

# Screening and Characterization of Drugs That Protect Corneal Endothelial Cells Against Unfolded Protein Response and Oxidative Stress

Eun Chul Kim,<sup>1,2</sup> Tetsuya Toyono,<sup>1,3</sup> Cynthia A. Berlinicke,<sup>1</sup> Donald J. Zack,<sup>1,4</sup> Ula Jurkunas,<sup>5</sup> Tomohiko Usui,<sup>3</sup> and Albert S. Jun<sup>1</sup>

<sup>1</sup>Wilmer Eye Institute, Johns Hopkins Medical Institutions, Baltimore, Maryland, United States

<sup>2</sup>Department of Ophthalmology & Visual Science, Catholic University of Korea, Seoul, Korea

<sup>3</sup>Department of Ophthalmology, University of Tokyo, Tokyo, Japan

<sup>4</sup>Departments of Molecular Biology and Genetics, Neuroscience, and Institute of Genetic Medicine, Johns Hopkins Medical Institutions, Baltimore, Maryland, United States

<sup>5</sup>Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, Massachusetts, United States

Correspondence: Albert S. Jun, Wilmer Eye Institute, Johns Hopkins Medical Institutions, 600 N. Broadway, Wilmer-Woods 376, Baltimore, MD 21287, USA; [aljun@jhmi.edu](mailto:aljun@jhmi.edu).

ECK and TT contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: June 18, 2016

Accepted: January 2, 2017

Citation: Kim EC, Toyono T, Berlinicke CA, et al. Screening and characterization of drugs that protect corneal endothelial cells against unfolded protein response and oxidative stress. *Invest Ophthalmol Vis Sci*. 2017;58:892-900. DOI:10.1167/iovs.16-20147

**PURPOSE.** To screen for and characterize compounds that protect corneal endothelial cells against unfolded protein response (UPR) and oxidative stress.

**METHODS.** Bovine corneal endothelial cells (BCECs) were treated for 48 hours with 640 compounds from a Food and Drug Administration (FDA)-approved drug library and then challenged with thapsigargin or H<sub>2</sub>O<sub>2</sub> to induce UPR or oxidative stress, respectively. Cell viability was measured using the CellTiter-Glo survival assay. Selected “hits” were subjected to further dose-response testing, and their ability to modulate expression of UPR and oxidative stress markers was assessed by RT-PCR, Western blot, and measurement of protein carbonyl and 8-hydroxydeoxyguanosine (8-OHdG) adducts in immortalized human corneal endothelial cells (iHCECs).

**RESULTS.** Forty-one drugs at 20 μM and 55 drugs at 100 μM increased survival of H<sub>2</sub>O<sub>2</sub>-challenged cells, and 8 drugs at 20 μM and 2 drugs at 100 μM increased survival of thapsigargin-challenged cells, compared with untreated control cells. Nicergoline, ergothioneine, nimesulide, oxotremorine, and mefenamic acid increased survival of both H<sub>2</sub>O<sub>2</sub>- and thapsigargin-challenged cells. Oxotremorine altered DNA damage inducible 3 (*CHOP*) gene expression, glucose-regulated protein 78 kDa (GRP78) and activating transcription factor 4 (ATF4) protein expression, and protein carbonyl and 8-OHdG levels. Mefenamic acid altered GRP78 protein expression and protein carbonyl and 8-OHdG levels.

**CONCLUSIONS.** Oxotremorine and mefenamic acid are potential survival factors for corneal endothelial cells under UPR and oxidative stress. The described assay can be further expanded to screen additional drugs for potential therapeutic effect in corneal endothelial diseases such as Fuchs' endothelial corneal dystrophy.

**Keywords:** drug screening, corneal endothelial cells, unfolded protein response, oxidative stress, Fuchs' endothelial corneal dystrophy

Fuchs' endothelial corneal dystrophy (FECD) is a common cause of corneal vision loss characterized by corneal endothelial (CE) cell loss and abnormalities in Descemet membrane.<sup>1-3</sup> Early disease stages are asymptomatic, but in the disease course corneal edema and loss of vision occur approximately 20 years after onset.<sup>4</sup> Fuchs' endothelial corneal dystrophy is among the leading causes of primary corneal transplant surgery in Europe and the United States, and so far there is no definitive conservative (nonsurgical) treatment.<sup>5</sup> Evidence of oxidative stress in the pathogenesis of FECD has been described.<sup>6,7</sup> Unfolded protein response (UPR) activation leads to endothelial cell apoptosis in Fuchs' dystrophy and may play a central pathogenic role in this disease.<sup>8,9</sup>

Koh and Waschek<sup>10</sup> have described the survival effects of vasoactive intestinal peptide for cultured corneal endothelial cells undergoing oxidative stress with H<sub>2</sub>O<sub>2</sub>. High-throughput

screening (HTS) is being used more commonly to identify novel therapeutic compounds.<sup>11</sup> Given the previous evidence for endoplasmic reticulum (ER) and oxidative stress in the pathogenesis of FECD, we sought to develop an efficient system to screen a commercially available drug library of Food and Drug Administration (FDA)-approved drugs for improved survival of cultured corneal endothelial cells undergoing ER and oxidative stress. Positive drugs were further tested for dose-response effects and alterations in ER and oxidative stress specific markers.

The approach described in the present work identified mefenamic acid as a drug with positive and specific survival effects against ER and oxidative stress in cultured corneal endothelial cells. This method could be extended to identify drugs with similar effects on known or yet to be elucidated pathophysiologic mechanisms in corneal endothelial diseases.

The overall purpose of this study was to apply a rational approach to drug discovery/screening, which could yield additional agents with the potential to alter the disease course and form the basis of nonsurgical treatments for these disorders.

## METHODS

### Cell Culture

Primary cultures of bovine corneal endothelial cells (BCECs) from freshly enucleated globes were established by scraping Descemet membrane of excised corneas with a surgical blade and resuspending cells in Dulbecco's modification of Eagle's minimum essential medium (DMEM; Cellgro, Manassas, VA, USA) supplemented with 10% fetal calf serum, antibiotic/antimycotic solution (10,000 units of penicillin [base], 10,000  $\mu$ g of streptomycin [base], and 25  $\mu$ g of amphotericin B/mL; Invitrogen, Grand Island, NY, USA) and 50  $\mu$ g/mL of gentamicin (Invitrogen). Cells were maintained at 37°C with 5% CO<sub>2</sub> in air on a 6-well plate (Cytoone, Orlando, FL, USA). Once confluence was reached (usually 7–10 days), the primary cultures were subcultured to 96-well plates (Cytoone) in the same medium.

Immortalized human corneal endothelial cell line (iHCECs)<sup>12</sup> was kindly provided by Ula Jurkunas, MD (Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, USA). Cells were grown in OptiMEM-1 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 8% fetal calf serum, antibiotic/antimycotic solution (10,000 units of penicillin [base], 10,000  $\mu$ g of streptomycin [base], and 25  $\mu$ g of amphotericin B/mL; Invitrogen), 50  $\mu$ g/mL of gentamicin (Invitrogen), 5 ng/mL of epidermal growth factor (EGF; Sigma-Aldrich Corp., St. Louis, MO, USA), 20 ng/mL of nerve growth factor (NGF; Alfa Aesar, Haverhill, MA, USA), 100  $\mu$ g of pituitary extract (Alfa Aesar), 20  $\mu$ g/mL of ascorbic acid (Sigma-Aldrich Corp.), 200 mg/L of calcium chloride (Sigma-Aldrich Corp.), and 0.08% of chondroitin sulfate (Sigma-Aldrich Corp.). Cells were maintained at 37°C with 5% CO<sub>2</sub> in air on a 6-well plate and subcultured every 2 days. Once confluence was reached, iHCECs were subcultured to 96-well plates (Cytoone) in the same medium.

