Effect of Ticrynafen on Aqueous Humor Dynamics in Monkeys

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Objective: To determine the effect of ticrynafen, a non–sulfhydryl-reactive compound similar to ethacrynic acid, on outflow facility in normotensive monkey eyes and on intraocular pressure (IOP) in monkey eyes with laser-induced glaucoma.

Methods: In normotensive eyes, facility (perfusion) was measured shortly before and after bolus or exchange intracameral infusion of ticrynafen or vehicle in opposite eyes, and 3.5 to 4.5 hours after 5 days of twice-daily 2% ticrynafen or vehicle ointment. In glaucomatous eyes, baseline and vehicle diurnal IOP curves were established, 2% ticrynafen ointment was given twice daily for 5 days, and IOP was measured immediately before and 0.5 to 6 hours after each morning treatment.

Results: In normotensive eyes, exchange 2-mL infusion of 0.2-, 1-, or 4-mmol/L ticrynafen increased facility by 33% ± 6% (mean ± SEM), 73% ± 18%, and 60% ± 11%, respectively. Day 5 posttreatment facility was higher in the ticrynafen group than in controls by 28% ± 9%. In glaucomatous eyes, maximum IOP decline, from approximately 35 mm Hg, was 7.5 ± 2.0 mm Hg on day 4 and 9.8 ± 2.4 mm Hg on day 5 of twice-daily ticrynafen treatment.

Conclusion: The facility-increasing, IOP-lowering action of ticrynafen, ethacrynic acid, and derivatives may not depend entirely on sulfhydryl reactivity.

Clinical Relevance: Whether such drugs as ethacrynic acid and ticrynafen prove valuable for glaucoma therapy, at the least they are useful probes to study aqueous outflow mechanisms.

MATERIALS AND METHODS

CYNOMOLGUS MONKEYS

Thirty-nine juvenile and adult cynomolgus monkeys (Macaca fascicularis) of both sexes were studied; 35 were bilaterally ocular normotensive with no biomicroscopically visible anterior chamber cells or flare or other ocular abnormalities, while 4 had stable unilateral ocular hypertension induced by repeated argon laser photocoagulation of the trabecular meshwork. All experiments were conducted in accordance with National Institutes of Health (Bethesda, Md) and institutional guidelines, and with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research.

ANESTHESIA

For applanation tonometry and administration of ticrynafen ointment, intramuscular ketamine hydrochloride, 10 to 12 mg/kg, supplemented at approximately 45 minute-intervals by 3 mg/kg, was used, supplemented in glaucomatous monkeys by topical 0.3% proparacaine hydrochloride. For anterior chamber perfusion, intramuscular ketamine hydrochloride, 10 mg/kg, was followed by intramuscular pentobarbital sodium, 35 mg/kg.

OUTFLOW FACILITY MEASUREMENT AND INTRACAMERAL DRUG DELIVERY

Total outflow facility was measured by 2-level constant-pressure (approximately 3 and 12 mm Hg above spontaneous IOP) perfusion of the anterior chamber (AC) with Barany mock aqueous humor. Two variations of the basic method were used. With a 1-needle technique (bolus intracameral infusion of drug and no drug in the reservoir), a second cannulation is avoided; however, the AC contents are not completely mixed or replaced, and drug concentration declines with a half-life of approximately 30 minutes to predrug baseline and adjusted for control eye washout increased significantly, by 33% ± 6% (0.2 mmol/L), 73% ± 18% (1 mmol/L), and 48% ± 11% or 60% ± 11% (4 mmol/L) (Table). In the group treated with 4 mmol/L ticrynafen, there were 2 animals in which the postexchange facilities in the control eyes nearly doubled compared with baseline, so that the facility increase in the control eyes averaged 30% ± 13% for 9 monkeys (Table). When these 2 animals were excluded, the perfusion-induced facility increase in the 7 remaining control eyes was a more typical 14% ± 8% and the facility increase in the contralateral ticrynafen-treated eyes remained at approximately 80%, so that the ticrynafen-vehicle ratios for postdrug facility and postdrug-predrug facility increased to 1.48 and 1.60, respectively (Table).

