Protective Effect of TEMPOL Derivatives against Light-Induced Retinal Damage in Rats

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PURPOSE. OT-551 (1-hydroxy-4-cyclopropanecarbonyloxy-2,2,6,6-tetramethylpiperidinyl hydrochloride), a TEMPOL-H (OT-674) derivative, is a new catalytic antioxidant. In the present study, the efficacy of OT-551 and OT-674 in retinal neuroprotection was tested in a model of light-induced photoreceptor degeneration.

METHODS. Albino rats were intraperitoneally injected with OT-551, OT-674, or water, approximately 30 minutes before a 6-hour exposure to 2700-lux white fluorescent light. Retinal protection was evaluated histologically by measuring the thickness of the outer nuclear layer (ONL) and functionally by electroretinogram (ERG) analysis, 5 to 7 days after exposure to light. Levels of protein modification by 4-hydroxyynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE), which are end products of the nonenzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids, respectively, were measured by Western dot blot analysis immediately after exposure to light.

RESULTS. After exposure to light, water-treated animals had a 77% loss of ERG b-wave amplitudes and a 76% and 56% loss of mean ONL thickness in the inferior and superior hemispheres, respectively. Compared with water-treated rats, ERG b-wave amplitudes in light-exposed eyes were significantly higher in 25 (P < 0.05), 50 (P < 0.05), and 100 (P < 0.001)-mg/kg OT-551-treated rats. Mean ONL thickness in the superior hemisphere was significantly higher in 25 (P < 0.01), 50 (P < 0.01), and 100 (P < 0.001)-mg/kg OT-551-treated, light-exposed eyes and in 100 mg/kg (P < 0.05) OT-674-treated eyes. No decrease of ONL thickness was observed in the light-protected covered fellow eyes in any animal. Increased levels of 4-HNE- and 4-HHE-protein modifications after exposure to light in water-treated eyes were completely counteracted by inhibiting lipid peroxidation. The protection by OT-551 was greater than OT-674. (Invest Ophthalmol Vis Sci. 2007;48: 1900–1905) DOI:10.1167/iovs.06-1057

EXcessive light may enhance the progression and severity of age-related macular degeneration (AMD) and some forms of retinitis pigmentosa.1,2 The hazards of light from the operating microscope used in ophthalmic practice can cause photic maculopathy.3 Acute exposure to light causes photoreceptor and retinal pigment epithelial (RPE) cell damage.4 Previous studies have clarified that exposure of the retina to intense light causes lipid peroxidation of retinal tissues,5–7 and lipid peroxidation is propagated by free radicals, especially lipid radicals.8 We recently reported that an increase in retinal proteins modified by reactive aldehydes, such as 4-hydroxynonenal (4-HNE) and 4-hydroxyhexenal (4-HHE), which are the end products of nonenzymatic oxidation of n-6 and n-3 polyunsaturated fatty acids, respectively,9 is a molecular event preceding retinal degeneration after exposure to light.10 Retinal damage caused by exposure to light can be reduced by various types of antioxidants, such as ascorbate,11 dimethylthiourea,12 thiodenoxin,12,13 and N4-nitro-l-arginine-methyl ester (L-NAME).14,15 We have previously shown that phenyl-N-nitro-butylinitrone (PBN), a potent free radical scavenger, administered systemically crosses the blood-retinal barrier and efficiently protects the albino rat retina from acute light-induced damage.16,17 Accordingly, oxidative stress is likely to be involved in the pathogenesis of light-induced retinal damage. TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidinyl-n-oxyl), a stable nitroxide-free radical, and its diamagnetic hydroxylamine form TEMPOL-H (TP-H or OT-674; 1,4-dihydroxy-2,2,6,6-tetramethylpiperidine) catalyze the dismutation of superoxide to H2O2 plus O2 (superoxide dismutase [SOD]-like activity), and the oxidation of Fe2+ to Fe3+ (ferrooxidase activity).18 OT-551 (1-hydroxy-4-cyclopropanecarbonyloxy-2,2,6,6-tetramethylpiperidinyl hydrochloride), a novel chemical entity that is a catalytic antioxidant, is converted to TP-H in the body. Preclinical studies have demonstrated the ability of OT-551 and TP-H (OT-674) to protect cells from free-radical damage (Othera Pharmaceuticals, Inc., Exton, PA). Previous studies have suggested that TP-H with its strong antioxidant activity and capacity for redox cycling prevents cataract formation in vivo19 and in vitro.20 TP-H is known to have high ocular bioavailability, penetrating both lens tissues and tissues in the posterior segment of the eye. As a result, OT-551 could represent a preventative treatment option for both cataract and age-related macular degeneration. However, the potential protective effects of OT-551 have not been tested in vivo in the retina.