### Cytotoxic Assay With H<sub>2</sub>O<sub>2</sub> and Thapsigargin

At 48-hours post-seeding BCECs and iHCECs in triplicate 96-well plates, cells were treated with 100- $\mu$ L medium containing thapsigargin (0, 6, 10, 20, 30, 50, and 100  $\mu$ M for BCECs; 0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 25.0, and 30.0  $\mu$ M for iHCECs; Sigma-Aldrich Corp.) for 24 hours or H<sub>2</sub>O<sub>2</sub> (0, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 mM for BCECs; 0, 0.1, 0.125, 0.15, 0.175, 0.2, 0.225, 0.25, 0.30, and 0.35 mM for iHCECs; Thermo Fisher Scientific) for 4 hours at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Thapsigargin was initially resuspended in dimethyl sulfoxide (DMSO; Sigma-Aldrich Corp.) at a stock concentration of 10 mM. Lethal dose (LD50) values of both thapsigargin and H<sub>2</sub>O<sub>2</sub> were determined by calculating mean cell viability (see below) of triplicate wells relative to untreated cells.

### Compound Library

Bovine corneal endothelial cells were used in these experiments. A commercially available FDA-approved drug library was obtained (Enzo Life Science, Inc., Farmingdale, NY, USA) consisting of 640 drugs and bioactive compounds. All drugs were dissolved in 100% DMSO at 10-mM concentration and all have known biological activity. Each drug was tested in

triplicate at a 1:100 dilution (100  $\mu$ M with 1% DMSO) or a 1:500 dilution (20  $\mu$ M with 0.2% DMSO) in cell culture medium. DMSO-treated control cells were tested in triplicate at 1% or 0.2% DMSO in cell culture medium. After pretreatment with drug for 48 hours, medium in all wells was replaced with thapsigargin (25  $\mu$ M) in fresh medium for 24 hours or H<sub>2</sub>O<sub>2</sub> (0.6 mM) in fresh medium for 4 hours at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. For the compound library screening and all subsequent experiments, drugs tested were dissolved in DMSO. Thus, no drug-treated control cells were incubated in equivalent concentrations of DMSO as experimental cells to avoid artifactual results from effect of the drug vehicle.

### Luminescence Assay

Bovine corneal endothelial cells were used in these experiments. At the end of incubation with drug and toxin (thapsigargin or H<sub>2</sub>O<sub>2</sub>), cell viability was determined by adding to each well 100  $\mu$ L of CellTiter-Glo luminescent reagent (Promega, Madison, WI, USA), which produces light in direct proportion to the amount of ATP and viable cells present. Luminescence was measured in relative light units using a FLUO star OPTIMA luminometer (Optima, Tokyo, Japan). Drugs associated with increased luminescence (cell survival) compared with no drug-treated controls exposed to H<sub>2</sub>O<sub>2</sub> and thapsigargin were selected for further study. Results were calculated as mean luminescence of treated replicates/mean luminescence of control replicates  $\times$  100. Control replicates were cultured in cell culture medium without H<sub>2</sub>O<sub>2</sub>, thapsigargin, or other additional agents. Level of cell protection for each agent (proportion of increased cell survival) against oxidative stress or UPR was assessed as grade 0 (no protection), less than 5%; grade 1 (low protection), 5% to 24%; grade 2 (medium protection), 25% to 44%; grade 3 (high protection), 45% to 69%; grade 4 (very high protection), 70% or more compared with cells with H<sub>2</sub>O<sub>2</sub> or thapsigargin and no additional agent.<sup>11</sup>

### Dose Response Assays

After initial selection of drugs with medium to high levels of protection (grade 2–3), additional dose response assays were performed with BCECs to determine the effective dose range for each protective drug and to determine the efficacy of an optimal dose of drug against oxidative stress and UPR. The following drugs were tested (all from Sigma-Aldrich Corp.): oxotremorine, mefenamic acid, nimesulide, and diclofenac. Each drug was resuspended in DMSO at a stock concentration of 10 mM and diluted in cell culture medium. Oxotremorine, mefenamic acid, and nimesulide were retested in quadruplicate at 0.0-, 0.3-, 1.0-, 3.0-, 10.0-, 30.0-, 60.0- (oxotremorine and mefenamic acid only), and 100- $\mu$ M (nimesulide only) concentrations for both H<sub>2</sub>O<sub>2</sub> and thapsigargin. Diclofenac was tested in quadruplicate at 0.0-, 0.05-, 0.1-, 0.2-, 0.3-, and 1.0- $\mu$ M concentrations for H<sub>2</sub>O<sub>2</sub> and 0.0-, 0.3-, 1.0-, 3.0-, 10.0-, 30.0-, 100.0-, and 300.0- $\mu$ M concentrations for thapsigargin.

Dose response assays were performed under the same H<sub>2</sub>O<sub>2</sub> or thapsigargin conditions as used above in the compound library screening. Negative control cells with no drug treatment and no H<sub>2</sub>O<sub>2</sub> or thapsigargin conditioning were also included to determine baseline cell viability (luminescence). Relative cell viability (%) for each drug and concentration was calculated as mean luminescence in H<sub>2</sub>O<sub>2</sub> or thapsigargin conditioned cells/mean luminescence in negative controls  $\times$  100.

Additionally, iHCECs were used to confirm the effectiveness of oxotremorine and mefenamic acid. Oxotremorine was tested in quadruplicate at 0.0- and 60.0- $\mu$ M concentrations

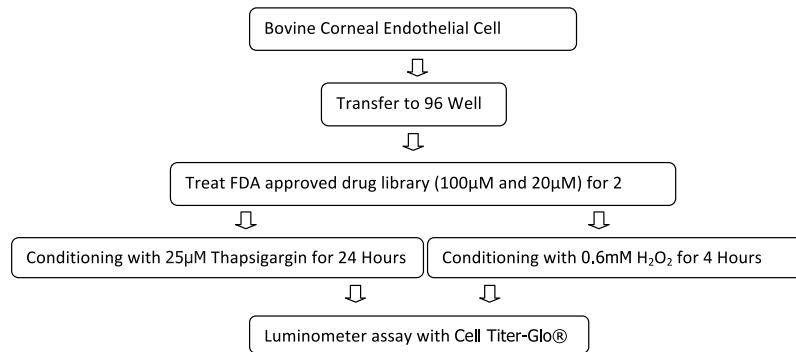


FIGURE 1. Protocol for screening drugs with corneal endothelial cell survival effect against unfolded protein response and oxidative stress.

for  $H_2O_2$  (0.18 mM) and 0.0- and 200.0- $\mu$ M concentrations for thapsigargin (27.5  $\mu$ M). Mefenamic acid was tested in quadruplicate at 0.0- and 30.0- $\mu$ M concentrations for  $H_2O_2$  (0.18 mM) and 0.0- and 20.0- $\mu$ M concentrations for thapsigargin (27.5  $\mu$ M).