Protocol 3

Average pretreatment IOP was 13.8 mm Hg in both eyes (Figure 2). The IOP in the control eyes 18 and 19 hours after the afternoon day 4 treatment (ie, immediately before and 1 hour after the day 5 morning treatment) was essentially identical to day 1 pretreatment baseline, but decreased by approximately 3 mm Hg at 3 hours on treatment day 5. The IOP in the ticrynafen-treated eyes averaged 1 to 1.5 mm Hg less to predrug baseline and adjusted for control eye washout increased significantly, by 33% ± 6% (0.2 mmol/L), 73% ± 18% (1 mmol/L), and 48% ± 11% or 60% ± 11% (4 mmol/L) (Table). In the group treated with 4 mmol/L ticrynafen, there were 2 animals in which the postexchange facilities in the control eyes nearly doubled compared with baseline, so that the facility increase in the control eyes averaged 30% ± 13% for 9 monkeys (Table). When these 2 animals were excluded, the perfusion-induced facility increase in the 7 remaining control eyes was a more typical 14% ± 8% and the facility increase in the contralateral ticrynafen-treated eyes remained at approximately 80%, so that the ticrynafen-vehicle ratios for postdrug facility and postdrug-predrug facility increased to 1.48 and 1.60, respectively (Table).

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2.5 mmol/L. For 1 monkey in group A and all 5 monkeys in group B, 82.8 mg of ticrynafen and 60.6 mg of TRIS (base) were dissolved in 8.0 mL of purified water, the pH adjusted to 7.0 with 1N hydrochloric acid, and then further diluted with purified water to 10 mL. The solution was then filtered through a 0.2-µm nylon or acetate filter. The vehicle solution was prepared identically, except that 20.0 mg of sodium chloride was substituted for the higher ticrynafen concentration. Solutions were made immediately before the experiment.

Protocol 2

Experimental Design. Total outflow facility was measured in both eyes for approximately 45 minutes, immediately before and beginning 0.5 hour after a 10-minute AC exchange with 2 mL of 0.04-, 0.2-, 1-, and 4-mmol/L ticrynafen in one eye and vehicle alone in the other in 4 groups of 4, 5, 7, and 9 monkeys, respectively. The system was closed to inflow during the interval between baseline and postdrug facility measurements. During postexchange facility measurements, the reservoirs contained the respective ticrynafen or vehicle solution.

Drug Preparation. TRIS, 242 mg, was dissolved in 7 mL of purified water; ticrynafen, 331 mg, was added and vortexed until dissolved; and water was added to 10 mL. A 1.0-mL aliquot of ticrynafen solution was added to 20 mL of Bârány solution and adjusted to pH 7.4 with 50-µL drops of 1N hydrochloric acid while being mixed, and Bârány solution was added to 25 mL. Vehicle solution was prepared identically, except for omission of ticrynafen and substitution of 5.0N for 1.0N hydrochloric acid. Both solutions were dissolved in a 0.2-µm nylon filter. These solutions are stable for 1 month, but were prepared 1 week immediately before the experiment.

Protocol 3

A 1-cm strip of ointment containing 2% ticrynafen was administered to the cul de sac of one eye and placebo ointment to the opposite eye, randomly selected, of 6 ketamine-anesthetized monkeys with bilaterally normotensive eyes (3 from one of the previous protocols, 5 with 1 previous AC perfusion) twice daily for 5 consecutive days at approximately 9:30 AM and 3:30 PM. Excess ointment was wiped away 15 minutes after application. On day 1, baseline IOP was measured immediately before treatment. On day 5, IOP was measured immediately before and 1 and 3 hours after morning treatment. Clinical biomicroscopy was performed by a trained ophthalmologist immediately before baseline and final IOP measurements on days 1 and 5. Total outflow facility was determined 3.5 to 4.5 hours after morning treatment on day 3.

