Tamoxifen Toxicity in Cultured Retinal Pigment Epithelial Cells Is Mediated by Concurrent Regulated Cell Death Mechanisms

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Published Version</td>
<td>10.1167/iovs.13-13662</td>
</tr>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:34902781">http://nrs.harvard.edu/urn-3:HUL.InstRepos:34902781</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>


Tamoxifen Toxicity in Cultured Retinal Pigment Epithelial Cells Is Mediated by Concurrent Regulated Cell Death Mechanisms

Leo A. Kim,1,2 Dhanesh Amarnani,1,2 Gopalan Gnanaguru,1,2 Wen Allen Tseng,1,2 Demetrios G. Vavvas,1 and Patricia A. D’Amore1–3

1Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, United States
2Schepens Eye Research Institute/Massachusetts Eye and Ear Infirmary, Boston, Massachusetts, United States
3Department of Pathology, Harvard Medical School, Boston, Massachusetts, United States

Correspondence: Patricia A. D’Amore, 20 Staniford Street, Boston, MA 02114, USA; patricia_damore@meei.harvard.edu.
Submitted: November 23, 2013
Accepted: June 23, 2014
Citation: Kim LA, Amarnani D, Gnanaguru G, Tseng WA, Vavvas DG, D’Amore PA. Tamoxifen toxicity in cultured retinal pigment epithelial cells is mediated by concurrent regulated cell death mechanisms. Invest Ophthalmol Vis Sci. 2014;55:4747–4758. DOI:10.1167/iovs.13-13662

PURPOSE. To evaluate the mechanism of tamoxifen-induced cell death in human cultured RPE cells, and to investigate concurrent cell death mechanisms including pyroptosis, apoptosis, and necroptosis.

METHODS. Human RPE cells were cultured until confluence and treated with tamoxifen; cell death was measured by detecting LDH release. Tamoxifen-induced cell death was further confirmed by 7-aminoactinomycin D (7-AAD) and annexin V staining. Lysosomal destabilization was assessed using lysosomal-associated membrane protein-1 (LAMP-1) and acridine orange staining. The roles of lysosomal enzymes cathepsin B and L were examined by blocking their activity. Caspase activity was evaluated by caspase-1, -3, -8, and -9 specific inhibition. Cells were primed with IL-1α and treated with tamoxifen; mature IL-1β production was quantified via ELISA. Caspase activity was verified with the fluorochrome-labeled inhibitor of caspases (FLICA) probe specific for each caspase. Regulated cell necrosis or necroptosis was examined with 7-AAD and inhibition of receptor-interacting protein 1 (RIP1) kinase using necrostatin-1 (Nec-1).

RESULTS. Cell death occurred within 2 hours of tamoxifen treatment of confluent RPE cells and was accompanied by lysosomal membrane permeabilization. Blockade of cathepsin B and L activity led to a significant decrease in cell death, indicating that lysosomal destabilization and cathepsin release occur prior to regulated cell death. Tamoxifen-induced toxicity was shown to occur through both caspase-dependent and caspase-independent cell death pathways. Treatment of RPE cells with caspase inhibitors and Nec-1 resulted in a near complete rescue from cell death.

CONCLUSIONS. Tamoxifen-induced cell death occurs through concurrent regulated cell death mechanisms. Simultaneous inhibition of caspase-dependent and caspase-independent cell death pathways is required to protect cells from tamoxifen. Inhibition of upstream activators, such as the cathepsins, may represent a novel approach to block multiple cell death pathways.

Keywords: apoptosis, necroptosis, pyroptosis, RPE, tamoxifen

Tamoxifen, a nonsteroidal estrogen receptor antagonist, has been widely used in low dosages as an adjuvant therapy for some forms of breast cancer. Although the current standard of care is 5 years of tamoxifen therapy, the global Adjuvant Tamoxifen: Longer Against Shorter (ATLAS) trial recently showed that 10 years of tamoxifen treatment reduced the risk of breast cancer recurrence, reduced breast cancer mortality, and reduced overall mortality.1 Thus, it is likely that the standard of care will change, resulting in a longer period of tamoxifen therapy with a likely increase in tamoxifen-induced ocular toxicity.

Tamoxifen can lead to corneal toxicity, progression of cataracts, retinopathy, and neuropathy. The reported incidence of ocular toxic side effects among patients receiving tamoxifen ranges from 6.3% to 12%. The most visually significant aspect of tamoxifen toxicity is a maculopathy.2–3 Tamoxifen is structurally similar to other drugs with well-known retinal effects including chloroquine, chlorpromazine, thioridazine, and tilorone. Chloroquine, specifically, has been shown by others to disrupt lysosomal function and disrupt phagocytosis in ARPE-19 cells.4 Although the RPE is thought to be the primary target of tamoxifen toxicity, recent reports have demonstrated that tamoxifen toxicity also affects photoreceptors.5 Ultrastructural lesions associated with these agents may appear as crystalloid inclusions in the neuroretina or as crystalloid bodies, within the RPE.6,7 which are thought to disrupt lysosomal function.

Retinal pigment epithelium cells serve a critical role in the maintenance of photoreceptors, phagocytizing the outer segment tips of photoreceptors, which are then digested within lysosomes. Retinal pigment epithelium dysfunction is thought to play a role in a variety of retinal diseases including AMD, tamoxifen retinopathy, chloroquine retinopathy, central...
serous retinopathy, as well as a variety of inherited retinal disorders. Since RPE cells are postmitotic, controlling or preventing cell death in these conditions may help to prevent subsequent photoreceptor degeneration and vision loss.

