Differential efficacy of small molecules dynasore and mdivi-1 for the treatment of dry eye epitheliopathy or as a countermeasure for nitrogen mustard exposure of the ocular surface

Jinhong Pan¹, Satyabrata Pany¹, Rafael Martinez-Carrasco¹, and M. Elizabeth Fini¹,²*

¹ New England Eye Center, Tufts Medical Center and Department of Ophthalmology, Tufts University School of Medicine, Boston, Massachusetts, 02111, USA. ² Program in Pharmacology and Drug Development, Tufts Graduate School of Biomedical Sciences, Tufts University, Boston, MA, USA
scRNA-seq: Single cell RNA sequencing

siRNA: small interfering RNA

sXBP1: alternatively spliced transcript of XBP1

tBHP: tert-butyl hydroperoxide

TBST: Tris-buffered saline with 0.1% Tween

UPR: unfolded protein response
Abstract

The ocular surface comprises the wet mucosal epithelia of the cornea and conjunctiva, the associated glands, and the overlying tear film. Epitheliopathy is the common pathological outcome when the ocular surface is subjected to oxidative stress. Whether different stresses act via the same or different mechanisms is not known. Dynasore and dyngo-4a, small molecules developed to inhibit the GTPase activity of classic dynamins DNM1, DNM2 and DNM3, but not mdivi-1, a specific inhibitor of DNM1L, protect corneal epithelial cells exposed to the oxidant tert-butyl hydroperoxide (tBHP). Here we report that, while dyngo-4a is a more potent inhibitor of endocytosis than dynasore, dynasore is a better cytoprotectant. Dynasore also protects corneal epithelial cells against exposure to high salt in an in vitro model of dysfunctional tears in dry eye. We now validate this finding in vivo, demonstrating that dynasore protects against epitheliopathy in a mouse model of dry eye. Knockdown of classic dynamin DNM2 was also cytoprotective against tBHP exposure, suggesting that dynasore’s effect is at least partially on-target. Like tBHP and high salt, exposure of corneal epithelial cells to nitrogen mustard upregulated the unfolded protein response (UPR) and inflammatory markers, but dynasore did not protect against nitrogen mustard exposure. In contrast, mdivi-1 was cytoprotective. Interestingly, mdivi-1 did not inhibit the nitrogen mustard-induced expression of inflammatory cytokines. We conclude that exposure to tBHP or nitrogen mustard, two different oxidative stress agents, cause corneal epitheliopathy via different pathological pathways.

Significance Statement

Results presented in this paper, for the first time, implicate the dynamin DNM2 in ocular surface epitheliopathy. The findings suggest that dynasore could serve as a new topical treatment for dry eye epitheliopathy and that mdivi-1 could serve as a medical countermeasure for epitheliopathy due to nitrogen mustard exposure, with potentially increased efficacy when combined with anti-inflammatory agents and/or UPR modulators.
Introduction

The ocular surface comprises the wet mucosal epithelia of the cornea and conjunctiva and the overlying tear film (Gipson, 2007). The epithelia are continually and rapidly renewed, with complete replacement in humans within ~5–7 days (Hanna et al., 1961; Hanna and O’Brien, 1960). Damaging stress disrupts the replication, maturation and turnover of ocular surface epithelial cells and leads to “epitheliopathy” characterized by barrier disruption and cell death (Ong and Dart, 2016). Epitheliopathy caused by tear dysfunction is a sign of dry eye, an affliction that affects 5% to 34% of all people globally (TFOS, 2007), in which epitheliopathy plays a driving role (Pflugfelder and de Paiva, 2017; Stern and Pflugfelder, 2017; Tsubota et al., 2020). Therapeutics that dominate the dry eye market are anti-inflammatories that promote only modest improvements in dry eye symptoms (Tsubota et al., 2020), and are inconsistent in reversing epitheliopathy (de Paiva et al., 2019; Holland et al., 2017; Holland et al., 2016; Perez et al., 2016).

Epitheliopathy is also caused by ocular surface exposure to toxic chemicals. Vesicants have been identified by the U.S. Department of Homeland Security and the Department of Health and Human Services as highly toxic chemicals of concern to public health security, and developing medical countermeasures is a critical health security and public preparedness need (Araj et al., 2022). Sulfur mustard, or its safer analogue nitrogen mustard, cause severe blistering and burns to the skin, mucous membranes and upper airways, and the eyes, with eyes being the most sensitive organ to the contact. Immediate damage to the ocular surface epithelia results in a biphasic response that includes photophobia, blistering and corneal edema in the early phase, and dry eye, persistent epithelial defects, limbal stem cell deficiency and corneal neovascularization in the later phase. These effects are the result of alkylation of major molecules, oxidative stress, lipid peroxidation, and induction of inflammatory responses (Fuchs et al., 2021; Goswami et al., 2016; Kadar et al., 2009; McNutt et al., 2021; Tewari-Singh et al., 2016). Current and proposed medical countermeasures, including anti-inflammatory, anti-
fibrotic, anti-neovascular and antioxidant agents, frequently result in incomplete or transient efficacy (Fuchs et al., 2021).

While low levels of reactive oxygen species (ROS) are beneficial, facilitating adaptation to stress via signaling, excessive ROS accumulation is damaging to the cell (Schieber and Chandel, 2014). Oxidative stress plays prominently in ocular surface epitheliopathy caused by a variety of damaging stresses, including dry eye (Buddi et al., 2002; Cejka and Cejkova, 2015; Dogru et al., 2018; Seen and Tong, 2017) and exposure to toxic mustards (Beigi Harchegani et al., 2018; Goswami et al., 2016; Shoham et al., 2008; Tahmasbpour et al., 2015; Yin et al., 2018). Whether these different stresses act via the same or different mechanisms is not known.