All monkeys had biomicroscopically clear ACs bilaterally at the time of the study. One monkey appeared in 4 groups, and 4 others appeared in 2 groups, but no monkey appeared twice in the same group.

MONKEYS WITH UNILATERAL LASER-INDUCED GLAUCOMA

The IOP was measured at approximately 9:30 AM and repeated 0.5, 1, 2, 3, 4, 5, and 6 hours later to establish a baseline diurnal curve in both eyes of 4 monkeys with unilateral glaucoma under ketamine anesthesia. The following day, IOP was measured in both eyes starting at approximately 9:30 AM, immediately before and then 0.5, 1, 2, 3, 4, 5, and 6 hours after administration of a 1-cm strip of placebo ointment to the glaucomatous eye only. Beginning 2 days later, 2% ticrynafen ointment was administered twice daily to the glaucomatous eye, at 9:30 AM and 3:30 PM, for 5 days. On each day, IOP was measured in both eyes immediately before the morning 2% ticrynafen dose in the glaucomatous eye and 0.5, 1, 2, 3, 4, 5, and 6 hours thereafter. Immediately after the last IOP measurement, the second dose of 2% ticrynafen was given. Slitlamp examination was performed immediately before the first IOP measurement of the day, and just before IOP measurement every 2 hours thereafter. The contralateral normotensive eyes were untreated throughout the experiment.

DATA ANALYSIS

Data are presented as mean ± SEM values. Differences between or ratios of ticrynafen-treated and contralateral vehicle-treated control eyes were tested against 0 or 1, respectively, by the 2-tailed paired t test. The Bonferroni t test was used for the analysis of the multiple-dose study in glaucomatous monkey eyes. The correlation between treated vs control IOP differences and treated/ control facility response was examined by least-squares linear regression, with and without adjusting for baseline IOP.

than in the control eyes at the day 3 pretreatment and the 1- and 3-hour posttreatment measurements, but these differences were not statistically significant. On day 5, 3-hour posttreatment IOP was significantly lower than day 1 pretreatment baseline in both ticrynafen-treated (−3.3 ± 0.8 mm Hg; n = 6; P = .004) and control (−3.0 ± 0.9 mm Hg; P = .02) eyes. Slitlamp examination disclosed only mild superficial punctate keratopathy associated with repeated tonometry, with no differences in frequency or severity between ticrynafen- and vehicle-treated eyes.

After 5 days of twice-daily unilateral topical administration of 2% ticrynafen ointment, facility in the ticrynafen-treated eyes averaged 28% ± 9% higher than in the contralateral vehicle-treated controls (Table; n = 6; P = .03). There was no significant correlation between treated vs vehicle eye IOP differences and treated-vehicle eye facility response, with or without adjusting for baseline IOP differences.

MONKEYS WITH UNILATERAL LASER-INDUCED GLAUCOMA

Six-hour baseline diurnal IOP (day −4) averaged between 34.0 ± 3.1 and 37.5 ± 4.0 mm Hg (Figure 3, A). The IOP immediately before and for 6 hours after
vehicle treatment the next day (day −3) averaged between 34.0 ± 2.6 and 37.3 ± 3.3 mm Hg (Figure 3, B). Since there was no apparent effect of the vehicle (Figure 3, H), the vehicle-treated IOP diurnal curve was used for comparison with ticrynafen treatment at the same time of the day in the same eye. The onset of IOP reduction after ticrynafen treatment did not occur until after the seventh dose beginning on day 4. The maximum IOP decline on day 4 averaged 7.5 ± 2.0 mm Hg (P < .05; n = 4) at hour 4, and on day 5 averaged 9.8 ± 2.4 mm Hg (P < .05; n = 4) at hour 2. The IOP in the untreated contralateral normotensive eyes displayed normal diurnal fluctuation during the course of the experiment, averaging between 15.8 ± 1.2 and 18.5 ± 0.7 mm Hg (Figure 4).