Cell death commonly occurs through pyroptosis, apoptosis, or necroptosis. Each of these cell death pathways is regulated by distinct molecular mechanisms. For instance, pyroptosis is an inflammatory-mediated form of cell death characterized by activation of caspase-1. During pyroptosis, there is an assembly of the inflammasome, a multiprotein complex that activates caspase-1 by facilitating the cleavage of procaspase-1 to active caspase-1. In turn, active caspase-1 mediates the proteolytic maturation of the pro-inflammatory cytokines IL-1β and IL-18 from their inactive precursors to their biologically active forms. The NLRP3 inflammasome can be activated by a diverse array of signals. Many of these signals activate NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes. For instance, crystalline or insoluble materials such as cholesterol crystals and amyloid-NLRP3 by destabilizing lysosomes.

In the case of apoptosis, coordinated activation of two groups of caspases: initiators (caspase-2, -8, -9, and -10) and effectors (caspase-3, -6, and -7) are responsible for apoptotic cellular degradation. This type of cell death is characterized by cell shrinkage, nuclear and cytoplasmic condensation, and chromatin fragmentation. Caspases are constitutively present in an inactive precursor form and are cleaved from procaspases to activated caspases; it is the effector caspases that are responsible for cellular degradation. Caspase-3 is the major effector caspase in apoptosis and is activated by several initiator caspases upstream in the signal cascade. Apoptosis can be further divided into various subcategories according to both the stimuli and the pathways leading to execution of cell death. In extrinsic apoptosis, cell death is initiated by caspase-8 after the binding of lethal ligands to their respective death receptor. Once activated, caspase-8 cleaves downstream procaspases by activation of BID (BH3-interacting domain death agonist) and inducing mitochondrial cytochrome c release. In intrinsic apoptosis, caspase-9 activation is triggered by intracellular stress, such as DNA damage, oxidative stress, or excitotoxicity, rather than binding of an extrinsic ligand to a death receptor. Activation of caspase-9 leads to mitochondria-mediated activation and cytochrome c release into the cytosol. Although caspase-8 and caspase-9 represent two distinct apoptotic signaling pathways, both have been shown to activate caspase-3.

Necroptosis is characterized by the activation of receptor-interacting protein 1 (RIP1) and RIPK kinase and is triggered by a variety of stimuli including TNE DNA damage, and viral infection. Cellular components or endogenous adjuvants, such as high-mobility group protein B1, uric acid, gallocateins, and thioredoxin, released as a consequence of cellular demise, promote an inflammatory response with activation of inflammasomes, cytokine production, inflammatory cell recruitment, and T-cell activation. Necroptosis has been defined as caspase-independent cell death with a necrotic phenotype that can be prevented by the specific RIP1 inhibitor necrostatin-1 (Nec-1). Necroptosis has been demonstrated to occur in T lymphocytes, photoreceptors, RPE cells, astrocytes, and neurons and has been suggested to be involved in myocardial infarction.

Tamoxifen toxicity of the retina is believed to be mediated by damage to the RPE through disruption of lysosomes. Our laboratory and others have recently demonstrated that RPE cells express components of the NLRP3 inflammasome, which is believed to play a role in AMD through lysosomal destabilization or accumulation of Abβ RNA resulting from DICER1 deficiency in the RPE. We hypothesize that prolonged use of medications such as tamoxifen can disrupt lysosomal membranes, leading to the activation of the NLRP3 inflammasome, release of the pro-inflammatory cytokine IL-1β, and pyroptosis. Here, we report on the involvement of multiple cell death mechanisms in tamoxifen-induced toxicity of the human RPE in culture. Specifically, we examined the roles of inflammasome-mediated pyroptosis, the extrinsic and intrinsic pathways of apoptosis, and RIP kinase-mediated necroptosis.

**MATERIALS AND METHODS**

**Cell Culture of Human ARPE-19 Cells**

Human ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), 2 mM L-glutamine (Lonza, Hopkinton, MA, USA), and 100 U/mL penicillin-100 μg/mL streptomycin (Lonza, Hopkinton) in a humidified incubator at 37°C, 10% CO₂, and passaged at a ratio of 1:2 to 1:4 using 0.25% trypsin-EDTA (Invitrogen, Carlsbad, CA). The ARPE-19 cells that were grown on cover slips were plated at approximately 6.0 × 10⁴ cells on 10 μg/mL laminin-coated 12-mm glass cover slips (Sigma-Aldrich Corp., St. Louis, MO, USA) and maintained in the above-mentioned medium until cells were confluent (usually 2 days post plating). The postconfluent cells were maintained in DMEM/F12 medium supplemented with 1% FBS, 2 mM L-glutamine, and 100 U/mL penicillin-100 μg/mL. Cells were used for experiments up to 2 weeks postconfluence.

**Cell Culture of Primary Fetal Human RPE (fhRPE)**

Primary fetal human RPE cells (Lonza, Walkersville) were cultured in RPE medium (RtEBM; Lonza, Walkersville) with 5% FBS, 2 mM L-glutamine, and 100 U/mL penicillin-100 μg/mL streptomycin in a humidified incubator at 37°C, 5% CO₂. These cells were plated at high density on laminin-coated 96-well plates or 0.4-μm polystyrene membrane Transwells coated with laminin (Costar; Corning, Inc., NY, USA) and maintained in RtEBM with 1% FBS medium, 2 mM L-glutamine, and 100 U/mL penicillin-100 μg/mL streptomycin. After 2 weeks of culture, the cells were used for the experiments.

