The selective estrogen receptor modulator raloxifene mitigates the effect of all-trans-retinal toxicity in photoreceptor degeneration

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ABSTRACT

The retinoid cycle is a metabolic process in the vertebrate retina that continuously regenerates 11-cis-retinal (11-cisRAL) from the all-trans-retinal (atRAL) isomer. AtRAL accumulation can cause photoreceptor degeneration and irreversible visual dysfunction associated with incurable blinding retinal diseases such as stargardt disease, retinitis pigmentosa (RP), and atrophic age-related macular degeneration (AMD). The underlying cellular mechanisms leading to retinal degeneration remain uncertain, although previous studies have shown that atRAL promotes calcium influx associated with cell apoptosis. To identify compounds that mitigate the effects of atRAL toxicity, here we developed an unbiased and robust image-based assay that can detect changes in intracellular calcium levels in U2OS cells. Using our assay in a high-throughput screen of 2400 compounds, we noted that selective estrogen receptor modulators (SERMs) potentlypotently stabilize intracellular calcium and thereby counteract atRAL-induced toxicity. In a light-induced retinal degeneration mouse model (Aβca4<sup>-/-</sup>Rdh8<sup>-/-</sup>), raloxifene (a benzothiophene-type scaffold SERM) prevented the onset of photoreceptor apoptosis and thus protected the retina from degeneration. The minor structural differences between raloxifene and one of its derivatives (Y 134) had a major impact on calcium homeostasis after atRAL exposure in vitro, and we verified this differential impact in vivo. In summary, the SERM raloxifene has structural and functional neuroprotective effects in the retina. We propose that the highly sensitive image-based assay developed here could be applied for the discovery of additional drug candidates preventing photoreceptor degeneration.

INTRODUCTION

Retinal degeneration associated with abnormal retinoid metabolism and exposure to bright light can cause visual dysfunction and retinal photoreceptor damage in humans and experimental animals (1). Vision at the molecular level relies on a metabolic pathway that continuously regenerates 11-cis-retinal (11-cisRAL) from its all-trans-retinal (atRAL) isomer (2). AIRAL is a photoproduct released from bleached rhodopsin and its efficient clearance from retinal rod outer segment (ROS) discs and isomerization to 11-cis-retinal by the visual cycle
enzymes are essential for the effective renewal of light-sensitive visual pigments and maintenance of rod photoreceptor homeostasis (3,4). Inadequate clearance of atRAL, for example, can result in the formation of directinoid-pyridinium-ethanolamine (A2E), a condensation product of atRAL and a surrogate marker for toxic retinoids (5). Humans affected by age-related macular degeneration (AMD), Stargardt disease, retinitis pigmentosa (RP) or other retinal diseases associated with the abnormal accumulation of atRAL condensation products, undergo retinal degeneration which eventually culminates in vision loss (5,6). Currently, there are only limited treatments for retinal degeneration caused by this metabolic defect. Even in the presence of a viable retinoid cycle, A2E and other toxic atRAL condensation products accumulate during normal aging (7,8). It is postulated that the mechanism of cell death induced by atRAL involves a prolonged elevation of intracellular calcium, which in turn triggers mitochondrial-associated cell death (9,10). Our previous studies showed pharmacological treatments that either scavenge free atRAL (11) or prevent rod photoreceptor cells from atRAL-induced intracellular calcium influx protect the retina from light damage (6,12). However, potent pharmacological targets and drug treatments that effectively protect photoreceptors from calcium influx are limited. Thus, the goal of this investigation was to identify food and drug administration (FDA) approved drugs that preserve calcium homeostasis and repurpose their known targeted mechanistic pathways to protect the retina from atRAL toxicity (9).