Slitlamp examination showed no abnormalities in any vehicle-treated eye or in 3 of 4 ticrynafen-treated eyes. Corneal epithelial edema was noted in 1 ticrynafen-treated eye 2 hours after dosing on days 1 and 4; the edema lessened by 4 hours and disappeared by 6 hours after dosing. However, a confluent epithelial defect occurred in the same eye on day 5 of treatment.

**COMMENT**

In normotensive cynomolgus monkeys, ticrynafen produced an increase in outflow facility when given by AC and reservoir exchange, but not at similar doses given by intracameral bolus injection. Anterior chamber and reservoir exchange, as performed here, allows more rapid administration of drug, more complete mixing of the AC, and better maintenance of the drug concentration during posttreatment facility measurements (as the perfusate in the reservoir contains the desired drug concentration). These pharmacodynamic differences may explain ticrynafen’s greater efficacy when given by AC-reservoir exchange.

In normotensive cynomolgus monkeys, ticrynafen administered by AC exchange infusion (with the drug concentration maintained during postdrug facility mea-
normalities in 2 of 4 eyes. In monkeys with bilaterally normotensive eyes (1 of 4 eyes; 1.5% ethacrynic acid: 8.5 mm Hg, corneal abnormalities in 3 of 4 eyes; 2.5% ticrynafen in normotensive monkeys is not surprising given the low baseline IOP. When IOP is low, even a substantial effect on inflow or outflow may have little effect on IOP; this is evident from the Goldmann equation.21 In our experiments, IOP of the control eye on day 5, 3 hours after the morning treatment (immediately before facility measurements) was 10.3 mm Hg. Assuming an episcleral venous pressure of 10 mm Hg, and episcleral venous pressure, aqueous formation, and uveoscleral outflow to be the same in both eyes, a 28% higher trabecular facility in the ticrynafen-treated eyes would predict an IOP of 10.2 mm Hg by the Goldmann equation, ie, essentially the same as in the control eyes. In fact, IOP of the ticrynafen-treated eyes on day 5, 3 hours after the morning treatment (immediately before facility measurements) averaged 9.3 mm Hg. As an even more striking example, assume an aqueous formation rate of 1.5 µL/min, IOP of 13 mm Hg, trabecular facility of 0.33 µL·mm Hg−1 min−1, episcleral venous pressure of 10 mm Hg, and uveoscleral outflow of 0.5 µL/min. By the Goldmann equation, even a 52% facility increase to 0.50 µL·mm Hg−1 min−1 would only yield a 1-mm Hg decrease in IOP.

Monkeys with normotensive eyes under ketamine anesthesia and receiving no other drug exhibit a time-dependent decrease in IOP during 6 to 8 hours, perhaps related to ketamine itself, to depth of anesthesia, or to the normal diurnal rhythm for IOP.22 This may partly or completely account for the small but significant contralateral IOP reduction in our ticrynafen-treated animals, rather than a contralateral effect of ticrynafen itself. As we did not measure baseline facility, we cannot say whether facility increased in the control eye. The contralateral (nonglaucomatous) eye in the unilaterally glaucomatous monkeys demonstrated only the normal diurnal IOP decline, with IOP returning to the morning baseline each day, ie, no contralateral effect was evident.