**Tamoxifen Treatment**

Tamoxifen (Sigma-Aldrich Corp.) stock solution (108 mM) was prepared in absolute ethanol, according to the manufacturer’s recommendation. Cells were treated with different doses of tamoxifen (10, 15, 20, 25, and 30 μM) or 4-OH-tamoxifen (Supplementary Fig. S1). Additionally, ARPE-19 cells were cultured and maintained in 12-well plates and treated with 2, 4, and 8 μM tamoxifen for a period of 2 weeks (Supplementary Fig. S2). The controls received serum free-media containing the same amount of absolute ethanol without tamoxifen. Cytotoxicity of tamoxifen was measured by LDH release.

**Assessment of Cytotoxicity**

The conditioned media were collected from ARPE-19 and fhRPE cells of each condition after 120 minutes of treatment, and percentage cell death was quantified by measuring the LDH release of the pro-inflammatory cytokine IL-1β, and pyroptosis.
the conditioned media were collected to measure the maximum LDH release. Percentage LDH release was calculated as 100% × (experimental LDH − spontaneous LDH)/(maximal LDH − spontaneous LDH). The early detection of LDH release was observed at 20 to 30 μM; therefore, these concentrations were used to perform all our experiments described below.

**Assessment of Cytoskeletal Changes**

Human ARPE-19 cells were seeded on cover slips and treated with or without tamoxifen. The cells were fixed at 15, 30, 45, 60, 90, 120, and 180 minutes using 3% paraformaldehyde, permeabilized using 0.1% Triton X-100, and blocked (3% goat serum, 3% BSA in PBS) for 60 minutes at room temperature (RT). Between each of the steps, the cells were washed with PBS. The cells were then incubated with AlexaFluor 594 phalloidin (1:100) in blocking buffer for 20 minutes, washed, and then the cover slips were mounted using mounting media with Prolong Gold Antifade Reagent with DAPI (Life Technologies, Carlsbad, CA, USA). Images were taken using a Leica AF6000 microscope (Leica, Wetzlar, Germany).

**Immunofluorescence**

Human ARPE-19 cells seeded on laminin-coated cover slips and fhrPE cells seeded on Transwells were treated with tamoxifen or control medium, washed, and fixed with 4% paraformaldehyde for 5 minutes. Cells were thoroughly washed in PBS and blocked (10% goat serum or PBS, 0.05% Triton X-100 in PBS) for 60 minutes at RT. Tight junctions and lysosomal integrity were assessed by incubating cells with anti-ZO-1 for the presence of zonula occludens (ZO-1; 1:250 dilution, Life Technologies) or anti-lysosomal-associated membrane protein-1 (LAMP-1; 1:100 dilution, Cell Signaling Technologies, Beverly, MA, USA), respectively. Each primary antibody was prepared in antibody dilution buffer (5% goat serum or PBS and 0.025% Triton X-100 in PBS) and incubated overnight at 4°C. After washing in PBS, cells were incubated with goat anti-rabbit secondary antibody (Life Technologies, Germany) at 1:250 dilution, Life Technologies (Carlsbad, CA, USA), respectively. Each primary antibody was prepared in antibody dilution buffer (5% goat serum or PBS and 0.025% Triton X-100 in PBS) and incubated overnight at 4°C. After washing in PBS, cells were incubated with goat anti-rabbit secondary antibody (Life Technologies) at 1:250 dilution for 2 hours at RT. The cover slips were washed and mounted onto slides using Prolong Gold Antifade Reagent with DAPI.

**Quantification of LAMP-1 Immunofluorescence**

In order to quantify LAMP-1 immunofluorescence, images of LAMP-1 staining in control and tamoxifen-treated ARPE-19 cells were taken at 0, 30, 90, and 120 minutes using a Leica AF6000 microscope. Within each image, 10 random fields were chosen, and the LAMP-1 fluorescence was measured using Imagej (Version 1.46; National Institutes of Health, Bethesda, MD, USA). A threshold value was set for red fluorescence intensity using Imagej; this value was kept constant for quantification of LAMP-1 fluorescence in control and tamoxifen-treated cells. The DAPI-positive nuclei were counted manually.

**Acridine Orange Staining (Supplementary)**

Acridine orange staining was performed as previously described to further confirm lysosomal destabilization. Cells were incubated with 5 μM acridine orange (Immunocytochemistry Technologies, Bloomington, MN, USA) for 30 minutes at 37°C. Cells were washed with PBS followed by treatment with tamoxifen or control medium for 120 minutes. Images of wells were taken over the course of 2 hours on a Nikon Eclipse TE2000 S-microscope (Nikon Instrumentations, Inc., Melville, NY, USA) to evaluate the progression of lysosomal destabilization with time.

**Detection of Necrosis and Apoptosis**

Human ARPE-19 cells were seeded onto laminin-coated cover slips at a density of 2.5 × 10⁴ cells/cover slip (day 0) and grown to confluence. After 2 to 3 weeks in culture, cells were treated with tamoxifen or control medium. Cells were washed with serum-free medium at 30, 60, 90, and 120 minutes and then incubated with fluorescent-conjugated 7-aminoactinomycin D (7-AAD), a dye for cell viability and early necrosis, (1:100; Enzo Life Sciences, Farmingdale, NY, USA) or the apoptosis marker annexin V (1:50; Life Technologies) for a period of 30 minutes at 37°C. The cells were washed and fixed with 2% paraformaldehyde for 5 minutes followed by washes with PBS. The cover slips were mounted onto slides using Prolong Gold Antifade Reagent with DAPI. Images were taken using a Leica AF6000 microscope for analysis and identification of 7-AAD and annexin V-positive cells.

