A Novel Neuroprotectant against Retinal Ganglion Cell Damage in a Glaucoma Model and an Optic Nerve Crush Model in the Rat

Kazumi Maeda,1,2 Akira Sawada,1 Masayuki Matsubara,1 Yoshiyuki Nakai,1 Akira Hara,5 and Tetsuya Yamamoto1

PURPOSE. To investigate the effects of repeated treatments with a neuroprotective compound, R(−)-1-(benzo[b]thiophen-5-yl)-2-[2-(N,N-diethylamino)ethoxy]ethanol hydrochloride (T-588), on retinal ganglion cell (RGC) survival in rat eyes with elevated intraocular pressure (IOP) or after optic nerve crush.

METHODS. An increase in IOP was induced by a single laser treatment to the trabecular meshwork in one eye of adult Wistar rats. Crush injury was unilaterally produced by clipping the optic nerve 2 mm behind the globe. RGC density was estimated by counting fluorescent dye-labeled cells in the flatmount of the retina. The optic nerve damage in the crush model was also evaluated histologically.

RESULTS. In the elevated IOP model, RGC survival decreased to 72.9% ± 3.4% (mean ± SEM) of that of the contralateral control eye on the eighth day after laser irradiation. Repeated treatments with T-588 at 30 mg/kg twice daily significantly enhanced RGC survival (86.0% ± 2.2%, P = 0.0242) without the reduction of IOP. In the optic nerve crush model, RGC survival diminished to 37.2% ± 8.4% of that of the contralateral control eye after 4 weeks. Repeated applications with T-588 at 10 mg/kg twice daily significantly enhanced RGC survival (77.8% ± 2.1%, P = 0.0038). Histologically, the rat optic nerve in the group treated with T-588 at 10 mg/kg retained a near-normal morphology.

CONCLUSIONS. T-588 has a neuroprotective effect against RGC death caused by elevated IOP and optic nerve crush in the rat. (Invest Ophthalmol Vis Sci. 2004;45:851–856) DOI:10.1167/iovs.03-0365

A novel compound, R(−)-1-(benzo[b]thiophen-5-yl)-2-[2-(N,N-diethylamino)ethoxy]ethanol hydrochloride (T-588; Toyama Chemical Co., Ltd., Toyama, Japan), was developed to treat the dementia associated with Alzheimer disease and is now undergoing clinical trials. This compound has a neuroprotective property that rescues rat cortical neurons and other neuronal cells from the damage induced by serum deprivation,1 β-amyloid protein 25-35,1 and glutamate (Ono S, unpublished data, 1996). It also potentiates the neurotrophic effects of nerve growth factor (NGF) in cultured rat astrocytes.2 Moreover, T-588 ameliorates cognitive dysfunction in various amnesia models in rodents.3 It also has a useful property of being efficiently transported into the central nervous system (CNS) after oral administration.4 Although the molecular mechanisms of T-588 are not fully understood, enhancement of the neuronal transmitter system, such as acetylcholine and noradrenaline, is considered to be one possible mechanism.5,6 Recently, Karim et al.7 reported that a single treatment of T-588 at 100 mg/kg significantly enhanced retinal ganglion cell (RGC) survival in a rat glaucoma model of elevated intracocular pressure (IOP).

Glaucoma is a chronic optic neuropathy with characteristic patterns of optic nerve head excavation and visual field damage. Because elevated IOP is the most recognized risk factor for the development and progression of this disease,8 the current glaucoma treatment has been primarily aimed at lowering IOP. Yet despite adequate IOP control, the glaucomatous damage progresses in many cases.9 Consequently, many investigators have become interested not only in stabilizing IOP at a lower level, but also in preventing the death of RGCs or even regenerating the RGCs.10,11

In the present study, we further explored whether T-588 could be used as a neuroprotective compound against RGC death and optic nerve damage, as well as brain damage. Another purpose of the study was to investigate the effects of T-588 with repeated administration of low dosages (3–30 mg/kg), to avoid adverse reactions in two rat models: an elevated IOP model and an optic nerve crush model.