### Real-Time PCR

Immortalized HCECs cultured in triplicate 6-well plates were pretreated with 50.0- $\mu$ M oxotremorine, 20.0- $\mu$ M mefenamic acid, or no drug (only 0.5% and 0.2% DMSO, respectively) for 48 hours followed by treatment with thapsigargin (2.5  $\mu$ M) for 24 hours at 37°C in a humidified 5%  $CO_2$  atmosphere. RNA samples were extracted from iHCECs with TRIzol (Ambion, Austin, TX, USA) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A 100-ng quantity of RNA was reverse transcribed using a High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real time-PCR was performed using the TaqMan Gene Expression Master Mix (2 $\times$ ; Applied Biosystems), preamplified cDNA products (diluted 1:20; 5  $\mu$ L), nuclease free water, and human-specific primers (Supplementary Table S1; Applied Biosystems) in a 20- $\mu$ L reaction volume. A no-template control was included in each experiment to confirm the absence of DNA contamination. All assays used similar amplification efficiency, and a  $\Delta C_T$  experimental design was used for relative quantification. Data analysis was performed using StepOne software (Version 2.2; Applied Biosystems). Data were normalized to *GAPDH* and are expressed as relative expression compared with cells untreated with thapsigargin, oxotremorine, or mefenamic acid.

### Western Blot

Immortalized HCECs cultured in triplicate 6-well plates were pretreated with 50.0- $\mu$ M oxotremorine, 20.0- $\mu$ M mefenamic acid, or no drug for 48 hours followed by treatment with thapsigargin (2.5  $\mu$ M) for 24 hours at 37°C in a humidified 5%  $CO_2$  atmosphere. Cells were lysed with ice-cold Tissue Protein Extraction Reagent (Thermo Fisher Scientific) containing protease inhibitor (1%) and EDTA (1%). Total protein concentration was measured using a protein assay kit (Thermo Fisher Scientific) and each sample was adjusted to 20  $\mu$ g/mL. Proteins were subjected to SDS-PAGE (Mini-PROTEAN TGX Gels; Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene fluoride membranes (The PerfectMembrane; GenHunter Corporation, Nashville, TN, USA) that had been soaked in methanol for 1 minute. After blocking with 5% milk for 1 hour, the membranes were then incubated overnight with rabbit anti-ATF4 antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), or

rabbit anti-GRP78 antibody (1:1000; Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibodies (1:10,000 dilution; Cell Signaling Technology) for 30 minutes. The membrane was washed with tris buffered saline with Tween 20 (TBST; Cell Signaling Technology) and antigen was detected using ECL solution (Pierce Biotechnology, Inc., Rockford, IL, USA). Membranes were stripped with Restore Western blotting stripping buffer (Thermo Fisher Scientific) using manufacturer's instructions, reblocked with 5% milk for 1 hour, and restained for GAPDH using rabbit anti-GAPDH antibody (HRP conjugate, 1:10000; Cell Signaling Technology) with 1-hour incubation at room temperature.

### ELISA of Oxidative Stress Markers

Immortalized HCECs cultured in triplicate 6-well plates were pretreated with 50.0- $\mu$ M oxotremorine, 20.0- $\mu$ M mefenamic acid, or no drug for 48 hours followed by treatment with  $H_2O_2$  (0.8 mM) for 4 hours at 37°C in a humidified 5%  $CO_2$  atmosphere. Protein carbonyls and 8-hydroxydeoxyguanosine (8-OHdG) were evaluated as oxidative stress markers. Protein carbonyls in iHCECs were measured using ELISA (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocols. Treated cells were lysed with ice-cold Tissue Protein Extraction Reagent (Thermo Fisher Scientific) containing protease inhibitor (1%) and EDTA (1%). Total protein concentration was measured using a protein assay kit (Thermo Fisher Scientific) and each sample was diluted to 10  $\mu$ g/mL. 8-OHdG in iHCECs was measured by DNA extraction (QIAamp DNA Mini Kit; Qiagen) followed by ELISA (OxiSelect Oxidative DNA Damage ELISA Kit; Cell Biolabs).

### Statistical Analysis

Statistical analysis was performed with two-tailed Student's *t*-test using SPSS (version 16.0; IBM, Armonk, NY, USA) or JMP (version 10.0.2; SAS, Cary, NC, USA) and *P* less than 0.05 was considered statistically significant.

## RESULTS

### Preliminary Cytotoxic Assay

Cytotoxic effects of BCECs exposed to  $H_2O_2$  for 4 hours and thapsigargin for 24 hours showed a dose-dependent response. The LD50 values for  $H_2O_2$  and thapsigargin against BCECs were 0.6 mM and 25.6  $\mu$ M, respectively (Supplementary Figs. S1A, S1B). In a similar way, the LD50s for  $H_2O_2$  and thapsigargin against iHCECs were 0.18 mM and 16.5  $\mu$ M (data not shown).

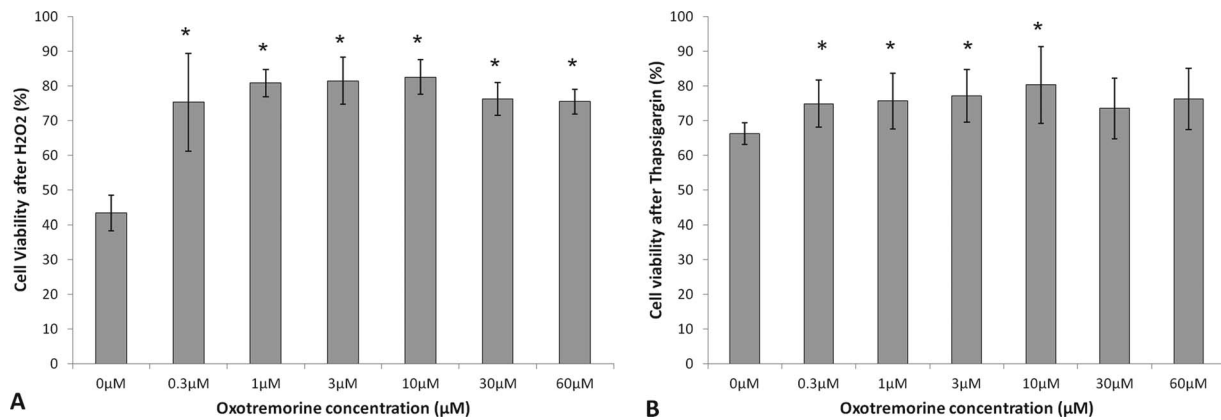


FIGURE 2. Cell viability of oxotremorine-treated BCECs after H<sub>2</sub>O<sub>2</sub> (A) or thapsigargin- (B) induced stress. Results are expressed as mean ± SD. \*P < 0.05.

**Initial Screen**

Bovine corneal endothelial cells were used in this screening test. For a flow chart of the initial screening process (Fig. 1). Initial screening of the 640 drug library using conditions established by the preliminary cytotoxic assay demonstrated 55 drugs at 100 μM and 41 at 20 μM with moderate to high levels of increased cell survival (grade 2-3) against H<sub>2</sub>O<sub>2</sub> conditioning. Against thapsigargin conditioning, two drugs at 100 μM and eight at 20 μM demonstrated moderate to high levels of increased cell survival. Overall, 14 drugs demonstrated increased cell survival with both concentrations of drug tested showing a grade 2 to 3 effect against either oxidative stress or UPR (Supplementary Table S2). Five drugs (ergothioneine, nicergoline, nimesulide, mefenamic acid, and oxotremorine) were found to demonstrate a grade 2 to 3 survival effect at one concentration against both oxidative stress and UPR (Supplementary Table S2).