**Detection of Caspase Activation**

Activation of various caspases was measured using the fluorochrome-labeled inhibitor of caspases (FLICA) probes for caspase-1 (FAM-YVAD-FMK), caspase-3 (FAM-DEVD-FMK), caspase-8 (FAM-IETD-FMK), and caspase-9 (FAM-LEHD-FMK) (Immuno histochemistry Technologies), as previously described. The cells were protected from light and pretreated with FLICA for 2 hours prior to incubation with tamoxifen or control media at 37°C, 10% CO₂. Following incubation with tamoxifen or control media for 60, 90, and 120 minutes, samples were washed, and the cell nuclei were stained with Hoechst 33342 (Immuno histochemistry Technologies). The samples were washed and fixed, using wash buffer and fixative supplied with the FLICA Kit, and imaged using a Leica AF6000 microscope.

**Inhibition of RIP1 Kinase, Caspases, and Cathepsins**

Human ARPE-19 cells maintained in 12-well plates were treated with tamoxifen alone or in combination with inhibitors as follows: RIP1 kinase inhibitor, necrostatin-1 (30 μM; Sigma-Aldrich Corp.); pan-caspase inhibitor, Z/VAD-FMK, caspase-1 inhibitor Z/YVAD-FMK, caspase-3 inhibitor Z/DEVD-FMK, caspase-8 inhibitor Z/IETD-FMK, caspase-9 inhibitor Z/LEHD-FMK (10 μM; all from BioVision, Mountain View, CA, USA); cathepsin B/L inhibitor Z-FFMK, specific cathepsin B inhibitor CA-074-Me, and/or cathepsin L inhibitor Z-FY(1-Bu)-FMK (20 μM; all from EMD Millipore, Billerica, MA, USA). After 120 minutes of treatment, cells were washed and imaged using the Nikon Eclipse TE2000 S-microscope.

**Quantification of IL-1β Secretion**

Human ARPE-19 cells were seeded onto 12-well plates in complete media and grown to confluence. The cells were primed for 48 hours with 10 ng/mL IL-1α as described previously. After 48 hours, the cells were treated with treated with 10, 20, and 30 μM tamoxifen. Dimethyl sulfoxide (DMSO) vehicle was used as negative control; and 1 mM L-leucyl-L-leucine methyl ester (Leu-Leu-OMe; Chem-Impex International, Wood Dale, IL, USA) was used as positive control, as it had been shown previously to induce release of mature IL-1β. Conditioned media were collected after 3 hours, and IL-1β was quantified via ELISA (BD Biosciences, Franklin, NJ, USA). Values were corrected for release of pro-IL-1β because of cytotoxicity using the method described by Miao and colleagues.
Western Blots (Supplementary)

Human ARPE-19 cells were lysed using 1× radio-immunoprecipitation assay (RIPA) buffer (Cell Signaling Technologies) containing Complete Mini EDTA-free Protease Inhibitor Tablet (Roche, Indianapolis, IN, USA), 2 mM phenyl methanesulfonyl fluoride (PMSF), and 2 mM sodium orthovanadate (NaOV), and then sonicated on ice. Protein concentrations were measured, and equal concentrations of protein were separated using 10% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked for 1 hour at RT in 3% BSA in Tris-buffered saline with 0.1% Tween 20 (TBS-T), followed by incubation with anti-RIP3 antibody (1:1000 in 1% BSA; Abcam, Cambridge, MA, USA) overnight at 4°C. After washes in TBS-T, membranes were incubated for 1 hour at RT in horseradish peroxidase (HRP)-linked secondary antibodies anti-mouse and anti-rabbit IgG (1:10,000 in blocking buffer; GE Healthcare, Pittsburgh, PA, USA). Following additional washes in TBS-T, proteins were visualized by enhanced chemiluminescence using SuperSignal substrates (Thermo Scientific, Tewksbury, MA, USA). Membranes were stripped and then reprobed with rabbit anti-RIP1 (1 μg per 1 mg of protein; BD Biosciences) overnight at 4°C, washed with TBS-T, and incubated for 1 hour at RT with HRP-linked secondary antibodies prior to visualization with enhanced chemiluminescence.

Data Analysis and Statistics

Data are presented as mean ± SEM of at least three independent experiments unless otherwise indicated. Statistical significance was evaluated using a one-way ANOVA followed by post hoc Tukey-Kramer multiple comparison tests, using the Prism 6.0 software package (GraphPad Software, Inc., La Jolla, CA, USA). Adjusted P values < 0.05 were considered statistically significant.