METHODS

Animals

Adult male Wistar/ST rats (7–8 weeks old) were obtained from the breeding colony of SLC Japan, Inc. (Shizuoka, Japan). Animal care and experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All manipulations were performed with animals under general anesthesia with intramuscular injection of a mixture of ketamine (40 mg/kg body weight [BW]) and xylazine (4 mg/kg BW), and topical 0.4% oxybuprocaine eye drops. We applied 0.3% ofloxacin ophthalmic ointment (Tarivid; Santen, Osaka, Japan) over the ocular surface to prevent corneal desiccation after surgery. The rats had free access to food and water and were maintained in cages in an environmentally controlled room with a 12-hour light–dark cycle (light period: 7 AM to 7 PM).

T-588 Administration

T-588 was provided by Toyama Chemical Co., Ltd., Toyama, Japan. It was dissolved in distilled water and administered orally through a gastric tube at doses of 3, 10, and 30 mg/kg BW as free base. In the elevated IOP model, T-588 (5 mL/kg BW) was administered 30 minutes before laser irradiation and twice daily for 8 days thereafter. In the optic nerve crush model, T-588 (5 mL/kg BW) was administered 30 minutes before laser irradiation and twice daily for 8 days thereafter.
minutes before optic nerve crush and twice daily for 4 weeks thereafter. The control rats were given distilled water on a similar schedule. All drugs were administered to each animal in a masked fashion.

Induction of Elevated IOP

The method for elevating IOP has been described previously. The anterior chamber of the right eye of each animal was injected with approximately 50 μL of 35% India ink immediately after aspiration of the same amount of aqueous humor. Four days later, argon laser photocoagulation was performed in the pigmented trabecular meshwork. Approximately 200 laser burns were delivered around the trabecular band at laser settings of 200 mW, 260 μm, and 0.2 seconds. In each animal, the contralateral eye served as the control. On the fifth day after laser irradiation, the RGCs were labeled in a retrograde fashion by fluorescent dye application (H-7599, hydroxyethylaminemethanesulfonate; Molecular Probes, Inc., Eugene, OR), as will be described later. Eight days after laser treatment, the rats were killed and the eyes were enucleated.

Optic Nerve Crush Model

Optic nerve crush injury was performed as described previously. Lateral canthotomy was performed in the right eye of each rat. The optic nerve was exposed and crushed for 30 seconds at 2 mm behind the globe with a 60-g clip (AM-1, experimental disposable clip; M. T. Giken Co., Ltd., Tokyo, Japan). Special care was taken to avoid damaging blood vessels around the posterior of the eye. The left eye underwent sham-surgery and served as the control. We examined the rat fundus ophthalmoscopically immediately after the injury, to confirm the patency of the central retinal artery. Five days before death, the RGCs were labeled with H-7599. Four weeks after crush injury, the rats were killed, and the eyes were enucleated.

IOP and Corneal Diameter Measurement

IOP was measured with a pneumotonometer (model Classic 30; Mentor, Norwell, MA) before and 5 and 8 days after laser treatment. Five consecutive readings were obtained in each eye, and the average of three measurements, excluding the maximum and minimum, was calculated. All IOP measurements were made at the same time of day (between 10 AM and 1 PM). When measuring the IOP, we simultaneously measured the corneal diameter with calipers.

RGC-Labeling Procedure

Rats were anesthetized and placed in a stereotactic device. H-7599 injections into the bilateral superior colliculi were made with the following coordinates: 6.0 mm posterior to bregma, 2.0 mm lateral to midline, and 4.0 mm from the skull. H-7599, 3% solution in saline (Molecular Probes, Inc.), was applied (2 μL, at a rate of 0.5 μL/min) through a Hamilton syringe.

Tissue Preparation

The rats were anesthetized, perfused through the ascending aorta with saline, and fixed with 10% formaldehyde in 0.1 M phosphate buffer (pH 7.4). The eyes were then enucleated, an incision was made in the cornea, and the eyes were fixed overnight. Each lens was removed, and the posterior segment was further fixed for 2 hours. The retinas were dissected, flattened by four radial cuts, and mounted with the vitreous side up on silicone-coated slides. The retina was examined through a fluorescence microscope (Axioskop; Carl Zeiss Meditec, Jena, Germany) equipped with an ultraviolet filter that permits the visualization of H-7599 fluorescence (excitation filter, G 365 nm; emission filter, LP 420 nm).

