macular degeneration. The mechanism of action of AL-3789 or other angiostatic steroids has not been fully elucidated. Recent data from other laboratories have suggested that some angiostatic steroids can decrease urokinase-type plasminogen activator (u-PA) in vitro by several possible mechanisms. These results are consistent with modulation of proteolytic activities of vascular endothelial cells by those angiostatic steroids and suggest that some compounds of this class can prevent the limited proteolytic digestion of extracellular matrix that precedes the “budding” of new capillaries.

Angiostatic agents of diverse chemical structures, isolated from many sources or prepared synthetically, have been described. Unfortunately, there is no consensus on the most appropriate or most predictive model for evaluating inhibitors of neovascularization for therapeutic use in the human eye. Indeed, the models for evaluating inhibitors of corneal neovascularization in vivo vary greatly among laboratories, with regard to animal species, method of delivery of drug, assessment of response, choice of positive drug control, treatment regimen and duration, and other parameters. The ophthalmic formulations and clinically feasible treatment regimens used in our study resulted in a reproducible and effective inhibition of neovascularization by hydrocortisone acetate and AL-3789. The significance of these results is that AL-3789 is a prototype of steroid structures, which, on the basis of study results to date, may be devoid of the known ocular side effects induced by the classic glucocorticoids but preserve their antiangiogenic properties. In conclusion, the findings of this study that AL-3789 is an efficacious inhibitor of rabbit corneal neovascularization warrant further investigation of the potential clinical utility of this angiostatic steroid for treating ocular neovascular diseases.

Key Words
AL-3789 ophthalmic formulation, angiostatic steroid, lipopolysaccharide, neovascularization inhibitor, rabbit corneal neovascularization

References