**Dose Response Assays**

After initial screening of the FDA drug library, dose-response assays with BCECs were performed for the five compounds with survival effects against both of oxidative stress and UPR. Nicergoline and ergothioneine did not exhibit a clear response (data not shown), but oxotremorine, mefenamic acid, and nimesulide exhibited a significant dose response. Cell viability of 0.3- (75.2 ± 14.1%, P = 0.002), 1.0- (80.8 ± 4.0%, P =

0.00001), 3.0- (81.4 ± 6.7%, P = 0.00005), 10.0- (82.5 ± 5.0%, P = 0.00003), 30.0- (76.2 ± 4.7%, P = 0.00004), and 60.0-μM (75.4 ± 3.6%, P = 0.00002) oxotremorine-treated cells against oxidative stress was significantly increased compared with 0.0-μM control (43.3 ± 5.1%; Fig. 2A). Cell viability of 0.3- (74.8 ± 6.7%, P = 0.01), 1.0- (75.6 ± 8.0%, P = 0.02), 3.0- (77.1 ± 7.6%, P = 0.02), and 10.0-μM (80.2 ± 11.0%, P = 0.01) oxotremorine-treated cells against UPR was significantly increased compared with 0.0-μM control (66.3 ± 3.1%; Fig. 2B). Overall oxotremorine demonstrated a maximal effect at 10.0 μM of 90.5% increased cell viability against H<sub>2</sub>O<sub>2</sub> and a maximal effect at 10.0 μM of 21.0% increased cell viability against thapsigargin.

Cell viability of 0.3- (69.1 ± 2.0%, P = 0.02), 1.0- (71.2 ± 8.2%, P = 0.01), 3.0- (73.6 ± 8.7%, P = 0.01), 10.0- (73.2 ± 3.5%, P = 0.005), and 30.0-μM (68.7 ± 3.4%, P = 0.03) mefenamic acid-treated cells against oxidative stress was significantly increased compared with 0.0-μM control (60.8 ± 6.0%; Fig. 3A). Cell viability of 10.0- (57.4 ± 2.4%, P = 0.04) and 30.0-μM (61.7 ± 3.1%, P = 0.04) mefenamic acid-treated cells against UPR was significantly increased compared with 0.0-μM control (51.9 ± 3.7%; Fig. 3B). Overall, mefenamic acid demonstrated a maximal effect at 3.0 μM of 21.1% increased cell viability against H<sub>2</sub>O<sub>2</sub> and a maximal effect at 30.0 μM of 18.9% increased cell viability against thapsigargin.

Cell viability of 1.0- (47.0 ± 3.3%, P = 0.01) and 3.0-μM (51.3 ± 3.8%, P = 0.001) nimesulide-treated cells against oxidative stress was significantly increased compared with 0.0-μM control (41.9 ± 1.5%; Fig. 4A). Cell viability of 1.0- (57.0 ±

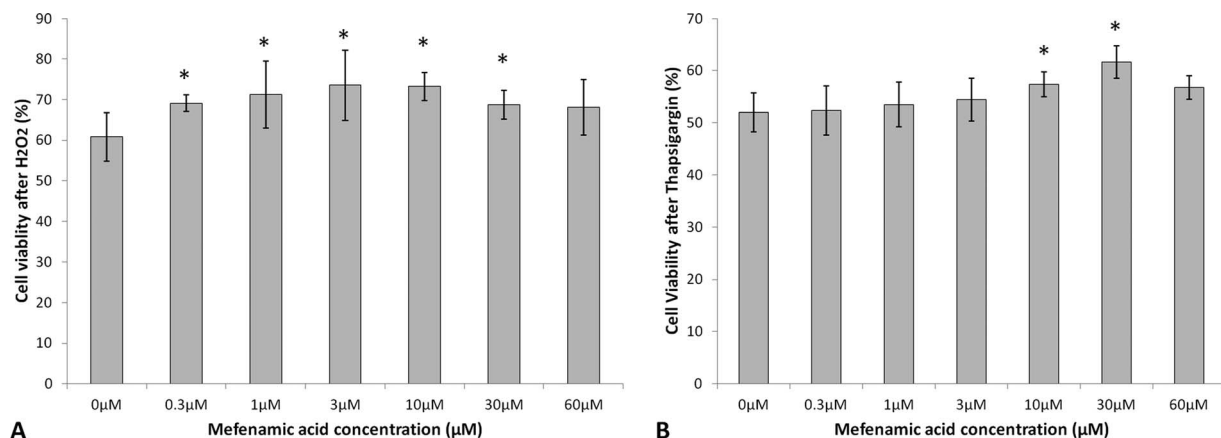


FIGURE 3. Cell viability of mefenamic acid-treated BCECs after H<sub>2</sub>O<sub>2</sub> (A) or thapsigargin- (B) induced stress. Results are expressed as mean ± SD. \*P < 0.05.

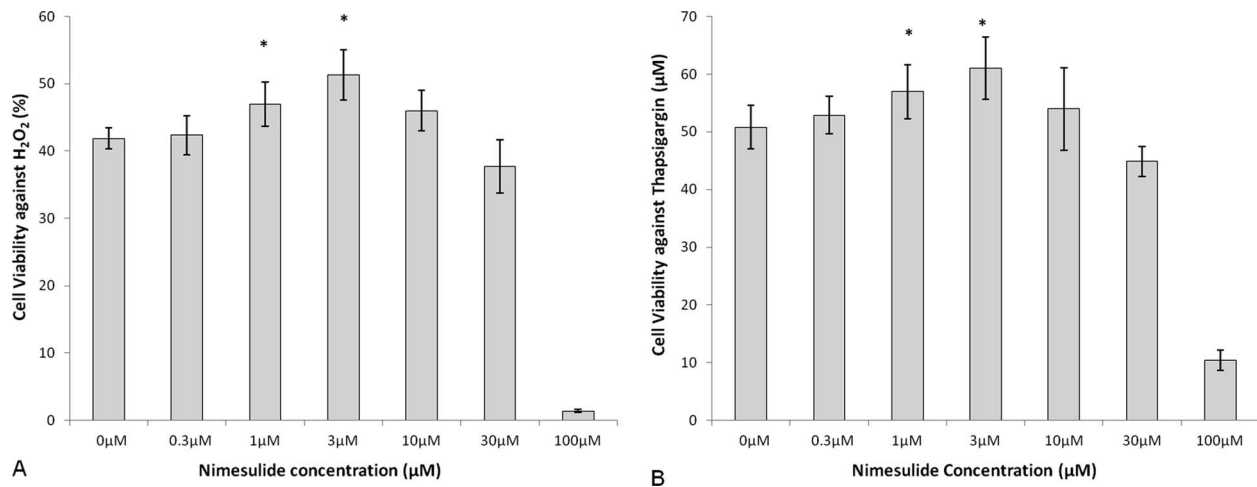


FIGURE 4. Cell viability of nimesulide-treated bovine corneal endothelial cells after H<sub>2</sub>O<sub>2</sub> (A) or thapsigargin- (B) induced stress. Results are expressed as mean ± SD. \*P < 0.05.

4.6%,  $P = 0.04$ ) and 3.0-μM ( $61.0 \pm 5.4\%$ ,  $P = 0.01$ ) nimesulide-treated cells against UPR was significantly increased compared with 0.0-μM control ( $50.8 \pm 3.8\%$ ; Fig. 4B). Overall, nimesulide showed a maximal effect at 3.0 μM of 22.4% increased cell viability against H<sub>2</sub>O<sub>2</sub> and 20.1% increased cell viability against thapsigargin.