Exposure to hydrogen peroxide and its analogues is widely used to cause oxidative stress in cellular models (Ransy et al., 2020). Recently, we made the unexpected discovery that dynasore and dyngo-4a, small molecules developed to target classic dynamins DNM1, DNM2 and DNM3 for the study of endocytosis (Macia et al., 2006; McCluskey et al., 2013), but not mdivi-1, a specific inhibitor of DNM1L (Cassidy-Stone et al., 2008), protect human corneal epithelial cells *in vitro* and the mouse corneal epithelium *ex vivo* against the damaging effects of exposure to tert-butyl hydroperoxide (tBHP) (Webster et al., 2018). In a follow-up study, we identified a novel pathway whereby dynasore protects tBHP-exposed cells by inhibiting Ca2+ influx, shifting activity of the unfolded protein response (UPR) towards homeostasis, and preventing opening of the mitochondrial transition pore (Martinez-Carrasco et al., 2020; 2022). We found that dynasore also protects these cells when incubated in high salt medium in a model of dysfunctional tears in dry eye. Much as for tBHP-exposed cells, dynasore maintained a homeostatic level of UPR activity in cells exposed to high salt (Martinez-Carrasco and Fini, 2023).

In this study, we determined the EC50 for cytoprotection against tBHP exposure by dynasore and dyngo-4a, and then investigated the *in vivo* significance of our cell culture findings for epitheliopathy in a mouse model of dry eye. With the knowledge that dynasore and dyngo-4a
target classic dynamins, we next determined the dynamin family members expressed by human corneal epithelial cells and then investigated the hypothesis that DNM2 mediates cell damage due to tBHP exposure. Finally, we investigated the hypothesis that dynasore can protect against oxidative stress more broadly, by evaluating its efficacy against nitrogen mustard exposure, comparing to mdivi-1, and by examining its effects on UPR and inflammatory markers.

Materials and Methods

Chemical reagents and antibodies. We purchased tBHP (Cat# 458139), dynasore (Cat# 324410), chloroquine (Cat# C6628-25G) and doxycycline (Cat# D5207) from Sigma-Aldrich, St. Louis, MO, USA. We purchased dyngo-4a (Cat# AB120689), N-acetyl cysteine (Cat# ab143032) and mdivi-1 (Cat# ab144589) from Abcam, Waltham, MA, USA. We purchased nitrogen mustard (mechlorethamine HCl) from MedChemExpress, Monmouth Junction, NJ, USA.

We purchased polyclonal antibodies against human DNM2 (Cat# PA5-29658) and human ATL3 (Cat# A303-312A) from ThermoFisher, Waltham, MA, USA. We purchased an ACTB polyclonal antibody (Cat# 3700) from Cell Signaling Tech Inc., Danvers, MA, USA.

Mouse model. We conducted our animal research in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, in adherence with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research, and as approved by the Tufts University Institutional Animal Care and Use Committee.

We purchased wild type C57Bl/6J female mice, 6-8 week old, from Jackson Labs (Bar Harbor, ME, USA). We used a single sex because dry eye is influenced by sex hormones (Krenzer et al., 2000). We housed mice at 25±1°C, relative humidity 60%±10%, with alternating 12 hour light/dark cycles. We performed euthanasia using compressed CO₂ gas, according to

We induced dry eye stress in mice by application of the air-draft-plus-scopolamine protocol for 5 days according to (Pflugfelder et al., 2005), as we have previously described (Bauskar et al., 2015). Briefly, we injected scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA) (0.5 mg/0.2 ml in PBS) subcutaneously in alternating hindquarters, 4 times/day (7 AM, 10 AM, 1 PM, and 4 PM), to inhibit tear secretion. At the same time, we exposed mice to an air draft for 18 hours/day in a room with 80±1°F and <40% humidity at all times.

We delivered dynasore eye drops according to standard methods (De Paiva et al., 2006; Pflugfelder et al., 2005) as we have previously described (Bauskar et al., 2015). To prepare dynasore eye drops, we diluted a 10 mM dynasore stock solution in DMSO to 20 µM with PBS. We formulated eye drops in PBS vehicle and delivered them topically to the unanesthetized mouse eye. We used the vehicle (containing an equal volume of DMSO as the diluted dynasore solution) as the control.

Our standard treatment protocol was 5 µL/eye, 4 times/day, delivered at the time of scopolamine injection. We performed fluorescein staining with Fluoresoft®-0.35% (Holles Laboratory, Cohasset, MA). We photographed the stained ocular surface using the Phoenix Micron IV (Phoenix Micron, Bend, OR, USA) equipped with a slit lamp. We quantified fluorescence intensity using National Institutes of Health ImageJ software.

Cell culture model. We obtained the immortalized human corneal limbal epithelial (HCLE) cell line from Drs. Ilene Gipson and Pablo Argüeso (Schepens Eye Research Institute, Massachusetts Eye & Ear and Harvard Medical School). These investigators developed the cell line (Gipson et al., 2003) according to methods described (Rheinwald et al., 2002). They routinely authenticate the cell line by marker analysis (Argueso et al., 2009) and by chromosomal analysis and use of polymorphic short tandem repeat loci (Dr. Pablo Argüeso,
personal communication). For experiments, we distributed HCLE cells into the wells of 24-well plates and used at sub-confluence.

*Cell culture stress and treatments.* In these studies, we stressed cell cultures by exposure to three different agents.

For tBHP exposure, we diluted a stock solution of tBHP in DMSO into the culture medium to 3 mM and incubated for 2 hours before performing the metabolic endpoint assay, as we have described (Martinez-Carrasco et al., 2020; 2022; Webster et al., 2018). We determined the optimal concentration and time empirically in our previous work by dose-response assessment, with the aim to achieve ~60-80% metabolic reduction (Webster et al., 2018). We selected the two hour time point because we performed other assessments in that study that required cells that were still alive (e.g., the rose bengal exclusion assay).

For high salt exposure, we supplemented cell cultures with NaCl to 69 mM to achieve 450 mOsM (cell culture medium is 312 mOsM), following a published method (Deng et al., 2015), as we have previously described (Martinez-Carrasco and Fini, 2023). According to this method, cells must be incubated in the specified media for 24 hours. In this case the endpoint assessed cell death.

For nitrogen mustard exposure, we diluted a stock solution of nitrogen mustard dissolved in DMSO into cell culture medium to a concentration of 100 or 200 µM. Cells were incubated for 2 hours for the quantitative polymerase chain reaction (qPCR) endpoint analysis to ensure cells remained alive, and 18-20 hours for the metabolic endpoint assay, in this case to assess cell death. We observed some variability in cell response to nitrogen mustard due to unknown variables, but always achieved at least a 65% reduction in metabolic activity.