RESULTS

Tamoxifen Induces Cell Death in Cultured ARPE-19 and fhRPE Cells

Several studies indicate that tamoxifen induces cytotoxicity that specifically affects RPE and photoreceptor cells. In order to investigate the mechanism of tamoxifen-induced RPE cell death, we treated ARPE-19 cells and primary fhRPE cells with different doses of tamoxifen. This study revealed that tamoxifen induced RPE cytotoxicity in a dose-dependent manner (Figs. 1A, 1B). Two-hour exposure of confluent ARPE-19 cells to 10 and 15 μM tamoxifen had little effect on cell viability, whereas exposure to 20, 25, and 30 μM tamoxifen induced 40% to 70% LDH release. Two-hour exposure of confluent fhRPE cells to 10 and 20 μM tamoxifen had little effect on cell viability, whereas exposure to 30 μM tamoxifen...
resulted in 85% LDH release (Fig. 1B). Exposure to 4-OH-tamoxifen, one of the primary active metabolites of tamoxifen metabolized by CYP2D6, induced LDH release in a dose-dependent fashion (Supplementary Fig. S1). Two-hour exposure of cells to 10 to 20 μM 4-OH-tamoxifen had limited LDH release, whereas exposure of confluent ARPE-19 cells to 30 to 60 μM 4-OH-tamoxifen induced 48% to 98% LDH release. Long-term exposure (over 2 weeks) of 2, 4, and 8 μM tamoxifen induced 19%, 19%, and 79% of LDH release, respectively (Supplementary Fig. S2).

In addition, tamoxifen-treated cells exhibited features of cell death including swollen cell bodies, shrinkage, blebbing, and detachment from the culture substrate (Fig. 1C). Visualization of actin cytoskeleton and ZO-1 distribution further validated these observations (Figs. 1D, 1E). Phalloidin staining displayed intact cytoskeletal organization in controls, whereas tamoxifen treatment resulted in a loss of actin stress fibers by 60 minutes of treatment (Fig. 1D). Similarly, differentiated 2-week-old ARPE-19 and fhRPE cells exposed to tamoxifen and stained with antibodies to ZO-1 revealed loss of ZO-1 staining upon exposure to tamoxifen (Fig. 1E).

Figure 2. Tamoxifen toxicity causes lysosomal destabilization. (A) LAMP-1 (red) was localized to lysosomes in ARPE-19 cells by immunofluorescence in control cells (top left), and a progressive loss of LAMP-1 staining was observed with 30, 90 and 120 minutes of tamoxifen treatment (top right and bottom). Scale bar: 25 μm. (B) Quantification of LAMP-1 staining showed a statistically significant decrease in LAMP-1 fluorescence after 90 minutes, with no significant difference in cell counts when compared with the control. After 120 minutes, there was a statistically significant decrease in both LAMP-1 fluorescence and cell count as determined by DAPI+ nuclei. Error bars: ±SEM. Adjusted P values: ***P < 0.005, ****P < 0.0001.

Tamoxifen-Induced Cell Death of the RPE

Our lab, as well as others, has demonstrated that disruption of lysosomal integrity induces RPE cell death through pyroptosis.36 To test if tamoxifen treatment induces RPE cell death through lysosomal destabilization, lysosomal integrity was assessed by analyzing LAMP-1 immunofluorescence staining in differentiated ARPE-19 cells. Time-course studies showed a statistically significant reduction of LAMP-1 staining at 90 minutes when compared with the initial control time point (252 vs. 625 AU, respectively), with no significant decrease in
DNA.44 There was a time-dependent increase in 7-AAD staining as an early necrosis marker that selectively binds to GC regions of DNA.44 The time course of 7-AAD staining was further assessed by acridine orange staining, which labels nucleic acids in green and lysosomes in red. Of interest, we found that at 60 minutes, only a subset of cells stained positive for 7-AAD.

Apoptotic and Necrotic Cell Death Occur Following Tamoxifen Treatment

The data thus far indicate that tamoxifen treatment leads to RPE cytoskeletal disruption resulting in cell death (Figs. 1, 2), but it is unclear whether tamoxifen triggers a specific cell death pathway. To investigate the mechanism of cell death, we used annexin V, which recognizes phosphatidylserine exposed on the cell surface during apoptosis, on ARPE-19 cells cultured for 2 weeks on laminin-coated coverslips.42 Staining with annexin V revealed apoptotic cell death with increasing fluorescence upon application of tamoxifen when compared with the control (Fig. 3, top row). Of interest, we found that at 60 minutes, only a subset of cells stained with annexin V. To test if tamoxifen induces other possible mechanisms of cell death, we analyzed for 7-AAD,43 an early necrosis marker that selectively binds to GC regions of DNA.44 There was a time-dependent increase in 7-AAD staining upon exposure to tamoxifen and no 7-AAD staining in the controls (Fig. 3, bottom row). As with annexin V staining, at 60 minutes, only a subset of cells stained positive for 7-AAD. Together, these data suggest that tamoxifen induces RPE cell death through multiple cell death pathways, as both necrosis and apoptosis appear to be activated following tamoxifen treatment.

Tamoxifen Activates Caspases That Mediate Pyroptosis and Apoptosis in ARPE-19 Cells

We showed that tamoxifen treatment destabilizes lysosomes (Fig. 2). Destabilization of lysosomes has been shown to induce the NLRP3 inflammasome through the activation of caspase-1,45 which has been shown to mediate pyroptosis.45 We therefore used a fluorescence-based FLICA assay to investigate if tamoxifen treatment activates caspase-1 or any other caspases that mediate cell death. Caspase-1 was activated within 60 minutes of tamoxifen exposure, with increasing levels of staining at 90 and 120 minutes (Fig. 4, row 1). No caspase-1 activation was observed in the controls (Fig. 4, row 1). We then evaluated the activation of apoptosis initiators caspase-8 and caspase-9, with and without tamoxifen treatment. The control cells showed no activation of either caspase-8 or caspase-9 during the experimental time period (Fig. 4, rows 2 and 3), whereas both caspase-8 and caspase-9 activation was observed after 60, 90, and 120 minutes of tamoxifen treatment (Fig. 4, rows 2 and 3). Activation of caspase-8 and caspase-9 leads to the activation of the effector caspase-3, which we observed to be minimally activated at 60 minutes, but more pronounced staining was observed at 90 and 120 minutes (Fig. 4, row 4). These results demonstrate that tamoxifen toxicity activates caspase-mediated cell death pathways including pyroptosis and apoptosis.