Based on the above results indicating a protective effect of the nonsteroidal anti-inflammatory drugs (NSAIDs) mefenamic acid and nimesulide on BCECs exposed to oxidative stress and UPR, we selected diclofenac, a highly potent NSAID<sup>13</sup> for additional dose-response testing. Cell viability of 0.3- ( $60.1 \pm 5.2\%$ ,  $P = 0.01$ ) and 1.0-μM ( $61.6 \pm 4.4\%$ ,  $P = 0.005$ ) diclofenac-treated cells against oxidative stress was significantly increased compared with 0.0-μM control ( $52.1 \pm 2.7\%$ ; Fig. 5A). However, cell viability of 3.0- ( $51.5 \pm 3.4\%$ ,  $P = 0.01$ ), 10.0- ( $50.6 \pm 4.6\%$ ,  $P = 0.02$ ), 30.0- ( $50.0 \pm 4.7\%$ ,  $P = 0.01$ ), 100.0- ( $45.2 \pm 2.9\%$ ,  $P = 0.0005$ ), and 300.0-μM ( $43.4 \pm 4.2\%$ ,  $P = 0.0007$ ) diclofenac-treated cells against UPR was significantly decreased compared with 0.0-μM control ( $57.8 \pm 3.1\%$ ; Fig. 5B). Overall diclofenac demonstrated a maximal effect at 1.0 μM of 18.2% increased cell viability against H<sub>2</sub>O<sub>2</sub> and a maximal effect at 300 μM of 24.9% decreased cell viability against thapsigargin.

In addition, we sought to characterize potential cell protective effects of oxotremorine and mefenamic acid on HCECs using iHCECs. Cell viability of 60.0-μM ( $78.4 \pm 7.3\%$ ,  $P = 0.0002$ ) oxotremorine-treated cells against oxidative stress was significantly increased compared with 0.0-μM control ( $40.9 \pm 6.3\%$ ; Fig. 6A). Cell viability of 200.0-μM ( $78.4 \pm 15.4\%$ ,  $P = 0.03$ ) oxotremorine-treated cells against UPR was significantly increased compared with 0.0-μM control ( $37.5 \pm$

4.5%; Fig. 6B). Cell viability of 30.0-μM ( $89.2 \pm 5.1\%$ ,  $P = 0.004$ ) mefenamic acid-treated cells against oxidative stress was significantly increased compared with 0.0-μM control ( $46.1 \pm 7.1\%$ ; Fig. 6C). Cell viability of 20.0-μM ( $79.0 \pm 11.0\%$ ,  $P = 0.009$ ) mefenamic acid-treated cells against UPR was significantly increased compared to 0.0-μM control ( $37.6 \pm 6.3\%$ ; Fig. 6D).

### Effects of Oxotremorine and Mefenamic Acid on UPR Markers

To assess mRNA level effects of oxotremorine and mefenamic acid on UPR markers in HCECs, iHCECs were pretreated with 50.0-μM oxotremorine or 20.0-μM mefenamic acid, exposed to thapsigargin, and real-time PCR was performed for *ATF4* and *CHOP* (Supplementary Table S1), two commonly used markers of UPR.<sup>14,15</sup> For thapsigargin-exposed iHCECs, oxotremorine treatment showed a trend for reduced *ATF4* mRNA expression to  $118.2 \pm 43.7\%$  ( $P = 0.23$ ) compared with untreated controls ( $148.3 \pm 28.3\%$ ) and reduced *CHOP* mRNA expression to  $112.3 \pm 60.6\%$  ( $P = 0.024$ ) compared with untreated controls ( $188.1 \pm 8.0\%$ ; Fig. 7A). For thapsigargin-exposed iHCECs, mefenamic acid treatment showed trends for reduced *ATF4* mRNA expression to  $117.0 \pm 13.6\%$  ( $P = 0.10$ ) compared with untreated controls ( $135.6 \pm 18.1\%$ ) and reduced *CHOP* mRNA expression to  $170.9 \pm 19.7\%$  ( $P = 0.06$ ) compared with untreated controls ( $190.9 \pm 6.5\%$ ; Fig. 7B).

To assess protein level effects of oxotremorine and mefenamic acid on UPR markers in HCECs, iHCECs were pretreated with 50.0-μM oxotremorine or 20.0-μM mefenamic

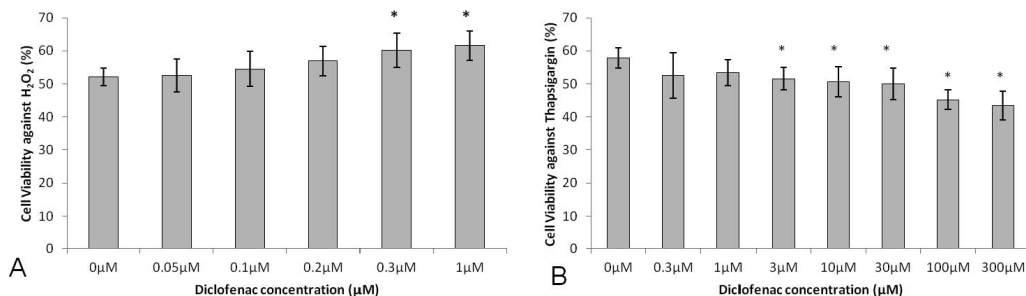


FIGURE 5. Cell viability of diclofenac-treated bovine corneal endothelial cells after H<sub>2</sub>O<sub>2</sub> (A) or thapsigargin- (B) induced stress. Results are expressed as mean ± SD. \*P < 0.05.

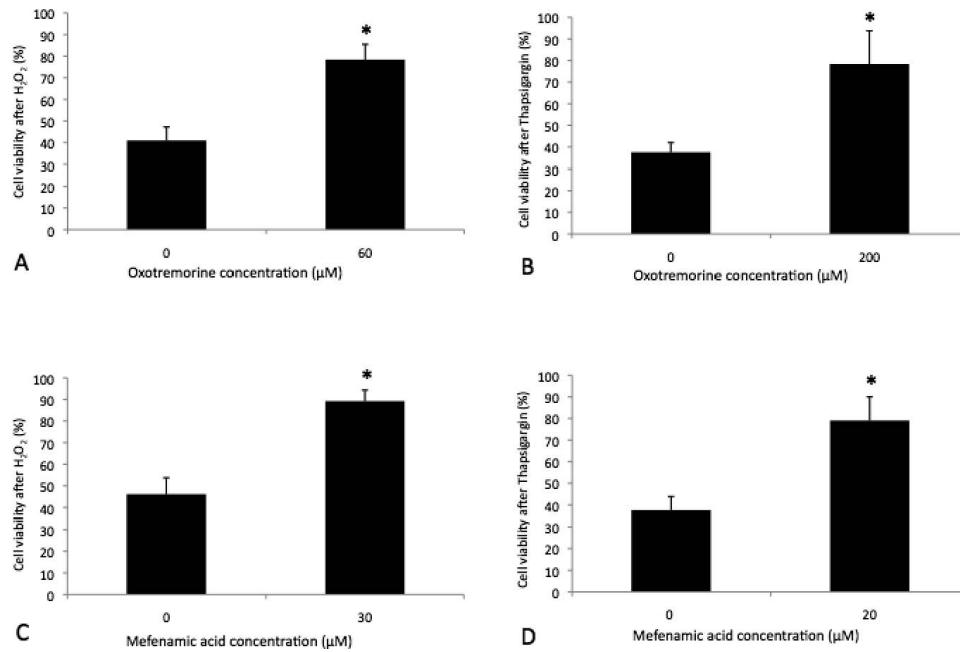


FIGURE 6. Cell viability of oxotremorine-treated iHCECs after H<sub>2</sub>O<sub>2</sub> (A) or thapsigargin- (B) and mefenamic acid-treated iHCECs after H<sub>2</sub>O<sub>2</sub> (C) or thapsigargin- (D) induced stress. Results are expressed as mean ± SD. \**P* < 0.05.