Therefore, we measured the efficacy of OT-551 and OT-674 in rat retinal neuroprotection using an in vivo model of light-induced photoreceptor degeneration. Both drugs were administered by intraperitoneal injection and protection was evaluated histologically by measuring the thickness of the outer nuclear layer (ONL) and functionally by electroretinogram (ERG) analysis. In addition, we measured the levels of modification of retinal proteins by 4-HNE and 4-HHE, to determine
whether the mechanism of protection by OT-551 was mediated by its antioxidant properties.

**MATERIALS AND METHODS**

**Animal Care**

All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of Oklahoma Health Sciences Center (OUHSC) Guidelines for Animals in Research. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the OUHSC and the Dean A. McGee Eye Institute. Sprague-Dawley (SD, Harlan Sera-Laboratory; Indianapolis, IN) rats were born and raised in our vivarium and kept under dim cyclic light (5 lux, 12 hours on/off, 7 AM–7 PM central time).

**Drug Injection and Exposure to Light**

Both OT-551 and OT-674 were provided by Othera Pharmaceuticals Inc. Rats (6–7 weeks of age) were intraperitoneally injected with OT-551 (25, 50, or 100 mg/kg dissolved in sterile water at the concentration of 50 mg/mL), OT-674 (100 mg/kg in sterile water at the concentration of 50 mg/mL), or an equivalent volume of sterile water (50 mg/mL), or an equivalent volume of sterile water (50 mg/mL), or an equivalent volume of sterile water (50 mg/mL). All exposures to light began at 30 minutes before exposure to light. The rats were exposed to damaging light as described previously.16,17 All exposures to light began at 9 AM. Briefly, unanesthetized rats were exposed to 2700-lux diffuse, cool, white fluorescent light for 6 hours in clear plastic cages with wire tops. Drinking water was supplied by a bottle attached to the side of the cage, so that there was no obstruction between the light and the animal. Each cage contained one rat. During the exposure to light, the right eye of each rat was covered with a black-painted polypropylene eye cup attached to the facial skin with an adhesive (no. 454; Loctite Corp., Hartford, CT), and it served as the non-light-damaged control (covered eye). The left eye of each rat was left uncovered and was considered the light-damaged eye (uncovered eye). For the morphology experiments, the rats were returned to the dim cyclic light environment after exposure to light, and 5 to 7 days later, retinal function was measured by ERG. The rats were then euthanatized and the eyes were taken for quantitative morphology. For the Western dot blot experiments, the rats were euthanatized immediately after the 6-hour exposure to light, and the eyes were enucleated. In Western dot blot experiment, covered and uncovered eyes from the rats that were not exposed to damaging light were considered dim-light–exposed control eyes. In this study, 80 rats (50 for electrophysiology and morphology and 30 for Western blot) were used for experiments. One rat died during the electroretinogram (ERG) testing and its data were eliminated from the study.

**Electroretinography**

Flash ERGs were recorded with an ERG recording system (UTAS-E3000, LKG Technologies Inc., Gaithersburg, MD). The rats were maintained in total darkness overnight and prepared for ERG recording under dim red light. They were anesthetized with ketamine (120 mg/kg body weight intramuscularly [IM]) and xylazine (6 mg/kg body weight IM). One drop of 10% phenylephrine was applied to the cornea to dilate the pupil, and one drop of 0.5% proparacaine HCl was applied for local anesthesia. A reference electrode was positioned in the mouth and a ground electrode on the foot, and the rat was placed inside of a Ganzfeld illuminating sphere. A single flash of saturating intensity (25 dB for 10 ms) was applied for each animal, and the ERG responses from both eyes were recorded simultaneously with gold electrodes placed on the cornea. The b-wave amplitudes from each eye were determined and used for the comparison of retinal function.