We treated stressed HCLE cells with the small molecules dynasore, dyngo-4a, chloroquine, doxycycline, N-acetyl cysteine or mdivi-1. For treatment, we diluted agents from
their DMSO stocks into the culture medium along with the stress agent. For the vehicle control, we used the same concentration of DMSO as in the least diluted treatment agent.

**Metabolic endpoint assay.** We used the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay (Thermo Fisher Scientific) as the metabolic endpoint for tBHP experiments, as we have previously described (Webster et al., 2018). Superoxide anions generated by NAD(P)H-dependent cellular oxidoreductase activity reduce WST-1 to a water-soluble formazan which absorbs visible light (Mosmann, 1983). WST-1 dye is cell-impermeable thus, reduction occurs outside the cell via plasma membrane electron transport (Berridge et al., 2005). We measured dye reduction by absorbance at 450 nm and quantified with a Biotek Synergy H1 Microplate Reader (Winooski, VT, USA).

**LDH endpoint assay.** We used the LDH assay as the cell death endpoint assay for high salt experiments, as we have previously described (Martinez-Carrasco and Fini, 2023). We quantified LDH activity in cell culture medium using the CyQUANT™ LDH Cytotoxicity Assay (Thermo Fisher Scientific, Waltham, Massachusetts). For this we collected cell culture medium from treated cells and centrifuged at 300 x g for 5 minutes to remove any cell debris. Then, we distributed 50 µL into triplicate well of a 96-well plate. We prepared LDH Reaction Mixture as indicated by the manufacturer and we added 50 µL per well. We then incubated samples for 30 minutes at ambient temperature, protected from light. We stopped the reaction by adding 50 µL of Stop Solution. We measured absorbance at 490 and 680 nm using a Biotek Synergy H1 Microplate Reader. We subtracted the 680-nm absorbance value from 490-nm absorbance to get the LDH activity value.

**Endocytosis inhibition assay.** We assessed endocytosis by quantifying uptake of Vesicular Stomatitis Virus (VSV)-G pseudovirus (Hu et al., 2020).
For the production of viral particles, we plated HEK293T/17 cells (American Type Culture Collection, Manassus, Virginia, USA) in poly-L-lysine precoated T75 flasks (3 x 10^6 per flask) and transfected with the plasmid constructs pUMVC (MLVgag-pol), pCMV-VSG-G, and pMKO.1 GFP. We transfected a total of 10 µg of DNA (3 µg of gag-pol and VSV-G plasmids and 4 µg of GFP plasmid), using Lipofectamine 3000 (Thermo Fisher Scientific). We collected cell culture media after 48 and 72 hours, and we concentrated viral particles using a Lenti Concentrator (Origene, Rockville, MD, USA). We determined the viral titer in cultures of HEK293T/17 cells using 96-well plates and serial dilution of virus for transduction.

For the endocytosis inhibition assay, we plated HEK 293T/17 cells in 96-well plates (10,000 cells/well). After 24 hours, we pretreated cells with dynasore or dyngo-4a for 30 minutes, in a dose-range, then added pseudovirus. In parallel, we performed infection with mock pseudovirus, which served as a negative control. After 72 hours, we quantified pseudoviral uptake. Infected cells express Green Fluorescent Protein (GFP). We viewed cells with a microscope equipped with epifluorescence, and counted green (infected) cells. We used Hoechst 33342 to stain the nuclei and we calculated the ratio of infected cells to uninfected cells.

**Gene expression quantification.** We performed quantitative polymerase chain reaction (qPCR) as we have described (Martinez-Carrasco et al., 2020) to quantify expression of dynamin family genes, UPR markers or inflammatory markers. We list the specific primers used in Table 1.

We extracted RNA using the RNeasy kit (Qiagen, Holden, Germany), following the manufacturer's instructions. We removed DNA contamination from columns with the PureLink® DNase Set (Invitrogen, Carlsbad, CA, USA). We synthesized first strand cDNA from 200 ng of total RNA by reverse transcription (Applied Biosystems, Waltham, MA). We performed qPCR using SYBR® Green reagents (iTaq Universal SYBR Green Supermix; Bio-Rad, Hercules, CA, USA). We used the following parameters: 30 seconds at 95°C, followed by 40 cycles of 5
seconds at 95°C and 30 seconds at 60°C. We normalized all samples to RNA levels of the housekeeping gene ACTB. We used the comparative CT method for relative quantification (Schmittgen and Livak, 2008), selecting the relative amount in control cells as the calibrator.

**Gene expression dataset mining.** We accessed a single-cell RNA sequencing (scRNA-seq) public dataset, National Center for Biotechnology Information (NCBI) accession number GSE155683 for gene expression mining. Datasets in this submission included scRNA-Seq of 17 human developing cornea/conjunctiva (10-21 post-conception weeks), 4 adult cornea/conjunctiva and 8 human cornea-scleral rings, as published (Collin et al., 2021). We analyzed the adult datasets for the current study. We downloaded processed read count matrices from the Gene Expression Omnibus. We processed the read count matrix according to the author’s procedures using the Seurat R package. Briefly, we filtered the data to remove cells with fewer than 1000 reads or 500 genes or more than 15% mitochondrial reads. We normalized the data using the “LogNormalize” method and we selected the top 2000 variable features using the “FindVariableFeatures”. We identified clusters by running a principal component analysis on these genes. We used the Louvain algorithm to generate cell clusters, using the “FindNeighbors” and “FindClusters” functions with 30 principal components and a resolution of 0.6. We identified clusters by attending to the author’s published markers. We then re-clustered these clusters based on the indicated categories: limbal epithelial cells, corneal epithelial cells, conjunctival epithelial cells, melanocytes, stromal cells, endothelial cells, vascular endothelial cells, and immune cells.

**Gene expression knockdown.** We used dicer-substrate small interfering RNAs (DsiRNAs; Integrated DNA Technologies, Coralville, Iowa) for gene expression knockdown. These are chemically synthesized 27mer duplex RNAs that have increased potency compared to 21mer small interfering RNAs (siRNAs). We confirmed the specificity for the various dynamin gene transcripts by searching with NCBI BLAST (Basic Local Alignment Search Tool) algorithm. The
negative control we used was a scrambled sequence (Cat# 51-01-14-03; Integrated DNA Technologies, Coralville, Iowa).