acid, exposed to thapsigargin, and Western blot was performed for ATF4 and GRP78, which are also commonly used markers of UPR.<sup>14,15</sup> For thapsigargin-exposed iHCECs, oxotremorine treatment increased GRP78 protein expression to 894.6 ± 123.7% (*P* = 0.013) compared with untreated controls (462.6 ± 121.9%) and decreased ATF4 protein expression to 3943.6 ± 307.7% (*P* = 0.039) compared with untreated controls 6465.3 ± 1413.3% (Fig. 8A). For thapsigargin-exposed iHCECs, mefenamic acid treatment increased GRP78 protein expression to 455.0 ± 154.9% (*P* = 0.038) compared with untreated controls (168.0 ± 52.7%) and showed a trend for decreased ATF4 protein expression to 19354.7 ± 10697.4% (*P* = 0.44) compared with untreated controls (27061.5 ± 11133.2%; Fig. 8B).

### Effects of Oxotremorine and Mefenamic Acid on Oxidative Stress Markers

To assess effects of oxotremorine and mefenamic acid on oxidative stress in HCECs, iHCECs were pretreated with 50.0-μM oxotremorine or 20.0-μM mefenamic acid, exposed to H<sub>2</sub>O<sub>2</sub>, and protein carbonyl and 8-OHdG levels were measured by ELISA. For H<sub>2</sub>O<sub>2</sub>-exposed iHCECs, mefenamic acid treat-

ment reduced protein carbonyl levels to 90.7 ± 7.1% (*P* = 0.037), and oxotremorine treatment reduced protein carbonyl levels to 83.8 ± 10.7% (*P* = 0.028) compared with untreated controls (131.5 ± 21.9%; Fig. 9A). For H<sub>2</sub>O<sub>2</sub>-exposed iHCECs, mefenamic acid treatment reduced 8-OHdG levels to 173.3 ± 15.7% (*P* = 0.0008), and oxotremorine treatment reduced 8-OHdG levels to 175.0 ± 31.2% (*P* = 0.0051) compared with untreated controls (275.0 ± 21.2%; Fig. 9B).

### DISCUSSION

Fuchs' endothelial corneal dystrophy results from primary loss of corneal endothelial cells and is a common cause of corneal vision loss. Current definitive therapy includes endothelial keratoplasty, and there is no nonsurgical treatment to delay or prevent disease progression. Multiple studies using keratoplasty tissue have shown evidence of oxidative stress in FECD.<sup>6,7</sup> We have shown evidence of unfolded protein response activation in FECD patient corneas and in a mouse model of FECD.<sup>8,9</sup> Thus, we sought to develop a system to screen for drugs with corneal endothelial cell survival effects against oxidative stress and UPR. The link between oxidative stress and UPR has been established in multiple reports. Holtz et al.<sup>16</sup>

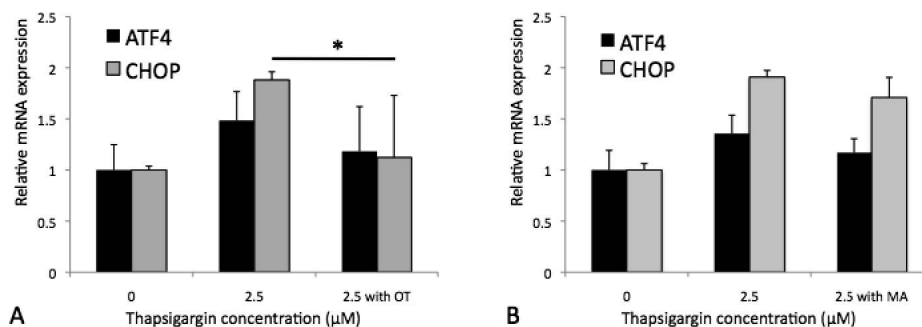
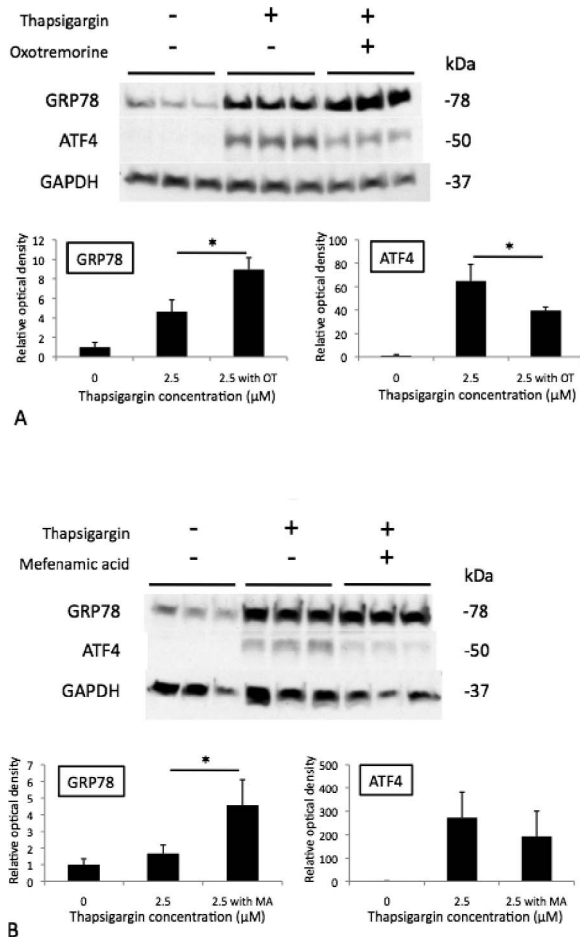


FIGURE 7. Reverse transcription-PCR results of 50-μM oxotremorine (OT) (A) and 20-μM mefenamic acid (MA)- (B) treated iHCECs after thapsigargin-induced stress. Results are expressed as mean ± SD. \**P* < 0.05.



**FIGURE 8.** Western blot of 50- $\mu$ M oxotremorine (OT) (A) and 20- $\mu$ M mefenamic acid (MA)- (B) treated iHCECs after thapsigargin-induced stress. Results are expressed as mean  $\pm$  SD. \* $P$  < 0.05.

described oxidative stress-triggered UPR in cell death evoked by Parkinson mimetics. Tumor necrosis factor- $\alpha$  has been shown to induce UPR in a reactive oxygen species (ROS) dependent fashion.<sup>17</sup> Tagawa et al.<sup>18</sup> demonstrated endoplasmic reticulum (ER) stress-mediated apoptosis is induced by cigarette smoke via an ROS-dependent mechanism.<sup>19</sup>