Combinatorial Inhibition of Necroptosis, Pyroptosis, and Apoptosis Prevents Tamoxifen-Induced Cell Death

Our results indicate that RPE cell death is mediated through caspase-dependent and caspase-independent mechanisms. To test the relative contribution of the various mechanisms to tamoxifen-induced RPE cell death, we treated ARPE-19 cells with inhibitors specifically targeted against pyroptosis, apoptosis, and necroptosis in the presence of 20 μM tamoxifen.

ARPE-19 cells express the mediators of necroptosis, RIP1, and RIP3 kinases (Supplementary Fig. S4), and treatment of tamoxifen activates the early necrosis marker 7-AAD (Fig. 5). Therefore, we tested the effect of Nec-1, a selective inhibitor of RIP1 kinase, on tamoxifen-induced ARPE-19 cell death. The treatment of Nec-1 in the presence of tamoxifen reduced LDH release by 80% when compared with the tamoxifen only–treated control, which showed 73% LDH release (Fig. 5A).

We next analyzed the role of the various caspases in tamoxifen-induced ARPE-19 cell death. Of interest, treatment...
with a pan-caspase inhibitor did not prevent tamoxifen toxicity (Fig. 5A); this could be due to a nonspecific or off-target effect, such as activation of alternate cell death pathways. In contrast, treatment of ARPE-19 cells with the caspase-1–specific inhibitor (Z-YVAD-FMK) and the caspase-3 inhibitor (Z-DEVD-FMK) partially rescued ARPE-19 cells from tamoxifen toxicity (Fig. 5A). Treatment of ARPE-19 with caspase-1 and -3 inhibitors led to a reduction in LDH release, with 50% and 40% LDH release, respectively. Concurrent treatment of Nec-1 with either pan-caspase, caspase-1, or caspase-3 inhibitors resulted in more effective inhibition of cell death when compared with the individual caspase inhibitors alone after 2 hours of treatment with tamoxifen (Figs. 5A, 5B; Supplementary Fig. S5). We also investigated whether the initiators of apoptosis, caspase-8 or -9 play a role in tamoxifen-induced RPE cell death. In contrast, treatment of ARPE-19 cells with the caspase-1–specific inhibitor (Z-YVAD-FMK) and the caspase-3 inhibitor (Z-DEVD-FMK) partially rescued ARPE-19 cells from tamoxifen toxicity (Fig. 5A). Treatment of ARPE-19 with caspase-1 and -3 inhibitors led to a reduction in LDH release, with 50% and 40% LDH release, respectively. Concurrent treatment of Nec-1 with either pan-caspase, caspase-1, or caspase-3 inhibitors resulted in more effective inhibition of cell death when compared with the individual caspase inhibitors alone after 2 hours of treatment with tamoxifen (Figs. 5A, 5B; Supplementary Fig. S5). We also investigated whether the initiators of apoptosis, caspase-8 or -9 play a role in tamoxifen-induced RPE cell death. Inhibition of caspase-8 did not decrease LDH release, while caspase-9 inhibition significantly decreased LDH release when compared with tamoxifen control (Fig. 5C). Treatment with a combination of Nec-1 and either caspase-8 or caspase-9 inhibitor more effectively blocked tamoxifen-induced LDH release than use of any of the inhibitors alone (Fig. 5C).

We speculate that the release of lysosomal cathepsins is the upstream activator of cell death pathways. This is supported by the observation that the dual cathepsin B/L inhibitor (Z-FF-FMK) significantly reduced tamoxifen toxicity, reducing LDH release by 90% (Fig. 5A). Next, we tested whether inhibition of cathepsin B or L alone could block tamoxifen toxicity. Treatment of tamoxifen-treated ARPE-19 cells with a cathepsin B (CA-074-Me) or cathepsin L (Z-FY[t-Bu]-DMK) inhibitor each significantly reduced LDH release (Fig. 5D), but inhibition of both cathepsin B and L almost completely blocked LDH release (Fig. 5D)46.

**Caspase-1 Activation Leads to Production of IL-1β**

The activation of caspase-1 can induce pyroptosis as well as lead to the release of the pro-inflammatory cytokine, mature IL-1β. To investigate if the activation of caspase-1 resulting from tamoxifen toxicity also leads to mature IL-1β release, ARPE-19 cells were primed with IL-1α for 24 hours to induce expression of IL-1β precursor, pro-IL-1β. We were unable detect mature IL-1β release in unprimed ARPE-19 cells treated with tamoxifen.
Treatment of primed ARPE-19 cells with different concentrations of tamoxifen resulted in the release of mature IL-1β in a dose-dependent manner (Fig. 6A). Maximal production of mature IL-1β (8.6 pg/mL) was observed with 30 μM tamoxifen treatment (Fig. 6A). Nec-1 treatment did not significantly alter IL-1β production, but inhibition of caspase-1 resulted in a 64% decrease in mature IL-1β secretion from 17.6 pg/mL to 6.4 pg/mL (Fig. 6B).