Estimation of RGC Density

H-7599-labeled RGCs were counted on fluorescent digital micrographs of eight standard areas in each retina. Each rectangular area measured 0.440 × 0.345 mm. Two areas were measured in each retinal quadrant at 1 and 3 mm (elevated IOP model) or 1 and 2 mm (optic nerve crush model) from the optic nerve head. The number of labeled cells in the eight photographs was divided by the area of the region and pooled to calculate the mean densities of labeled neurons/mm² for each retina. Cell counts were conducted by automated analysis with image-analysis software (Optimas, Bothell, WA). Changes in the number of RGCs were expressed as a ratio compared with the count in the contralateral control retina.

Histologic Procedure

Four weeks after the optic nerve crush, the optic nerves were removed 2 mm posterior to the globe and were fixed in 0.1 M phosphate-buffered 10% formaldehyde. The fixed optic nerves were embedded in paraffin and cut as longitudinal sections at a thickness of 4 to 6 μm. The specimens were used for nervous-system-specific staining by the Bodian method and immunohistochemical staining for glial fibrillary acidic protein (GFAP) was conducted (primary antibody against GFAP; Dako, Carpinteria, CA).

Statistical Analysis

The results were expressed as the mean ± SEM. Comparisons were made using the Dunnett test. Differences were considered significant at P < 0.05.

RESULTS

Effect of T-588 in the Elevated IOP Model

In the control group, the RGC survival rate was 72.9% ± 3.8% of that of the contralateral control eyes by 8 days after laser irradiation. T-588 at doses of 3, 10, and 30 mg/kg twice daily for 8 days enhanced RGC survival in a dose-dependent manner (Fig. 1). RGC survival in the animals treated with T-588 at 30 mg/kg significantly increased to 86.0% ± 2.2% of that of the contralateral control eyes (P = 0.0242; Dunnett test). On day 55 after laser exposure, the IOP had increased to 16.4 ± 0.7 mm Hg (mean ± SEM) in the laser-treated eyes (right eye), but remained at 10.1 ± 0.3 mm Hg in the untreated contralateral eyes (Fig. 2A). The mean corneal diameter in the control group

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933435/ on 04/15/2017)
was 7.0 ± 0.2 mm in the right eyes and 6.0 ± 0.1 mm in the left eyes (Fig. 2B). On the eighth day, the IOP and the corneal diameter in the right eye decreased to 14.7 ± 0.7 mm Hg and 6.7 ± 0.1 mm, respectively (Fig. 2). T-588 did not influence the IOP or corneal diameter of eyes with elevated IOP.

**Effect of T-588 in the Optic Nerve Crush Model**

In the control group, RGC survival decreased to 37.2% ± 8.4% of that of the contralateral control eyes by 4 weeks after optic nerve crush (Fig. 3). In animals treated with 10 mg/kg of T-588 twice daily for 4 weeks, the RGC survival rate was significantly higher than that in the control group (77.8% ± 2.1%, P = 0.0038; Dunnett test). However, 30 mg/kg of T-588 failed to improve RGC survival further. In other words, we observed a bell-shaped dose–response curve for RGC survival (Figs. 3, 4).

A strong contrast between the neuronal axon and surrounding myelin was demonstrated by the Bodian method, which was suitable for the assessment of damage to the axonal structure (Figs. 5A, 5B). GFAP immunostaining clearly revealed the localization of astrocytes (Figs. 5C, 5D). In the control group, many axons were damaged and disappeared, and those that remained showed an irregularity of fiber arrangement (Figs. 5B, 5D). Glial cells, such as astrocytes and oligodendrocytes, were scattered around the crushed axon (Figs. 5B, 5D). In the T-588–treated rats at 10 mg/kg, the structure of the optic nerves was well preserved and showed a nearly intact morphology (Fig. 6D). In this group, glial cell proliferation was not remarkable, and glial fibers were localized regularly along with the optic nerve axons. In the T-588–treated rats at doses of 3 or 30 mg/kg, the axons were damaged and proliferating glial cells were observed around the crushed area (Figs. 6C, 6E).