In this study, we developed an image-based high-throughput screen (HTS) assay to identify hit compounds that maintain normal calcium homeostasis after exposure to atRAL, without impairing the retinoid cycle required by the mammalian visual system. Such an image-based HTS assay with a unique measurement of calcium as an indicator of insult from reactive aldehydes is of substantial value not only to this study, but also a useful tool to the vision community for studies related to the calcium pathway. Followed by comprehensive medicinal chemistry and in vivo studies, we identified a group of potent compounds that effectively protect the retina from light-induced atRAL toxicity. More importantly, the common triphenylethylene parent compounds revealed an important target for therapies maintaining calcium homeostasis in the retina. Nonsteroidal selective estrogen receptor modulators (SERMs), clomiphene (13-15) and toremifene (16,17), well-established and widely used drugs that are known to exert agonist and antagonist effects (18), were identified as potential therapeutics against photoreceptor degeneration. While these drugs have shown to be estrogenic in some species, they are anti-estrogenic in others leading to conflicting effects regarding the exact mechanism of action. There are currently two main chemical classes of SERMs approved for clinical use, triphenylethylene (19) and a benzothiopene derivative (20). However, the effect of SERMs on photoreceptors is not completely understood. Estrogen receptors (ERs) consist of two subtypes: nuclear receptors which mediate its effects via gene transcriptional regulation (21), and non-nuclear receptors which are membrane estrogen receptors (mERs) (22) that alter cell signaling via the modulation of intracellular signaling cascades associated with calcium homeostasis (23). In this study, we subjected these drugs to an in vitro calcium homeostasis evaluation and an in vivo light-induced damage assessment. Our findings likely will yield new targets for drug intervention in the prevention and/or progression of retinal degenerative diseases.

**RESULTS**

**AtRAL induced cell death**

To ensure the robustness and sensitivity of our HTS assay, we initially evaluated the optimal concentration and treatment time of atRAL, whereby an insult was sufficient to elevate intracellular calcium levels without causing extreme cytotoxicity. We treated human bone osteosarcoma epithelial (U2OS) cells with 7.5, 15, 30, 45 and 90 µM of atRAL and monitored cell viability over 72 h. Cell viability was analyzed by quantifying dying and live cells by nuclear morphology and a surrogate marker for toxic retinoids (24). We showed that atRAL decreased U2OS cell survival as a function of both exposure time and atRAL concentration. At a higher concentration (45 or 90 µM), atRAL induced severe cellular toxicity even with shorter exposure time (8h). In contrast, at a lower concentration (7.5, 15 or 30 µM), atRAL showed a minor effect on cell survival even with longer periods of exposure (72 h). To confirm this result, we used a CellTiter-Glo luminescent cell viability assay, which measures the number of viable cells in culture based on the quantification of
adrenosine triphosphate (ATP). Cells were exposed to atRAL concentrations of 15, 30 and 60 µM for 1-24 h (Supplementary Figure 1). With 1 h incubation, ATP levels remained stable at all concentration levels, but a longer incubation with atRAL dramatically decreased the ATP concentrations in a dose-dependent manner, indicating a reduced number of viable cells with longer than 1 h atRAL incubation. Based on these results, we proceeded with our HTS assay using atRAL at a concentration of 30 µM with an incubation time of less than 1 h.