**DISCUSSION**

It has been shown that tamoxifen toxicity can lead to visual defects and is likely associated with damage to RPE and photoreceptors. The goal of this study was to investigate the mechanism by which tamoxifen induces RPE cell death and identify targets that can be used as a potential therapy. We examined the mechanism of tamoxifen toxicity in cultured RPE cells and demonstrated that tamoxifen-induced lysosomal destabilization is associated with the activation of a number of cell death pathways. Initial lysosomal destabilization in ARPE-19 cells was followed by release of cathepsins, which have been shown to play a major role in initiating multiple cell death mechanisms: pyroptosis, apoptosis, and necroptosis. Dual inhibition of cathepsins B and L completely prevented tamoxifen-induced RPE cell death. Cell death can occur through several different mechanisms. One event that may initiate cell death is lysosomal destabilization. Permeabilization of lysosomal membranes leads to the release of hydrolases such as the cathepsins, which have been...
necroptosis can arrest with application of cathepsin-B inhibitor CA-074-OME.55 Similarly, we have shown that combined cathepsin B and L inhibition leads to complete inhibition of cell death, and that cathepsin release occurs upstream of multiple cell death mechanisms. Necroptosis depends on the activity of the serine/threonine kinase RIP1, an adaptor kinase that functions downstream of death domain receptors and forms a complex with RIP3 to activate necroptosis. It is interesting to note that inhibition of RIP3 kinase activity will re-shuttle cell death toward apoptosis as catalytically inactive RIP3 kinase mutant engages RIP1, Fas-associated death domain (FADD), and caspase-8 complex and leads to caspase-8 activation, suggesting redundancy of cell death pathways.56 Exposure of RPE cells to tamoxifen led to the activation of RIP1 and the initiation of necroptosis. As previously demonstrated in photoreceptors in a model of retinal detachment, cotreatment with caspase inhibitors and Nec-1 effectively suppresses photoreceptor cell death.57,58 Accordingly, we found that suppression of tamoxifen cytotoxicity of the RPE requires concurrent treatment with caspase inhibitors and Nec-1.

Pathologic activation of the NLRP3 inflammasome and pyroptosis is characteristic of a variety of disease conditions in which lysosomal destabilization results from phagocytosis of crystals or insoluble aggregates.16,17 For instance, silicosis and asbestosis are mediated by NLRP3 inflammasome activation in myeloid-derived cells.18 Our observations that tamoxifen induces lysosomal destabilization prior to cell death and that inhibition of cathepsins B and L lead to almost complete blockade of tamoxifen-induced cytotoxicity are consistent with these previous reports.

In addition, cathepsins have been implicated in the process of apoptosis. Cytosolic cathepsins cleave the pro-apoptotic Bcl-2 family member BID, resulting in its activation, and degrade anti-apoptotic protein Bcl-2 proteins, triggering the intrinsic pathway of apoptosis.50,51 Caspase-8 and -9 mediate the extrinsic and intrinsic pathways of apoptosis, respectively; and both of these initiator caspases can activate caspase-3. We demonstrate, as shown by others,5 a significant role for the effector caspase-3 in tamoxifen cytotoxicity. Further, we have shown a primary role for the caspase-9–dependent intrinsic pathway, an observation that is indicative of mitochondrial dysfunction. This is in agreement with the finding that necroptosis can induce mitochondrial dysfunction, reactive oxygen species (ROS) formation,53,54 and release of pro-apoptotic proteins of the Bcl-2 family.55 Although the extrinsic pathway, which is caspase-8 dependent, does not seem to play a primary role in tamoxifen-mediated toxicity, others have reported that inhibition of caspase-8 activates RIP1 kinase.55,56 Consistent with this finding, cotreatment with Nec-1 and a caspase-8 inhibitor led to near complete inhibition of cell death, greater than Nec-1 alone.

Caspase-8 and -9 mediate the intrinsic and extrinsic pathways of apoptosis, respectively; and both of these initiator caspases can activate caspase-3. We demonstrate, as shown by others,5 a significant role for the effector caspase-3 in tamoxifen cytotoxicity. Further, we have shown a primary role for the caspase-9–dependent intrinsic pathway, an observation that is indicative of mitochondrial dysfunction. This is in agreement with the finding that necroptosis can induce mitochondrial dysfunction, reactive oxygen species (ROS) formation, and release of pro-apoptotic proteins of the Bcl-2 family. Although the extrinsic pathway, which is caspase-8 dependent, does not seem to play a primary role in tamoxifen-mediated toxicity, others have reported that inhibition of caspase-8 activates RIP1 kinase.55,56 Consistent with this finding, cotreatment with Nec-1 and a caspase-8 inhibitor led to near complete inhibition of cell death, greater than Nec-1 alone.

Caspase-8 and -9 mediate the intrinsic and extrinsic pathways of apoptosis, respectively; and both of these initiator caspases can activate caspase-3. We demonstrate, as shown by others,5 a significant role for the effector caspase-3 in tamoxifen cytotoxicity. Further, we have shown a primary role for the caspase-9–dependent intrinsic pathway, an observation that is indicative of mitochondrial dysfunction. This is in agreement with the finding that necroptosis can induce mitochondrial dysfunction, reactive oxygen species (ROS) formation, and release of pro-apoptotic proteins of the Bcl-2 family.55 Although the extrinsic pathway, which is caspase-8 dependent, does not seem to play a primary role in tamoxifen-mediated toxicity, others have reported that inhibition of caspase-8 activates RIP1 kinase.55,56 Consistent with this finding, cotreatment with Nec-1 and a caspase-8 inhibitor led to near complete inhibition of cell death, greater than Nec-1 alone.