**DISCUSSION**

In the present study, repeated treatment with T-588 at doses of 3 to 30 mg/kg enhanced RGC survival without affecting IOP in an elevated IOP model in the rat. Moreover, T-588 prevented the death of RGCs in an optic nerve crush model in the rat.

We propose that at least three kinds of mechanisms underlie the neuroprotective action of T-588 in these experimental models. First, T-588 may promote the actions of endogenous neurotrophins in the retina after optic nerve crush. Gao et al. showed that the upregulation of brain-derived neurotrophic factor (BDNF) occurred in the ganglion cell layer after optic nerve crush. T-588 does not stimulate neurotrophic factor production in astrocytes, whereas T-588 potentiates the effect of NGF and BDNF (Ukai W, unpublished data, 2002) in cultured cells. Second, T-588 may protect RGCs from glutamate...
neurotoxicity. The glutamate toxicity to mammalian RGCs has been well documented in various experimental models. T-588, which is not a glutamate receptor antagonist, rescues rat cerebellar granular cells and hippocampal neurons from glutamate neurotoxicity (Ono S, unpublished data, 1996). Third, T-588 may act on glial cells. Recently, many investigators have been interested in the interrelations between RGCs and glial cells such as Müller cells. Incidentally, Takuma et al. demonstrated that T-588 protects cultured astrocytes against Ca\(^{2+}\)/H\(_{11001}\) reperfusion injury, and the effect is expressed through the ERK/mitogen-activated protein (MAP) kinase pathway. It is possible that the effects of T-588 in the retina are due to specific upregulation of survival pathways through activation of ERK MAP kinase in glial cells.

It has been reported that a single treatment with T-588 at 100 mg/kg significantly minimizes the death of RGCs that is induced by elevation of IOP in the rat. In the present study, we tried to determine whether repeated administrations of T-588 at lower doses could alleviate neuronal damage induced by two different kinds of initial insults, because it is well known that some neuroprotectants have diverse systemic side effects, including psychological and gastrointestinal symptoms. However, at all study dosages, T-588 caused no observable systemic side effects.

In this study, we found a difference between optimal doses in the elevated IOP model and the crush model. We wonder whether this difference was caused by the change of glutamate homeostasis in these models. Martin et al. reported that optic nerve transection causes rapid, massive RGC death with consequent acute retinal glutamate release and glutamate transporter (GT) upregulation, whereas IOP elevation may primarily affect GT (downregulation), with any effect on glutamate levels in the retina or vitreous occurring as a secondary phenomenon. Mawrin et al. reported that mRNA levels of the retinal glutamate transporter GLT-1 increase in the early phase after optic nerve crush and decrease toward control level with time. These reports suggest that the change in glutamate homeostasis in a glaucoma model may be deeply implicated in RGC death. The ability of GT to maintain appropriate glutamate levels in a glaucoma model could be much lower than in optic nerve injury models. We conclude that the protection of retina from excitotoxicity is quite important for neuroprotection in a glaucoma model. Also, MK-801 showed neuroprotective properties in both an experimental glaucoma model and an optic nerve crush model. The optimal doses of the agent in

![Figure 5](image1.png)

**Figure 5.** Micrographs of the rat optic nerve 1 to 1.5 mm behind the globe. Optic nerves were stained using the Bodian method (A, B) and by the GFAP immunolabeling method (C, D). Representative micrographs are of intact (A, C) and injured (B, D) nerves 4 weeks after optic nerve crush injury. Bar, 50 μm.