The glaucomatous monkeys had received laser treatment in the midtrabecular meshwork of all 4 quadrants, and we did not measure their perfusion or tonographic measurements) and ethacrynic acid given by bolus intracameral infusion (with initial drug concentration declines during postdrug measurements) (M.A.C., P.L.K., unpublished data, 1997) both increase facility dose-dependently. Ticrynafen, 1 mmol/L, appears maximal and increased facility by a washout-corrected 73% ± 18% (n = 7) relative to baseline; ethacrynic acid, 0.25 mmol/L, which is also maximal (M.A.C., P.L.K., unpublished data, 1997), produced a nearly identical baseline- and washout-corrected 71% ± 15% (n = 6) facility increase.2

Topical 2% ticrynafen ointment, administered twice daily for 5 days, produced a 28% ± 9% facility increase and no ocular abnormalities, while 1.5% ethacrynic acid ointment, administered once daily for 5 days, produced a 40% ± 15% increase in facility and ocular abnormalities in all eyes (12 of 12 eyes).3 In glaucomatous monkey eyes, topical 2% ticrynafen ointment, administered twice-daily for 5 days, produced an IOP drop similar in magnitude and with fewer corneal abnormalities than once-daily 2.5% or 1.5% ethacrynic acid ointment in a similar base of mineral oil and petrolatum (ticrynafen: 9.8 mm Hg, corneal abnormalities in 1 of 4 eyes; 2.5% ethacrynic acid: 8.5 mm Hg, corneal abnormalities in 3 of 4 eyes; 1.5% ethacrynic acid: 6.5 mm Hg, corneal abnormalities in 2 of 4 eyes4). In monkeys with bilaterally normotensive eyes, IOP after unilateral 2% ticrynafen ointment, administered twice daily for 5 days, averaged 1 to 1.5 mm Hg less in the ticrynafen-treated eyes than in the control eyes at the day 3 pretreatment and the 1- and 3-hour posttreatment measurements, but these differences were not statistically significant. Ethacrynic acid, 1.5% ointment, given once daily for 5 days, significantly lowered IOP by 2.8, 1.7, and 3.7 mm Hg before and 1 and 3 hours, respectively, after treatment on day 5 compared with contralateral control eyes.3

The small IOP reduction induced by topical ticrynafen in normotensive monkeys is not surprising given the low baseline IOP. When IOP is low, even a substantial effect on inflow or outflow may have little effect on IOP; this is evident from the Goldmann equation.21 In

Figure 1. Chemical structures of ethacrynic acid and ticrynafen. Although the right hand portions of the molecules are identical, ethacrynic acid has substantial sulfhydryl reactivity, whereas ticrynafen does not.

Figure 2. Intraocular pressure (IOP) in 6 cynomolgus monkeys under ketamine hydrochloride anesthesia during twice-daily topical application of ticrynafen in one eye and vehicle in the opposite eye. Data are mean ± SEM IOP (A) in ticrynafen-treated and control eyes, or IOP difference (B) between eyes. BL indicates baseline. Time 0 hours on both days is at 9 AM. Time 0 hours on day 5 is 18 hours after the afternoon treatment on day 4 and immediately precedes the morning treatment on day 5. Asterisk indicates significantly different from ipsilateral pretreatment BL by the 2-tailed paired t test (P < .05).
outflow facility after they received ticrynafen. However, pilocarpine\textsuperscript{23} does produce a substantial IOP decrease and an outflow facility increase in this model, indicating that the meshwork can still respond functionally to mechanical distortion\textsuperscript{24} or a direct drug effect.\textsuperscript{25-27} Epinephrine also lowers IOP in the glaucomatous monkey model,\textsuperscript{23,28} but it is not known whether this effect is caused by enhanced trabecular or uveoscleral outflow.\textsuperscript{29-33} Some trabecular meshwork between the burns may have been unaffected by the laser treatment or subsequent scarring and remained responsive to the drug, or even damaged trabecular meshwork could be affected functionally by ticrynafen.

Ethacrynic acid produced a small but significant transient IOP rise after the day 5 treatment compared with day 5 baseline values in cynomolgus monkeys with normotensive eyes.\textsuperscript{5} A similar trend was seen with ticrynafen in our normotensive monkeys, but the magnitude was even smaller and not statistically significant.