We trialed three different DsiRNAs for each gene and assessed the specificity and effectiveness of knockdown by qPCR. We show the oligo sequences trialed in Supplementary Table 1. To determine knockdown efficiency, we compared specific mRNA expression to the scramble control. We transfected HCLE cells at 60% confluency by incubating with 20 nM of DsiRNA and Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Thermo Fisher Scientific) at 37°C. HCLE cells are resistant to transfection, thus we transfected HCLE cells once, and then a 2nd time a day later, according to a previously described technique (Taniguchi et al., 2017; Uchino et al., 2018). Then we incubated cells for 72 hours at 37°C prior to analysis of knockdown efficiency. For an experiment, we transfected cells with the knockdown construct selected as the most efficacious, along with the scramble control.

**Protein extraction and western blot analysis.** We lysed cells in lysis buffer (RIPA Lysis and Extraction Buffer, 5X, ThermoFisher Cat# 89900) containing protease and phosphatase inhibitor [Halt™ Protease and Phosphatase Inhibitor Cocktail (100X), ThermoFisher, Cat#78442] and harvested using a cell scraper. We then centrifuged lysates at 14,000 x g for 15 minutes at 4°C. We collected supernatants and assessed protein concentration using the BCA protein assay method (Pierce™ BCA Protein Assay Kit, ThermoFisher, Cat#23250).

We loaded protein extracts (60 µg per sample) on SDS-PAGE gels (Bio-Rad, Hercules, CA) separated the proteins by electrophoresis under reducing conditions, then blotted the gels onto membranes. We blocked the membranes in TBST buffer (Tris-buffered saline with 0.1% Tween) containing 5% bovine serum albumin or every-blot blocking buffer (Biorad, Hercule, CA) for 1 hour at room temperature, and incubated with primary antibodies overnight at 4°C. We then exposed the blots to IRDye®-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) for 1 hour at room temperature. We visualized binding of antibodies to the
membranes using a Li-Cor Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE, USA), photographed the results, and assessed the density of the different bands with Image Studio™ Acquisition Software (Li-Cor Biosciences).

**Statistical analysis.** To ensure scientific rigor, we repeated each experiment 2-3 times. We performed cell culture assays with at least 3-4 biological replicates (n=3-4), for statistical power at 80%. Since statistical comparison of experimental groups involved multiple comparisons, we calculated significance by Analysis of Variance (ANOVA) using Graphpad Prism software. We determined statistical significance as $P<0.05$.

For EC50/IC50 determination, we performed dose-response experiments with 10-12 different concentrations and at least 3 biological replicates of each (n=3). We drew a dose-response curve using Prism software and fitted with a non-linear regression program to determine the EC50 or IC50 value. To ensure scientific rigor, we repeated each experiment at least 3 times and calculated the final EC50 or IC50 as the mean of replicates.

In mouse experiments, we assumed an approximate effect size (mean group differences divided by SD) of 2.5. We determined that six eyes per group allowed detection of an effect size of 2.5, testing at 2-sided alpha=0.05, 80% power. To ensure experimental rigor, we randomly distributed animals to experimental groups and masked group identity to the observer. Since analysis of experimental groups involved multiple comparisons, we calculated significance by ANOVA using Graphpad Prism software. We determined statistical significance as $P<0.05$.

**Chemical safety.** The Institutional Environmental Health & Safety team reviewed the studies proposing to use nitrogen mustard and approved them. We used nitrogen mustard in an externally ducted hood, with access to exposure rooms restricted to necessary personnel. Standard operating procedures included wearing of double gloves, laboratory coats, glasses with side shields and face masks. We purchased nitrogen mustard in small quantities, stored in a secure location according to manufacturer’s recommendations, and opened only within the
hood. Liquid and solid waste was processed through a dedicated waste stream managed by Environmental Health & Safety.

**Results**

*Dynasore protects corneal epithelial cells exposed to tBHP or high salt in vitro, and also when subjected to a dry eye protocol in vivo.*

We previously showed that the closely-related small molecules dynasore and dyngo-4a protect cells of the immortalized human corneal limbal epithelial (HCLE) line against damaging stress caused by exposure to the oxidant tBHP (Webster et al., 2018). We replicate this finding in Figure 1A. In a new set of experiments, we compared the potency of dynasore and dyngo-4a to inhibit clathrin-mediated endocytosis or to protect cells against tBHP exposure in our HCLE cell culture model. Representative and summary results are shown in Figure 1B. We found that dyngo-4a was the more potent inhibitor of clathrin-mediated endocytosis, confirming previously published results (McCluskey et al., 2013). We calculated an IC50 for endocytosis of 3.8±0.7 µM (n=3) for dyngo-4a versus an IC50 of 11.1±5.3 µM (n=4) for dynasore. However, we found that dynasore was the more potent cytoprotectant, with an EC50 of 6.2±1.5 µM (n=33) versus an EC50 of 10.7±1.5 µM (n=3) for dyngo-4a.

We also previously showed that dynasore protects against exposure to high salt in an *in vitro* cell culture model of dysfunctional tears in dry eye (Martinez-Carrasco and Fini, 2023). We replicate this finding in Figure 2A. In a new set of experiments, we investigated protection of the corneal epithelium by dynasore *in vivo* using the “air-draft-plus-scopolamine” mouse model that mimics mixed aqueous-deficient/evaporative dry eye (Pflugfelder et al., 2005). We diagram the method in Figure 2B. Following the stress, we measured corneal epitheliopathy by fluorescein staining. We show representative results in Figure 2C. Corneal epitheliopathy was significantly lower in stressed mice treated with dynasore, as compared to vehicle control.
DNM2 contributes to corneal epitheliopathy due to tBHP exposure.