**Measurement of the ONL Thickness**

After ERG testing, animals were euthanatized by an overdose of carbon dioxide, and the eyes were enucleated, fixed, and embedded in paraffin. Sections (5 μm thick) were taken along the vertical meridian, to allow for comparison of all regions of the retina in the superior and inferior hemispheres.21 In each hemisphere, the ONL thickness was measured at 480-μm intervals in nine defined areas, starting at the optic nerve head and extending along the vertical meridian toward the superior and inferior ora serrata. The mean ONL thickness was calculated for the inferior and the superior regions of the retina.

**Western Dot Blot for 4-HNE- and 4-HHE-Modified Proteins**

Western dot blot analysis was performed as previously described.21 Mouse monoclonal anti-4-hydroxynonenal (+HNE)- and anti-4-hydroxyhexenal (+HHE)-modified protein antibodies were purchased from NOF Corp. (Tokyo, Japan). These antibodies recognize +HNE- and +HHE-histidine adducts, respectively.22 Animals were euthanatized by an overdose of carbon dioxide, and the eyes were enucleated. For each eye, the cornea and the lens were removed and the retina was separated from the eye cup. The retinas were sonicated in radioimmuno-precipitation (RIPA) buffer (Upstate Biotechnology, Lake Placid, NY) containing a protease inhibitor cocktail (Upstate Biotechnology). 1 mM dithiothreitol (Bio-Rad, Hercules, CA), 2 mM diethylenetriaminepenta-acetic acid (Sigma-Aldrich, St. Louis, MO), and 100 μM butylated

![Figure 1](image-url)
hydroxytoluene (Sigma-Aldrich) and centrifuged at 10,000 g for 15 minutes at 4°C. The supernatants were collected, and equal aliquots (5 μg) of retinal proteins were applied to a 96-well dot blot apparatus (Bio-Rad) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) by vacuum filtration. Equivalent sample loading was monitored by staining a membrane loaded in parallel with Coomassie brilliant blue R-250 (CBB). After the reaction was blocked with 10% nonfat dry milk for 30 minutes at room temperature, the membrane was incubated with the anti-4-HNE (1:5000) or anti-iHHE (1:5000) antibodies for 1 hour at room temperature, followed by incubation with peroxidase-linked anti-mouse IgG (GE Healthcare, Buckinghamshire, UK) for 1 hour at room temperature. Chemiluminescence was developed (SuperSignal West Dura Extended Duration Substrate; Pierce, Rockford, IL) and detected with a digital imaging system (IS4000R; Kodak, New Haven, CT). Care was taken to ensure that the intensities of detected spots were within the linear range of the camera and that no pixels were saturated. Intensities of dots stained with CBB, anti-iHNE, and anti-iHHE were determined using Image J 1.32j software (available at http://rsb.info.nih.gov/ij/, developed by Wayne Rasband and provided in the public domain by National Institutes of Health, Bethesda, MD). Coefficient of variance (CV) of this method was calculated as 12.7% ± 7.3% (mean ± SD), based on analysis of quadruplicate spots of six independent samples.21

RESULTS

Functional Evaluation by ERG

After exposure to 2700-lux white fluorescent light for 6 hours, a 77% loss of ERG b-wave amplitude was observed in the uncovered eyes (P < 0.001) compared with the covered eyes of the water-treated rats. In the 100-mg/kg OT-674-treated rats, there was a 44% loss of ERG b-wave amplitude in the uncovered eyes (P < 0.001) when compared to the covered eyes. A 35% loss b-wave amplitude was observed in the uncovered eyes of both the 25 (P < 0.05)- and the 50 (P < 0.01)-mg/kg OT-551–treated rats compared with their respective covered eyes, whereas the b-wave amplitude was identical between the uncovered and covered eyes in the 100-mg/kg OT-551–treated rats.

![Figure 2. ONL thickness.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932942/)
Comparisons among water-, OT-674-, and OT-551-treated groups demonstrated that a loss of b-wave amplitude in uncovered eyes was significantly inhibited in the 25 (P < 0.05), 50 (P < 0.05), and 100 (P < 0.001)-mg/kg OT-551–treated eyes. In addition, there was a significant difference between the b-wave amplitudes in the uncovered eyes of the 100-mg/kg OT-674–treated rats and the 100-mg/kg OT-551–treated rats (P < 0.01; Fig. 1, right).