Some intracameral doses of some sulfhydryl-reactive compounds may produce an acute facility decrease in enucleated calf and primate eyes,\textsuperscript{34} perhaps because of trabecular cell swelling. No postticrynafen IOP rise was seen on any day in the glaucomatous monkey eyes. In 4 other glaucomatous monkeys, IOP was measured hourly for 6 hours on the third day before, and then daily for 5 days during, once-daily treatment with normal saline. The IOP tended to decline over 5 days, but the change from baseline was not statistically significant at any time point (R.F.W., unpublished data).

Four- or 5-fold excess concentrations of cysteine block the ethacrynic acid–induced facility increase in enucleated calf eyes\textsuperscript{1} and living cynomolgus monkeys (M.A.C., P.L.K., unpublished data, 1997) and the ethacrynic acid–induced shape change in cultured human trabecular meshwork cells.\textsuperscript{35} This suggests that the ethacrynic acid effects might result from sulfhydryl reactivity. However, ticrynafen, which is structurally similar to

**Figure 3.** Intraocular pressure (IOP) in 4 monkey eyes with laser-induced glaucoma at baseline (BL) before (A) and after topical application of vehicle (B) or 2% ticrynafen ointment (C-G). All data are mean ± SEM. H, Vehicle-treated minus ipsilateral BL IOP at the corresponding time point. I through M, Ticrynafen-treated minus ipsilateral vehicle-treated IOP at the corresponding time point. Time 0 (always 9:30 AM) vehicle and all ticrynafen days immediately precedes topical treatment; on treatment days 2 through 5, time is 18 hours after the afternoon ticrynafen treatment on the previous day. Significantly different from 0.0 by the 2-tailed paired t test: asterisk indicates P<.10; dagger, P<.02; double dagger, P<.05.
Ethacrynic acid but putatively lacks sulfhydryl reactivity, significantly lowers IOP in glaucomatous and increases outflow facility in normotensive monkey eyes. In addition, a recently published study reported that, in excised bovine eyes, 0.125-mmol/L ticrynafen increases facility by 102% relative to baseline, significantly more than the 50% washout-induced facility increase in control eyes, and that, in contrast to ethacrynic acid, the effect is maintained in the presence of a 5-fold excess concentration of cysteine. Cytoskeletal drugs such as cytochalasins, latrunculins, and certain protein kinase inhibitors such as H-7 and staurosporine may exert their facility-increasing effect by altering the shape and adhesion of endothelial cells in the meshwork and along the inner canal wall. Ethacrynic acid and ticrynafen both induced shape changes in cultured calf pulmonary artery endothelial cells and human trabecular meshwork cells, but ethacrynic acid had a greater effect at comparable doses. This might explain why, in our present study, a 4-fold higher concentration of ticrynafen delivered via AC exchange (\(\text{[ticrynafen]}_{\text{AC}}\) maintained during facility measurements) was required to produce a facility increase similar to that with ethacrynic acid given via bolus injection. Nonetheless, given that ticrynafen and ethacrynic acid are of reasonably comparable potency and efficacy, and that the drugs likely lower IOP and increase outflow facility by similar mechanisms (eg, cell shape change; ethacrynic acid, ticrynafen), these data suggest that the facility-increasing, IOP-lowering action of ethacrynic acid and derivatives may not depend entirely, if at all, on sulfhydryl reactivity.

Despite evidence of changes in cell shape, volume regulation, and adhesion consequent to drug effects on cytoskeletal proteins such as actin and tubulin or sodium-potassium-chloride ion cotransport, the cellular biophysical mechanism by which ethacrynic acid and related compounds such as ticrynafen lower IOP and increase outflow facility remains elusive. Whatever the mechanism, it is intriguing to note that ticrynafen increases facility and lowers IOP, apparently in conjunction with a change in cell shape, as does ethacrynic acid, but apparently without microtubule disruption, and does so in the absence of sulfhydryl reactivity. Whether drugs such as ethacrynic acid and ticrynafen prove valuable for glaucoma therapy, they are useful probes for studying aqueous outflow mechanisms.

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