Classic dynamins DNM1, DNM2, and DNM3 belong to the dynamin superfamily, which contains several other evolutionarily-related subfamilies, all mechanochemical large GTPases that function in membrane scission or tubulation (Praefcke and McMahon, 2004; Santos et al., 2018). The original paper describing dynasore (Macia et al., 2006) showed it to be relatively selective for the classic dynamins, but it also inhibited the GTPase activity of the closely-related dynamin DNM1L at a higher concentration. However, in a later study, dynasore at an even higher concentration was found to inhibit GTPase activity of ATL1 (Muriel et al., 2009). Thus dynasore could potentially target many different dynamin family members, depending on the dose used. Based on our finding that dynasore is cytoprotective, we hypothesized that one or more dynamin GTPase family members mediate corneal epithelial cell damage due to tBHP exposure.

First we determined which member(s) of each dynamin subfamily is expressed by cells of the HCLE cell line using qPCR. We show representative results in Figure 3A. As observed in most other non-neuronal tissues (Cao et al., 2007), DNM2 was by far the predominant classic dynamin expressed by HCLE cells. Relative amounts of the mitochondrial-localized mitofusins, MFN1 and MFN2, vary among tissues examined (Santel et al., 2003); we found ~4-fold more MFN2, as compared to MFN1, was expressed by HCLE cells. Of the three atlastins, ATL1 is reported to be neuronal-specific, while ATL2 and ATL3 are the major atlastins expressed in other tissues (Rismanchi et al., 2008); in agreement with this, we found that ATL2 and ATL3 were the predominant atlastins expressed by HCLE cells, with ~3-fold more ATL3 than ATL2.

To compare these results to the corneal epithelium in vivo, we mined an scRNA-seq NCBI public dataset, accession number GSE155683 (Collin et al., 2021). We show results in Figure 3B. Similar to the HCLE cell line, DNM2 was the only classic dynamin expressed detectably by the corneal epithelium. MFN1 and MFN2 were similarly expressed in the corneal epithelium. We observed a similar pattern in the corneal epithelium and the HCLE cell line for
atlastins, with low expression of ATL1 and higher expression of ATL2 and ATL3. DNM1L was expressed in all epithelial cell types.

In our next set of experiments, we used gene knockdown technology to identify dynamin superfamily members that contribute to tBHP exposure pathology. We decided to test the role of the most highly-expressed member of each subfamily, and we included DNM1L. To identify the best knockdown reagent, we made three DsiRNA knockdown constructs for each gene, then tested for efficacy (Suppl. Table 1 and Suppl. Fig. 1). For DNM2, we also assessed protein knockdown by immunoblotting (Suppl. Fig. 1).

Once we had selected the most effective knockdown construct for each gene, we transfected HCLE cells with each construct individually, then transfected cells were exposed to tBHP. Then we assessed cytoprotection by comparing metabolic activity to the scramble control using the WST-1 assay. We show representative results in Figure 3C. We observed significant cytoprotection only in DNM2 knockdown cells. These results are consistent with the idea that dynasore protects against tBHP exposure, at least in part, by on-target inhibition of DNM2.

Interestingly, knockdown of ATL3 significantly increased cell damage due to tBHP exposure (Fig. 3C), suggesting that the role of ATL3 is cytoprotective. The atlastins are membrane-anchored proteins that function in endoplasmic reticulum (ER) fusion (Hu et al., 2009; Orso et al., 2009). To bring membranes into contact, two atlastin molecules in adjacent membranes must dimerize in a GTP-dependent process (Orso et al., 2009). We performed an experiment to learn whether ATL3 dimerization activity is inhibited at the dose of dynasore that we use for cytoprotection. Western blotting was used to compare ATL3 protein dimerization in unstressed HCLE cells and cells stressed by exposure to tBHP, with or without dynasore treatment. Representative results are shown in Figure 3D. Dimerization would be expected to increase as new ER is formed following stress and we observed this to be the case; ATL3 dimers were clearly visible in stressed cells. However, dynasore at 80µM, which is 2-4 times higher than we use for cytoprotection, did not inhibit dimerization.
The DNM2 inhibitor dynasore does not protect against nitrogen mustard exposure.

It was reported that sulfur mustard exposure of skin in the mouse ear vesicant model activates the UPR (Chang et al., 2013), and sulfur mustard exposure of skin epithelial cells in culture increased intracellular Ca2+ (Ray et al., 1995). Since these mechanisms are also activated when HCLE cells in culture are exposed to tBHP (Martinez-Carrasco et al., 2020; 2022), we hypothesized that dynasore might similarly protect against nitrogen mustard exposure. We show representative results of experiments to investigate this idea in Figure 4.

First we examined expression of UPR markers in cells exposed to nitrogen mustard (Fig. 4A). As predicted, nitrogen mustard exposure increased expression of DDIT3 (also known as CHOP) as well as the alternatively spliced form of XBP1 (sXBP1). Nitrogen mustard exposure also increased expression of TNFA and IL6, encoding inflammatory cytokines.

We next performed a series of dose-response experiments to find a useful range of concentration for nitrogen mustard exposure, and to investigate the cytoprotective effect of dynasore. The representative experiment in Figure 4B shows that exposure of HCLE cell cultures to even the lowest nitrogen mustard concentration, significantly decreased cell metabolism. Moreover, as nitrogen mustard concentration was increased, cell metabolism decreased in parallel. As a positive control, we used chloroquine (CQ), which protects against nitrogen mustard exposure by inhibiting autophagy through impairment of autophagosome fusion with lysosomes (Chen et al., 2021). Chloroquine effectively protected against nitrogen mustard exposure, with increased efficacy as the dosage increased, as in the representative results shown in Figure 4C. Contrary to our prediction however, dynasore did not protect cells against nitrogen mustard exposure at any concentration, as in the representative results shown in Figure 4D.
The DNM1L inhibitor mDivi-1 protects against nitrogen mustard exposure.