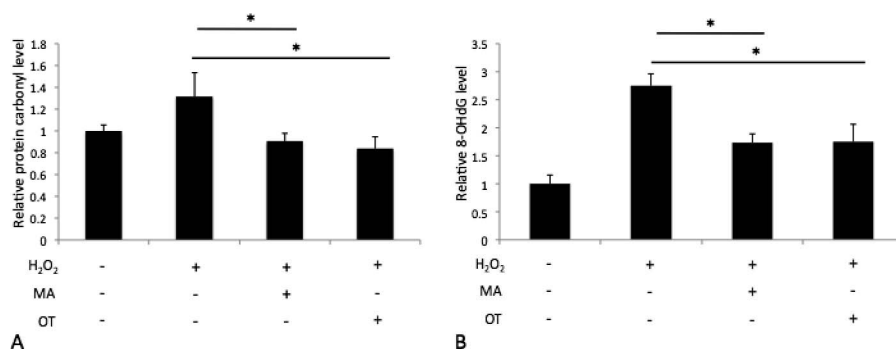
Our results demonstrate a dose response effect of the muscarinic receptor agonist, oxotremorine, and the NSAIDs, mefenamic acid and nimesulide, against oxidative stress and UPR in a BCEC culture model. Although bovine cells are commonly used in studies of the corneal endothelium,<sup>20-22</sup>

their use is a potential shortcoming of our system. However, our previous experience with primary cultured human corneal endothelium<sup>23-25</sup> indicated potential difficulties obtaining adequate numbers of phenotypically consistent cells for the high throughput scale of these experiments. Thus, we used immortalized corneal endothelial cells<sup>12</sup> to confirm dose-responsive survival and further characterize the effects of oxotremorine and mefenamic acid against ER and oxidative stress in human cells. Another potential shortcoming of our system is the use of pretreatment with pharmacologic agents prior to the application of cellular stress. We chose this design expecting it to maximize effects on cell survival. However, clinical situations would likely include the possibility of initiating drug therapy in patients with moderate to advanced disease (i.e., after intermediate to severe levels of cell stress were already established). Thus, follow-up studies in cultured HCECs with pre- and post-stress application of candidate drugs are warranted prior to drawing further implications in diseases such as FECD. A further shortcoming of our work involves short-term responses to relatively high doses of cytotoxic agents. Such a model, though convenient for high throughput analysis, may have limited relevance to FECD, which more likely involves lower levels of chronic cellular stress.

Stimulation of muscarinic receptors provides substantial protection from DNA damage, oxidative stress, and mitochondrial impairment.<sup>26</sup> Oxotremorine prevents camptothecin-induced mitochondrial cytochrome c release, Bcl-2 depletion, and Bax accumulation.<sup>26</sup> In addition, activation of muscarinic receptors blocks caspase 3 activation.<sup>27</sup> The mechanism by which oxotremorine increases survival of cultured corneal endothelial cells treated with the UPR inducer, thapsigargin, is not well understood. Thapsigargin blocks the ability of the cell to pump calcium into the ER, which results in ER damage.<sup>28</sup> Oxotremorine has also been shown to cause an increase in intracellular free Ca<sup>2+</sup>.<sup>29</sup> Thus, one could speculate that oxotremorine would synergize with thapsigargin to cause increased cytotoxicity mediated by intracellular calcium levels. This result was not observed in our cell survival data for reasons yet to be elucidated.

Mefenamic acid decreases production of the free radical nitric oxide and reduces cytochrome c release from mitochondria induced by A $\beta$ <sub>1-42</sub>, Swe-APP, or APP-CTs in neuronal cells.<sup>30</sup> Similarly, our results show significantly decreased oxidative stress markers in mefenamic acid-treated iHCECs exposed to H<sub>2</sub>O<sub>2</sub>. Thus, our results indicate, for the first time to our knowledge, the ability of mefenamic acid to upregulate the UPR marker GRP78 and to alleviate cytotoxicity caused by the UPR inducer, thapsigargin.

COX-2 inhibitors such as nimesulide act as antioxidants to correct lipid peroxidation products such as malondialdehyde (MDA).<sup>31</sup> Unfolded protein response due either to expression



**FIGURE 9.** Protein carbonyl (A) and 8-OHdG (B) semiquantification of 50- $\mu$ M OT and 20- $\mu$ M MA-treated iHCECs after H<sub>2</sub>O<sub>2</sub>-induced stress. Results are expressed as mean  $\pm$  SD. \* $P$  < 0.05.

of viral surface proteins or drugs can stimulate the expression of COX-2 through the nuclear factor kappa-B and pp38 kinase pathways.<sup>32</sup> Thus, NSAIDs may represent an attractive agent to reduce both oxidative stress and UPR in corneal cells in vivo. Additional NSAIDs in our drug library showed a partial protective effect against either H<sub>2</sub>O<sub>2</sub> (celecoxib, diclofenac, fenoprofen, ketoprofen, niflumic acid, piroxicam, and tolfe-namic acid) or thapsigargin (aceclofenac). Diclofenac was selected as a potent NSAID for additional follow-up studies in our ER and oxidative stress model system.<sup>13</sup> Our results indicate positive cell survival effects against oxidative stress, but decreased survival against UPR (Fig. 5). Diclofenac previously has been shown to reduce superoxide and peroxide production as well as to reduce glutathione depletion in human lens epithelial cells exposed to oxidative stress.<sup>13</sup> However, diclofenac also activates the PERK pathway of the UPR, which induces the expression of the proapoptotic GADD153/CHOP.<sup>33</sup> Thus, our results indicate differential effects of individual NSAIDs on the responses to ER versus oxidative stress in corneal endothelial cells.

Chiu et al.<sup>34</sup> recently used a high throughput assay system and elegant mechanistic studies to identify the NSAID, glafenine, as being able to reduce retention of SLC4A11 mutant protein in the ER and increase post-ER trafficking to the cell membrane. Properly trafficked mutant SLC4A11 was further shown to retain partial function.<sup>34</sup> These authors suggested that prostaglandin synthesis may contribute to ER retention of mutant proteins, and a further inference of this work would be that NSAIDs could reduce UPR levels for certain misfolded mutant proteins. Our work using a high throughput drug library screen to identify the positive survival effects of multiple NSAIDs (Supplementary Table S2) against the UPR inducer thapsigargin supports these earlier findings by Chiu et al.<sup>34</sup>

To our knowledge, this is the first system for screening drugs with corneal endothelial cell survival effect against ER and oxidative stress. Based on our results, it is tempting to speculate that NSAIDs such as mefenamic acid, nimesulide, or others may have potential efficacy as a survival factor for corneal endothelial cells under these stressors in vivo. Our screening system also could be used as the basis of an expanded approach to identify additional therapeutic targets for corneal endothelial diseases.

### Acknowledgments

Supported by grants from the J. Willard and Alice S. Marriott Foundation (Bethesda, MD, USA), Margaret Andrews, Edward Colburn, Lorraine Collins, Richard Dianich, Mary Finegan, Barbara and Peter Freeman, Stanley Friedler, MD, Ida Jeffries, Herbert Kasoff, Diane Kemker, Jean Mattison, Florenz Ourisman, Lee Silverman, Norman Tunkel, PhD, the National Eye Institute EY 019874 (all to ASJ; Bethesda, MD, USA), Japan Eye Bank Association (Tokyo, Japan) and EBAA Richard Lindstrom Research Grant (TT; Washington, DC, USA), and generous support from the Guerrieri Family Foundation (Salisbury, MD, USA).