![Figure 6](image2.png)

**Figure 6.** Longitudinal sections of rat optic nerves 4 weeks after a crush injury. The optic nerves were stained by the Bodian method for detection of nerve fiber and cells. The eye is on the left with arrows showing the crush region. Representative micrographs are of normal (A), injured (B), and treated retinas at T-588 doses of 3 (C), 10 (D), and 30 (E) mg/kg. Bar, 100 μm.
each model are different: higher in the glaucoma model than in the crush model, as was the case with T-588. Furthermore, the effective dose of T-588 was higher in glutamate-induced neuronal cell death than in neurotoxic-factor-related cell damage such as serum-deprivation-induced neuronal cell death. Consequently, we believe that the highest dose (30 mg/kg) of T-588 may be necessary for neuroprotection in the elevated IOP model.

In the crush model, the primary insult is transient. The secondary degeneration, followed by initial insult, occurs gradually over several weeks.\(^{28,29}\) It may be that apoptosis and/or elevation in the level of extracellular glutamate are the major contributors to the spread of secondary degeneration after CNS injury. Various pharmacologic agents such as N-methyl-D-aspartate (NMDA)-receptor antagonist, neurotrophic factors, and immunophilin ligand (e.g., FK506) have been tested as a neuroprotective approach.\(^{28,29,30}\) Accordingly, the long-term treatment of T-588 in the crush model may protect RGCs from these various pathologic events, including release of glutamate. We believe that T-588 at 10 mg/kg may show multiple activities in the crush model.

In contrast, T-588 at 30 mg/kg did not enhance RGC survival. Such a bell-shaped curve has been reported for BDNF,\(^{29,51}\) \(\alpha_2\) agonists (brimonidine, clonidine),\(^{52}\) and HU-211 (nonpsychotropic cannabinoid)\(^{53}\) in an optic nerve crush model and in other free models. It is thought that BDNF may limit its own neuroprotective potential by downregulation of TrkB activation and enhancement of free radical release and nitric oxide (NO) production after excessive BDNF application.\(^{27}\) Although the true cause of the negative reaction of T-588 at 30 mg/kg for 4 weeks is not known, it may be due to loss of balance caused by some excessive reactions.

RGC survival rate after the rat optic nerve crush depends on diverse conditions such as severity and time of injury. Yoles and Schwartz\(^{24}\) showed that moderate rat optic nerve crush injury leads to more than 90% loss of surviving RGCs within 5 days. Gelrich et al.\(^{55}\) reported that neuron death directly correlates with both the force applied and the duration of the optic nerve lesion. In this study, RGC survival at 28 days after crush was approximately 40%. We believe that RGC survival in this study is appropriate for estimation of the effect of the agent.

In the optic nerve crush control group, remarkable changes in the optic nerve structure were seen around the crush site. Many glial cells had proliferated, sending irregular GFAP-positive processes into the crushed area, whereas a decrease in neuronal axons was seen in the whole optic nerve. In contrast, the optic nerves in the group treated with 10 mg/kg of T-588 remained nearly intact compared with the crushed control group. In this group, neither optic nerve atrophy nor glial cell proliferation was seen, and the GFAP-localizing pattern of the glial cells was regular as in the normal optic nerve. These results indicate that T-588 may have protective effects against crush damage to the optic nerve.

Astrocytes are the main cells that respond to various injuries in any neuronal system. In general, CNS trauma causes rapid swelling of the astrocytes, which hampers their normal functions, such as the regulation of extracellular ion levels, pH, and glutamate uptake.\(^{56,59}\) These phenomena, in turn, are thought to lead to further damage to the neural tissue.\(^{26,29}\) Furthermore, many studies indicate that glial cell proliferation, the formation of a glial scar, and the production of many different growth-inhibitory proteins contribute to regeneration failure after injury to the CNS.\(^{40}\) These results suggest that T-588 may have some effect on astrocytes, may inhibit axonal degeneration, and may then promote axonal regeneration.

In conclusion, the results of the present study suggest that T-588 has a neuroprotective effect in an elevated IOP model and an optic nerve crush model in the rat, and we believe that T-588 may act on both RGCs and astrocytes.

Acknowledgments

The authors thank Toyama Chemical Co., Ltd. for providing technical assistance with the histology.

References