Next we compared dynasore to other small molecules known to protect against nitrogen or sulfur mustard exposure. Doxycycline (DOX) is an antibiotic that inhibits inflammatory cytokine (Solomon et al., 2000) and matrix metalloproteinase (Golub et al., 1991) production and bioactivity, used at doses ranging from 10 µg/mL to 2 mg/mL (Horwitz et al., 2014; Kadar et al., 2009; Sobrin et al., 2000; Solomon et al., 2000). N-acetyl cysteine (NAC) is an antioxidant and matrix metalloproteinase inhibitor (Slansky et al., 1970), used at doses between 50 µM and 50 mM (Kadar et al., 2009). We also investigated efficacy of mdivi-1, which (as noted above) is thought to specifically target DNM1L. The EC50 for mdivi-1 is 10 µM and it known to be effective at doses up to 100 µM (Cassidy-Stone et al., 2008). Since the effective dose ranges for all four agents overlap, we compared them at the same concentration of 50 µM. Representative results are shown in Figure 5A. Dynasore was again observed to be ineffective. Doxycycline, at the dose used, did not significantly protect against nitrogen mustard exposure. However, N-acetyl cysteine and mdivi-1 were both cytoprotective.

To our knowledge, mdivi-1 has never been tested for efficacy against mustard exposure, thus we performed dose-response experiments. We show representative results in Figure 5B. Mdivi-1 maximally protected against nitrogen mustard at 20 µM, which is close to the IC50 for inhibition of DNM1L (10 µM) (Cassidy-Stone et al., 2008).

Mdivi-1 does not inhibit UPR activation or inflammatory marker expression induced by nitrogen mustard exposure.

In our final set of experiments, we investigated the hypothesis that mdivi-1 protects against nitrogen mustard exposure by its effect on UPR activation or inflammation. We show representative results in Figure 6. At a concentration that we showed is cytoprotective, mdivi-1 did not inhibit nitrogen mustard exposure-induced expression of UPR markers, and also had no effect on nitrogen mustard exposure-induced expression of inflammatory markers.
Discussion

Excessive accumulation of ROS induces oxidative stress, which plays prominently in ocular surface epithelial damage from a variety of causes, including the hyperosmotic and desiccating conditions of dry eye (Chi et al., 2017; Dai et al., 2019; Deng et al., 2015; Dogru et al., 2018; Navel et al., 2022; Roy et al., 2017; Seen and Tong, 2017; Shoham et al., 2008; Yin et al., 2018), and exposure to highly toxic mustard (Beigi Harchegani et al., 2018; Buddi et al., 2002; Goswami et al., 2016; Tahmasbpour et al., 2015; Yin et al., 2018). Recently, we made the unexpected discovery that dynasore and dyngo-4a, small molecules that targets the GTPase activity of the so-called classic dynamins, are remarkably protective of corneal epithelial cells exposed to tBHP (Webster et al., 2018). Most recently, we showed that dynasore protects against exposure to high salt in an in vitro cell culture model of dysfunctional tears in dry eye (Martinez-Carrasco and Fini, 2023). We now validate this result in vivo, demonstrating that dynasore also protects against epitheliopathy in a mouse model that mimics mixed aqueous-deficient/evaporative dry eye (Pflugfelder et al., 2005).

The classic members of the dynamin family, DNM1, DNM2 and DNM3, take part in clathrin-mediated and clathrin-independent endocytosis, intracellular trafficking, and actin assembly and reorganization (Harper et al., 2013). Dynasore was developed as a tool for the study of endocytosis. The closely-related small molecule dyngo-4a was identified later as a more potent endocytosis inhibitor (Kirchhausen et al., 2008; Macia et al., 2006). Interestingly, we show here that dynasore is a better tBHP cytoprotectant than dyngo-4a, despite published evidence that dyngo-4a has lower overall toxicity (McCluskey et al., 2013). One possible explanation for our finding is the difference in mechanisms of action reported for the two small molecules. Dynasore has greater affinity for dynamin oligomers assembled into the ring structure. In contrast, dyngo-4a appears to target dynamin assembly into helices (McCluskey et al., 2013).
Dynasore inhibits the GTPase activity of classic dynamins with greatest potency towards DNM2; at a higher concentration, it also inhibits activity of DNM1L, the most closely-related member of the dynamin superfamily (Kirchhausen et al., 2008; Macia et al., 2006). We found that DNM2 is the predominant classic dynamin expressed by the immortalized human corneal epithelial cell line (HCLE) used in this study, as reported for other non-neuronal tissues (Cao et al., 2007). Moreover, DNM2 was the only classic dynamin gene expressed in human corneal epithelium in vivo, based on our analysis of an scRNA-seq dataset (Collin et al., 2021). DNM2 knockdown protected HCLE cells against tBHP exposure, suggesting that dynasore’s cytoprotective effect is at least partially on target. Significantly, our findings, for the first time, implicate a dynamin family member in the pathophysiology of ocular surface epitheliopathy.

DNM1L plays an important role in mitochondrial fission (Breitzig et al., 2018; Smirnova et al., 2001), and it has been shown to contribute to oxidative stress-induced cell death in other systems (Lackner and Nunnari, 2010). However, DNM1L knockdown in our study did not significantly protect against tBHP exposure. The same was true for MFN2, which controls homotypic docking and fusion of outer mitochondrial membranes (Cohen and Tareste, 2018). Interestingly, we found that knockdown of ATL3 enhances damage due to tBHP exposure. In support of this finding, it has been reported that triple knockdown of ATL1/2/3 increased sensitivity to endoplasmic reticulum (ER) stress (Zhao et al., 2016).

The atlastins are plasma membrane-anchored dynamins that mediate the fusion of endoplasmic reticulum membranes into a network of tubules and sheets fusion (Hu et al., 2009; Orso et al., 2009). ATL3 also functions as a receptor for ER-phagy, promoting tubular ER degradation under conditions of stress (Chen et al., 2019a). Both of these activities provide protection against stress. To bring membranes into contact, two atlastin molecules in adjacent membranes must dimerize in a GTP-dependent process (Orso et al., 2009); dimerization generates an enzymatically active protein that drives membrane fusion after nucleotide hydrolysis and conformational reorganization (Moss et al., 2011). Dynasore, at the dose used...
for cytoprotection in our studies, did not inhibit dimerization. Thus we would not expect that the beneficial activity of ATL3 would be impaired at the concentration of dynasore that we use for cytoprotection.