Disclosure: E.C. Kim, None; T. Toyono, None; C.A. Berlinicke, None; D.J. Zack, None; U. Jurkunas, None; T. Usui, None; A.S. Jun, None

### References

- Borderie VM, Baudrimont M, Vallée A, Ereau TL, Gray F, Laroche L. Corneal endothelial cell apoptosis in patients with Fuchs' dystrophy. *Invest Ophthalmol Vis Sci.* 2000;41:2501-2505.
- Jun AS. One hundred years of Fuchs' dystrophy. *Ophthalmology.* 2010;117:859-860.
- Vedana G, Villarreal G, Jun AS. Fuchs endothelial corneal dystrophy: current perspectives. *Clin Ophthalmol.* 2016;10:321-330.
- Elhalis H, Azizi B, Jurkunas UV. Fuchs endothelial corneal dystrophy. *Ocul Surf.* 2010;8:173-184.
- Afshari NA, Pittard AB, Siddiqui A, Klintworth GK. Clinical study of Fuchs corneal endothelial dystrophy leading to penetrating keratoplasty: a 30-year experience. *Arch Ophthalmol.* 2006;124:777-780.
- Jurkunas UV, Bitar MS, Funaki T, Azizi B. Evidence of oxidative stress in the pathogenesis of Fuchs endothelial corneal dystrophy. *Am J Pathol.* 2010;177:2278-2289.
- Azizi B, Ziaei A, Fuchsluger T, Schmedt T, Chen Y, Jurkunas UV. p53-regulated increase in oxidative-stress-induced apoptosis in Fuchs endothelial corneal dystrophy: a native tissue model. *Invest Ophthalmol Vis Sci.* 2011;52:9291-9297.
- Engler C, Kelliher C, Spitze AR, Speck CL, Eberhart CG, Jun AS. Unfolded protein response in Fuchs endothelial corneal dystrophy: a unifying pathogenic pathway? *Am J Ophthalmol.* 2010;149:194-202.
- Jun AS, Meng H, Ramanan N, et al. An alpha 2 collagen VIII transgenic knock-in mouse model of Fuchs endothelial corneal dystrophy shows early endothelial cell unfolded protein response and apoptosis. *Hum Mol Genet.* 2011;21:384-393.
- Koh SW, Waschek JA. Corneal endothelial cell survival in organ cultures under acute oxidative stress: effect of VIP. *Invest Ophthalmol Vis Sci.* 2000;41:4085-4092.
- Zhang J, Chung T, Oldenburg K. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen.* 1999;4:67-73.
- Schmedt T, Chen Y, Nguyen TT, Li S, Bonanno JA, Jurkunas UV. Telomerase immortalization of human corneal endothelial cells yields functional hexagonal monolayers. *PLoS One.* 2012;7:e51427.
- Petersen A, Carlsson T, Karlsson JO, Zetterberg M. Intracellular effects of NSAIDs/ASA in oxidatively stressed human lens epithelial cells in culture. *Ophthalmic Res.* 2008;40:77-85.
- Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. *J Hepatol.* 2011;54:795-809.
- Wang C, Jiang K, Gao D, et al. Clusterin protects hepatocellular carcinoma cells from endoplasmic reticulum stress induced apoptosis through GRP78. *PLoS One.* 2013;8:e55981.
- Holtz WA, Turetzky JM, Jong Y-JI, O'Malley KL. Oxidative stress-triggered unfolded protein response is upstream of intrinsic cell death evoked by parkinsonian mimetics. *J Neurochem.* 2006;99:54-69.
- Xue X, Piao J-H, Nakajima A, et al. Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha. *J Biol Chem.* 2005;280:33917-33925.
- Tagawa Y, Hiramatsu N, Kasai A, et al. Induction of apoptosis by cigarette smoke via ROS-dependent endoplasmic reticulum stress and CCAAT/enhancer-binding protein-homologous protein (CHOP). *Free Radic Biol Medicine.* 2008;45:50-59.
- Huang S-S, Cheng H, Tang C-M, et al. Anti-oxidative, anti-apoptotic, and pro-angiogenic effects mediate functional improvement by sonic hedgehog against focal cerebral ischemia in rats. *Exp Neurol.* 2013;247:680-688.
- Teo BKK, Goh KJ, Ng ZJ, Koo S, Yim EKF. Functional reconstruction of corneal endothelium using nanotopography for tissue-engineering applications. *Acta Biomater.* 2012;8:2941-2952.
- Wang T-J, Wang I-J, Lu J-N, Young T-H. Novel chitosan-polycaprolactone blends as potential scaffold and carrier for



- corneal endothelial transplantation. *Mol Vis.* 2012;18:255-264.
22. Nguyen TT, Bonanno JA. Bicarbonate, NBCe1, NHE, and carbonic anhydrase activity enhance lactate-H<sup>+</sup> transport in bovine corneal endothelium. *Invest Ophthalmol Vis Sci.* 2011;52:8086-8093.
  23. Engler C, Kelliher C, Speck CL, Jun AS. Assessment of attachment factors for primary cultured human corneal endothelial cells. *Cornea.* 2009;28:1050-1054.
  24. Engler C, Kelliher C, Wahlin KJ, Speck CL, Jun AS. Comparison of non-viral methods to genetically modify and enrich populations of primary human corneal endothelial cells. *Mol Vis.* 2009;15:629-637.
  25. Suh LH, Zhang C, Chuck RS, et al. Cryopreservation and lentiviral-mediated genetic modification of human primary cultured corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 2007;48:3056-3061.
  26. De Sarno P, Shestopal SA, King TD, Zmijewska A, Song L, Jope RS. Muscarinic receptor activation protects cells from apoptotic effects of dna damage, oxidative stress, and mitochondrial inhibition. *J Biol Chem.* 2003;278:11086-11093.
  27. Leloup C, Michaelson DM, Fisher A, Hartmann T, Beyreuther K, Stein R. M1 muscarinic receptors block caspase activation by phosphoinositide 3-kinase- and MAPK/ERK-independent pathways. *Cell Death Differ.* 2000;7:825-833.
  28. Rogers TB, Inesi G, Wade R, Lederer WJ. Use of thapsigargin to study Ca<sup>2+</sup> homeostasis in cardiac cells. *Biosci Rep.* 1995; 15:341-349.
  29. Grudt TJ, Usowicz MM, Henderson G. Ca<sup>2+</sup> entry following store depletion in SH-SY5Y neuroblastoma cells. *Mol Brain Res.* 1996;36:93-100.
  30. Joo Y, Kim H-S, Woo R-S, et al. Mefenamic acid shows neuroprotective effects and improves cognitive impairment in in vitro and in vivo Alzheimer's disease models. *Mol Pharmacol.* 2006;69:76-84.
  31. Yesilot S, Ozer MK, Bayram D, Oncu M, Karabacak HI, Cicek E. Effects of aspirin and nimesulide on tissue damage in diabetic rats. *Cytokine.* 2010;52:163-167.
  32. Hung J-H, Su I-J, Lei H-Y, et al. Endoplasmic reticulum stress stimulates the expression of cyclooxygenase-2 through activation of NF-kappaB and pp38 mitogen-activated protein kinase. *J Biol Chem.* 2004;279:46384-46392.
  33. Franceschelli S, Moltedo O, Amodio G, Tajana G, Remondelli P. In the Huh7 hepatoma cells diclofenac and indomethacin activate differently the unfolded protein response and induce er stress apoptosis. *Open Biochem J.* 2011;5:45-51.
  34. Chiu AM, Mandziuk JJ, Loganathan SK, Alka K, Casey JR. High throughput assay identifies glafenine as a corrector for the folding defect in corneal dystrophy-causing mutants of SLC4A11 assay for SLC4A11 folding correctors. *Invest Ophthalmol Vis Sci.* 2015;56:7739-7753.