Caspase-8 and -9 mediate the intrinsic and extrinsic pathways of apoptosis, respectively; and both of these initiator caspases can activate caspase-3. We demonstrate, as shown by others,5 a significant role for the effector caspase-3 in tamoxifen cytotoxicity. Further, we have shown a primary role for the caspase-9–dependent intrinsic pathway, an observation that is indicative of mitochondrial dysfunction. This is in agreement with the finding that necroptosis can induce mitochondrial dysfunction, reactive oxygen species (ROS) formation, and release of pro-apoptotic proteins of the Bcl-2 family.55 Although the extrinsic pathway, which is caspase-8 dependent, does not seem to play a primary role in tamoxifen-mediated toxicity, others have reported that inhibition of caspase-8 activates RIP1 kinase.55,56 Consistent with this finding, cotreatment with Nec-1 and a caspase-8 inhibitor led to near complete inhibition of cell death, greater than Nec-1 alone.

Caspase-8 and -9 mediate the intrinsic and extrinsic pathways of apoptosis, respectively; and both of these initiator caspases can activate caspase-3. We demonstrate, as shown by others,5 a significant role for the effector caspase-3 in tamoxifen cytotoxicity. Further, we have shown a primary role for the caspase-9–dependent intrinsic pathway, an observation that is indicative of mitochondrial dysfunction. This is in agreement with the finding that necroptosis can induce mitochondrial dysfunction, reactive oxygen species (ROS) formation, and release of pro-apoptotic proteins of the Bcl-2 family.55 Although the extrinsic pathway, which is caspase-8 dependent, does not seem to play a primary role in tamoxifen-mediated toxicity, others have reported that inhibition of caspase-8 activates RIP1 kinase.55,56 Consistent with this finding, cotreatment with Nec-1 and a caspase-8 inhibitor led to near complete inhibition of cell death, greater than Nec-1 alone.
mitochondrial gene and protein expression,\(^5\) and \(E2\) is considered an anti-apoptotic agent, including pro-survival actions on human RPE challenged with oxidative stress.\(^6\) In contrast, others have demonstrated ER-independent autophagic cell death by tamoxifen in breast cancer cells\(^6\) and RPE cells.\(^1\) In addition, \(E2\)-mediated cytoprotection of ARPE-19 cells was inhibited by ER antagonists ICI (ER\(z\)) and ER\(\beta\) and THC (ER\(\beta\)) but not by tamoxifen (ER\(\alpha\)).\(^6\) Our results are consistent with these previous findings that tamoxifen cytotoxicity occurs through an ER-independent mechanism of cell death.

Tamoxifen is a prodrug that is processed in the liver, yielding up to 40 different metabolites.\(^6\) Analysis of human plasma using liquid chromatography-high resolution mass spectrometry shows that a patient who received a very low dosage of tamoxifen for 5 years had 446 ng/ml or 1.2 \(\mu\)M of the parent drug. The potential CYP2D6 by-products \(\alpha\)-OH-tamoxifen and \(4\)-OH-tamoxifen represented only a small percentage of the prodrug (0.06% and 1.32%, respectively).\(^6\) Thus, we have focused primarily on the tamoxifen prodrug, but we believe that the metabolic by-products of the parent drug, such as \(\alpha\)-OH-tamoxifen, can initiate cell death (Supplementary Fig. S1). We believe that both the tamoxifen prodrug and its metabolites can induce RPE death but that the degree of toxicity may vary in patients depending on dosage. Patients who take higher doses of tamoxifen may show relatively rapid signs of maculopathy, whereas patients who take low doses of the drug for a prolonged period of time show delayed signs of maculopathy owing to the accumulation of tamoxifen and its metabolites over time. The absolute quantification of the prodrug and all of its metabolites was thought to be unrealistic by Dahmane and colleagues.\(^6\) We have shown that using relatively high concentrations of tamoxifen in vitro induces rapid RPE cell death, while lower concentrations of tamoxifen incite cell death over 2 weeks (Supplementary Fig. S2), demonstrating a cumulative insult to the RPE by tamoxifen.

Simultaneous inhibition of multiple cell death pathways may not be practical for therapeutic intervention. However, targeting factors upstream of all pathways involved would likely yield better outcomes with regard to safety and efficacy. As we have found that cathepsins B and L are key mediators of tamoxifen-induced RPE cytotoxicity, these proteases may be viable candidates for the development of pharmacologic inhibitors.

**Acknowledgments**

The authors thank Magali Saint-Geniez, PhD (Schein Eye Research Institute–Massachusetts Eye and Ear), for valuable discussion and advice and Christina Kaiser Marko, PhD, for editorial support.

Supported by National Eye Institute/National Institutes of Health (NEI/NIH) Grant K12-EY16355 and by the Massachusetts Lions Eye Research Fund (LAK); NIH Grants GM07226 and AG039245 (WAT); NEI Grant R21EY023079-01/A1, the Yeatts Family Foundation, a 2013 Macula Society Research Grant Award, and anResearch to Prevent Blindness (RPB) Physician-Scientist Award (DGJ); and NIH Grant EY015455 (PAD).

Disclosure: \(L.A.\) Kim, None; \(D.\) Amaranni, None; \(G.\) Gnanaguru, None; \(W.A.\) Tseng, None; \(D.G.\) Vavvas, P.A. D’Amore, None

**References**


60. Marin-Castano ME, Striker GE, Alazar O, Catanuto P, Espinosa-Heidmann DG, Cousins SW. Repetitive nonlethal oxidant injury to retinal pigment epithelium decreased extracellular...