We previously identified a novel pathway whereby dynasore protects cells by inhibiting Ca2+ influx, shifting activity of the UPR towards homeostasis and preventing opening of the mitochondrial transition pore (mPTP) (Martinez-Carrasco et al., 2020; 2022; Martinez-Carrasco and Fini, 2023). We show here that nitrogen mustard exposure also activates the UPR in our cell culture model. Despite this similarity in mechanism, we found that dynasore does not protect cells against nitrogen mustard exposure. In contrast, mdivi-1, another small molecule that is widely regarded as a specific inhibitor of DNM1L (Cassidy-Stone et al., 2008), does protect. Significantly, mdivi-1 did not shift the UPR activated by nitrogen mustard exposure in our cell culture model, and did not protect against tBHP exposure (Martinez-Carrasco et al., 2020; 2022). In contrast, DRP1i27, a small molecule shown to be a potent and specific inhibitor of DNM1L is cytoprotective in a cell culture model of hydrogen peroxide exposure similar to our own (Rosdah et al., 2022). Together these findings suggest that mdivi-1 acts to protect cells against nitrogen mustard exposure by a different mechanism than the targeting of DNM1L.

The more relevant mechanism of cytoprotection against nitrogen mustard exposure by mdivi-1 may be its recently identified effect on mitochondrial bioenergetics (Bordt et al., 2017; Manczak et al., 2019a; Manczak et al., 2019b). When respiration is uncoupled from ATP production by mitochondrial damage, electrons from ubiquinol are transferred back to respiratory complex I, reducing NAD+ to NADH. This process, called reverse electron transport (RET), generates a substantial amount of ROS and is implicated in various pathological events (Andreyev et al., 2005; Scialo et al., 2017). Rotenone and other complex I inhibitors attenuate pathological RET-ROS production (Chinta et al., 2009; Tretter et al., 2007). Similarly, mdivi-1 also inhibits respiratory complex I. This could explain why mdivi-1 does not protect against
tBHP, as use of tBHP to create oxidative stress circumvents the need for mitochondrial ROS production.

Importantly, mdivi-1 is a relatively unusual complex I inhibitor in that it is not only weak and reversible but also attenuates pathological ROS production at the complex I Q site, while having only limited impact on ROS in healthy cells (Bordt et al., 2017). Our observation that mdivi-1 did not inhibit nitrogen mustard exposure-induced expression of inflammatory markers could possibly reflect the incompleteness of complex 1 attenuation by mdivi-1. Alternatively, our finding may indicate that the ROS involved in stimulating inflammation come from another source. Our future studies will address these proposed mdivi-1 mechanisms.

Evidence has been accumulating for the cytoprotective roles of dynasore in studies utilizing various in vivo challenge models (Chen et al., 2019b; Gao et al., 2013; Li et al., 2017). We now add dry eye epitheliopathy to the growing list of indications for which dynasore might be therapeutic. Similarly, there is currently much interest in mdivi-1 as a potential therapeutic in numerous degenerative diseases, and it has been shown to be protective in several preclinical disease models, including heart/brain ischemia-reperfusion injury (Grohm et al., 2012; Ong et al., 2010), traumatic brain injury (Wu et al., 2016), and Parkinson’s disease (Rappold et al., 2014). The fact that mdivi-1 might selectively target pathological ROS production makes it a uniquely attractive therapeutic drug candidate. Our results reported here suggest the possibility that mdivi-1 could serve as a medical countermeasure for toxic mustard exposure.

Conclusions

We demonstrate here that exposure to tBHP or nitrogen mustard cause corneal epitheliopathy via different pathological pathways. Our results implicate the dynamin DNM2 in ocular surface epitheliopathy for the first time, to our knowledge. The findings suggest that dynasore, which targets DNM2, could serve as as a new topical treatment for dry eye epitheliopathy. We also identified mdivi-1 as a potential medical countermeasure for
epitheliopathy due to nitrogen mustard exposure. No existing medical countermeasures for mustard toxicity target mitochondrial damage. Our results suggest that mdivi-1 might show increased efficacy when combined with anti-inflammatory agents and/or UPR modulators and this should be investigated further.

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The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship Contributions

Participated in research design: Pan, Pany, Martinez-Carrasco, Fini

Conducted experiments: Pan, Pany, Martinez-Carrasco

Performed data analysis: Pan, Pany, Martinez-Carrasco

Wrote or contributed to the writing of the manuscript: Pan, Pany, Martinez-Carrasco, Fini

References


Footnotes

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The authors have declared no conflict of interest exists.

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Correspondance to: M. Elizabeth Fini, Tufts Medical Center, 800 Washinton St., Boston, MA 02111; elizabeth.fini@tuftsmedicine.org

1 New England Eye Center, Tufts Medical Center and Department of Ophthalmology, Tufts University School of Medicine, Boston, Massachusetts, 02111, USA.

2 Program in Pharmacology and Drug Development, Tufts Graduate School of Biomedical Sciences, Tufts University, Boston, MA, USA
### Table 1. Primer sequences for qPCR.

<table>
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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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HUGO nomenclature used for genes; NCBI: National Center for Biotechnology Information; *sXBP1*: alternative spliced form of *XBP1* mRNA
**Figure Legends**

**Figure 1.** Dynasore and Dyngo-4a protect corneal epithelial cells exposed to tBHP *in vitro*:
EC50 determinations and comparison to IC50 for endocytosis.

A. tBHP exposure stress *in vitro*. Left) Chemical structure of dynasore and dyngo-4a. Right) HCLE cells were left unstressed or exposed to 3mm tBHP for 2 hours (stressed). Dynasore (40 µM), dyngo-4a (40 uM) or vehicle were added at the same time as treatment. Cytoprotection was measured by the WST-1 assay. N=3; statistical significance was determined by ANOVA; ns=nonsignificant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

B. Comparison of the EC50 for cytoprotection and the IC50 for endocytosis in HCLE cells treated with dynasore or dyngo-4a.

Top) Examples of EC50 determinations in HCLE cells stressed by exposure to 3 mM tBHP for 2 hours while being treated with dynasore or dyngo-4a at escalating doses. Cytoprotection was measured by the WST-1 assay. N=3.

Middle) Examples of IC50 determinations for clathrin-mediated endocytosis of pseudovirus in HEK293T cells treated with dynasore or dyngo-4a. N=3.

Bottom) Mean EC50 and IC50 determinations. The ‘N’ for each determination is indicated.