**Morphologic Evaluation by Quantitative Histology**

There was no apparent decrease in the ONL thickness of covered fellow eyes 5 to 7 days after exposure to light as a result of the systemic administration of water, OT-674, or OT-551 (Fig. 2A), indicating that the administered doses of OT-674 and OT-551 did not cause the loss of photoreceptor cells. A decrease in ONL thickness was observed in both the inferior and the superior hemispheres in the uncovered eyes of water-treated rats, although the superior hemisphere was more damaged than the inferior hemisphere (Fig. 2A). The decrease in ONL thickness in the uncovered eyes was apparently inhibited in the OT-674- and OT-551-treated rats (Fig. 2A).

A 26% (P < 0.01) and 56% (P < 0.001) loss of mean ONL thickness was observed in the inferior and the superior hemispheres, respectively, in the uncovered eyes compared to the covered eyes of the water-treated rats. A 13% (P < 0.01) and 28% (P < 0.01) loss of the mean ONL thickness was observed in the inferior and the superior hemispheres, respectively, in the 100 mg/kg OT-674–treated uncovered eyes (Figs. 2B, 2C, left). There was no significant difference in the mean ONL thickness between the uncovered and the covered eyes in either the inferior or the superior hemispheres from rats treated with any dose of OT-551 (Figs. 2B, 2C, left).

Decreases in ONL thickness in the inferior hemispheres of the uncovered eyes were significantly inhibited in 25 (P < 0.05), 50 (P < 0.05), and 100 (P < 0.001)-mg/kg OT-551–treated rats compared with water-treated rats (Fig. 2B, right). Likewise, rats treated with 100 mg/kg OT-674 (P < 0.05) or 25 (P < 0.01), 50 (P < 0.01), or 100 (P < 0.001)-mg/kg OT-551
lost fewer nuclei in the superior hemispheres compared with water-treated, uncovered eyes (Fig. 2C, right). Collectively, the results clearly indicate that both drugs protect the structure and function of the retina from light-induced damage, and the protection is greater with OT-551 than OT-674 (at equivalent doses).

**Effects of OT-551 on 4-HNE- and 4-HHE Protein Modifications in the Retina**

The effects of the OT-551 on light-induced protein modifications by the lipid oxidation products 4-HNE and 4-HHE were tested by Western dot blot analysis to explore a possible mechanism of protection. Figure 3A shows representative dot blots of two retinas from rats treated either as dim-light-reared controls or given 0, 25, 50, or 100 mg/kg OT-551 30 minutes before light-induced stress. The CBB spots were used to control for protein loading. In the water-treated groups, the levels of 4-HNE- and 4-HHE-modified proteins were identical between the uncovered and the covered eyes from non-light-exposed animals (Figs. 3B, 3C, left, dim), whereas the levels of 4-HNE- and 4-HHE-modified proteins were significantly higher in the uncovered eyes than in the covered eyes after exposure to light (P < 0.01 and P < 0.05, respectively; Figs. 3B, 3C, left, 0 mg/kg). Thus, exposure to light increased retinal levels of both modifications, which is consistent with our previous report. Radial reactions appear to be involved in the initiation of these modifications, which are early events that precede photoreceptor cell apoptosis. The radical trapping agent PBN effectively prevented white-light-induced protein modifications by reactive aldehydes and subsequent photoreceptor cell apoptosis. In the present study, the levels of both modifications were significantly lower in the OT-551-treated uncovered eyes than in the water-treated eyes, suggesting that a reduction in photooxidative stress was involved in the retinal protection as described earlier. Of note, levels of both modifications also increased in the water-treated covered eyes, and these increases were inhibited by OT-551 treatment. General stress caused by exposure to light (i.e., increases of body temperature, respiration, and metabolism, may explain the increased levels of protein modifications in the covered eyes) and thus the results suggest that OT-551 may also reduce oxidative stress resulting from processes other than photochemical reactions. Retinal protection evaluated by ERG and morphology was not significantly different between the 25- and the 50-mg/kg OT-551-treated eyes, whereas inhibition of protein modifications was more apparent in the 50-mg/kg OT-551-treated rats than 25-mg/kg OT-551-treated rats. The elimination of general oxidative stress by systemically administrated OT-551 may explain this discrepancy.

No apparent decrease in ONL thickness was observed in the covered eyes as a result of systemic administration of water, OT-674, or OT-551 (Fig. 2A), demonstrating that the dose of these drugs that we administered was not toxic to retinal cells.

Clinical trials using OT-551 as an agent to delay nuclear cataract formation and treat geographic atrophy in AMD are in progress. Our results may provide a theoretical basis for the use of OT-551 in humans.

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**References**