**Figure 2.** Dynasore protects corneal epithelial cells exposed to high salt *in vitro*, and also when subjected to a dry eye protocol *in vivo*.

A. High salt exposure stress *in vitro*. HCLE cells were incubated in normal cell culture medium or in hyperosmolar medium (stressed) made by increasing the NaCl concentration to 69 mM in a model of dysfunctional tears. Dynasore (40 µM) or vehicle were added as treatment. After 24 hours, the medium was collected for the LDH assay. N=12

B. Dry eye stress *in vivo*. Left) Schematic of the air-draft-plus-scopolamine dry eye protocol. Right) Mice were left unstressed or subjected to the dry eye stress protocol (stressed), while being treated 4 times per day with vehicle alone or dynasore (20 µM). On the morning of the day
6, clinical fluorescein staining was used to assess epitheliopathy. Stained corneas were photographed and staining was quantified using ImageJ software. A representative image of a stained cornea for each of the experimental groups is shown beneath the graph bar for that group. N=6

Statistical significance was determined by ANOVA; ns=nonsignificant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 3. DNM2 contributes to corneal epitheliopathy due to tBHP exposure

A. Dynamin superfamily gene expression in HCLE cells. RNA was extracted from cultured HCLE cells and qPCR was performed to quantify mRNAs for the following dynamin family subgroups: classic dynamins (DNM1, DNM2, DNM3), mitofusins (MFN1, MFN2) and atlastins (ATL1, ATL2, ATL3). Results were normalized to ACTB. N=3.

B. Dynamin superfamily gene expression at the ocular surface. A public scRNA-seq dataset derived from adult human corneal and conjunctival tissues was mined for expression of dynamin superfamily genes. The violin plot shown depicts comparative quantitative assessment of dynamin gene expression in cells for which markers indicated identity as limbal epithelial cells (Limb. Ep.), corneal epithelial cells (Cor. Ep), conjunctival epithelial cells (Conj. Ep.), melanocytes (Melan.), stromal cells (Stromal), endothelial cells (Endot.), vascular endothelial cells (Vascular), and immune cells (Immune).

C. Assessment of cytoprotection after knockdown. HCLE cells were transfected with 20 nM of one of the cognate DsiRNAs or scramble control and incubated for 3 days. Cells were then stressed by exposure to tBHP (3 mM) or vehicle alone for 2 hours while being treated with 40 µM dynasore or vehicle alone. The cytoprotection was measured by WST-1 assay. N=4; statistical analysis was determined by ANOVA; ns=nonsignificant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

D. Immunoblot. Cells were stressed by exposure to tBHP (3 mM) or vehicle alone (control) for 2
hours while being co-treated with dynasore (80 µM; stress/dynasore) or vehicle alone (stress). Then proteins were extracted and samples were loaded onto a SDS-PAGE gel. The resolved gel was transferred to a nitrocellulose membrane and the blot was probed with ATL3 antibody. Equal loading of gel lanes was determined by probing for ACTB. N=3.

**Figure 4.** The DNM2 inhibitor dynasore does not protect against nitrogen mustard exposure. A) HCLE cells were stressed with nitrogen mustard (NM) at 100 µM or 200 µM, or vehicle alone, for 2 hours. Then RNA was extracted and first strand cDNA was synthesized by reverse transcription. qPCR was performed with cDNA and cognate primers for the mRNAs indicated using SYBR®Green. N=3. B) HCLE cells were stressed by exposure to increasing concentrations of nitrogen mustard (NM; 20-100 µM) or vehicle alone (NM; 0 µM) for 18 hours. Then metabolic activity was measured by WST-1 assay. N=3. C) HCLE cells were stressed by exposure to nitrogen mustard (NM; 100 µM) or vehicle alone, while being cotreated with increasing concentrations of chloroquine (CQ; 5-40 µM) for 20 hours. Then metabolic activity was measure by WST-1 assay. N=3. D) HCLE cells were stressed by exposure to increasing concentrations of nitrogen mustard (50-200 µM NM) or vehicle alone (0 µM NM), while being cotreated with dynasore (40 µM) or vehicle alone for 18 hours. Then metabolic activity was measured by WST-1 assay. N=3. Statistical significance was determined using ANOVA. ns=nonsignificant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

**Figure 5.** The DNM1L inhibitor mDivi-1 protects against nitrogen mustard exposure. A) HCLE cells were stressed by exposure to nitrogen mustard (NM; 100 µM) or vehicle alone, and cotreated with dynasore, mdivi-1, doxycycline (DOX) or N-acetyl cysteine (NAC), each at 50 µM, or vehicle alone. After 20 hours, cytoprotection was measured by WST-1 assay. N=3.
B) HCLE cells were pretreated with mdivi-1 (5-40 µM) or vehicle alone for 1 hour. Then the cells were stressed by exposure to nitrogen mustard (NM; 200 µM) or vehicle alone. After 20 hours, metabolic activity was measured by WST-1 assay. N=3. Statistical significance was determined using ANOVA; ns=nonsignificant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

**Figure 6.** Mdivi-1 does not inhibit UPR activation or inflammatory marker expression induced by nitrogen mustard exposure.

HCLE cells were stressed by exposure to nitrogen mustard (NM; 200 µM) or vehicle alone, while also being treated with mdivi-1 (50 µM) or vehicle alone. Then RNA was extracted and first strand cDNA was synthesized by reverse transcription. qPCR was performed with cDNA and cognate primers for the mRNAs indicated using SYBR®Green. N=3; statistical significance was determined using ANOVA; ns=nonsignificant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Figure 1

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<td>Dynasore</td>
<td>6.2 ± 1.5 µM (n=33)</td>
<td>11.1 ± 5.3 µM (n=4)</td>
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<tr>
<td>Dyngo-4a</td>
<td>10.7 ± 1.5 µM (n=3)</td>
<td>3.8 ± 0.7 µM (n=3)</td>
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Figure 2

A. High Salt Stress; LDH Assay

- Vehicle
- Dynasore
- Vehicle
- Dynasore

Stressed

Relative absorbance

B. Dry Eye Stress; Fluorescein Staining Assay

- Not stressed
- Vehicle
- Dynasore

Stressed

Fluorescein Staining
Figure 3
Figure 4
Figure 5
Figure 6