AtRAL induced cellular calcium influx

To quantify atRAL-induced calcium influx, which triggers cell apoptosis (24,25), we visualized intracellular calcium in U2OS cells by using the cell-permeable dye, fluo-3 AM with a high-content imaging system (Figure 2). AtRAL increased the fluorescent intensity of cells loaded with fluo-3 in a dose-dependent manner after only 15 min exposure time, indicating a rapid increase in intracellular calcium concentration. Next, we measured the effects of other aldehyde-derived retinoids: 11-cisRAL, 9-cis-retinal (9-cisRAL), all-trans-retinol (atROL) and A2E on intracellular calcium levels (Figure 3A). All aldehyde derivatives and A2E demonstrated elevated intracellular calcium, whereas atROL had no significant effect showing normal calcium homeostasis, suggesting that all free aldehyde derivatives of retinoids are cytotoxic. To confirm that atRAL toxicity is indeed associated with its aldehyde moiety, we tested whether free-aldehyde scavenger compounds, primary amine containing drugs MB-001 and emixustat, affect the atRAL induced calcium influx. Primary amine drugs formed a Schiff base with the aldehyde derivative of atRAL (Figure 3B), preventing the reactivity of atRAL from reaching toxic levels causing that trigger calcium influx (26). Our assay showed MB-001 and emixustat lowered the intracellular calcium levels in a dose-dependent manner, whereas MB-002 lacking a primary amine moiety had no protective effect (Figure 3C). In addition, we tested atRAL-dependent cell viability on six different cell lines, namely human retinal pigment epithelial cells (ARPE-19), mouse embryonic fibroblast cells (NIH-3T3), human embryonic kidney 293 cells (HEK-293), human breast cancer cells (MCF-7), Chinese hamster ovary cells (CHO) and U2OS cells. Each of these demonstrated a rise in intracellular calcium levels after atRAL exposure and dose-dependent cellular apoptosis (Supplementary Figure 2), suggesting that the atRAL-dependent induction of cytotoxicity through elevation of intracellular calcium levels occurs in a broad range of cell types.

HTS assay monitoring intracellular calcium influx

To discover effective compounds that mitigate atRAL toxicity, we applied an image-based HTS assay that measures intracellular levels of calcium using cell-permeable fluo-3 AM fluorescence as an indicator of cellular stress. We optimized our HTS assay with the time course and dose-response evaluations for calcium channel blocker (CCB) treatment prior to atRAL exposure (Figure 4A). As a positive control, we selected nilvadipine, a CCB that demonstrated protective effects on photoreceptor degeneration in animal models of RP (27-30). In our system, pretreatment with nilvadipine a CCB acting as positive control to obtaining normal calcium levels after atRAL exposure significantly reduced the intracellular calcium responses as compared to the DMSO control tested in a dose-dependent manner due to the ability of CCB to reduce calcium excess to cells (Figure 4B). A total of 2,400 compounds, including 1,600 FDA-approved drugs and 800 natural substances and pharmacologically active compounds formatted as 10 mM stocks in dimethyl sulfoxide were screened at a final calibrated concentration of 11 µM (Figure 4C and D). The screen identified an initial 37 hit compounds, from which we selected 25 compounds that best represented the desired structural diversity and drug-like properties. All compounds were tested over a range of 10 concentrations in triplicate to determine their EC50 values. Twelve dose-dependent hits were identified with EC50 values of 1 to 45 µM (Supplementary Figure 3).

SERMs effect on intracellular calcium influx

Interestingly, two out of the twelve identified hit compounds from our HTS assay shared a triphenylethylenie parent scaffold acting as SERMs. Clomiphene and toremifene in Figure 5A, exhibiting a positive calcium balance output after treatment with EC50 values of 25.5 and 29.2 µM respectively, were of particular interest due to their structural similarity to tamoxifen (31,32). Tamoxifen, another member of the triphenylethylenie SERMs, has previously been reported for its photoreceptor protective effect by significantly improving retinal structure, light responses and visual behavior (33). To validate that the dose-dependent response for both clomiphene and toremifene were not due to the interaction of compounds with the calcium dye,
Raloxifene is described to be essential for activity. In the environment we used fluo-3 as an alternative to the activity of the dye in a non-cellular calcium sensitive acidic form, in order to probe as an ester and is hydrolyzed in the cell to its benzothiophene based SERMs have been structure activity relationship (SAR) studies on the activity of raloxifene (Figure 5E). Wide structure activity relationship (SAR) studies on benzothiophene based SERMs have been performed (36-39), and the piperidin side chain of raloxifene is described to be essential for activity.

A minor structural substitution of the raloxifene piperidin ring with the alkylated piperazine ring, Y 134 (35), dramatically abolished the dose-dependent effect of raloxifene (Figure 5E). Wide structure activity relationship (SAR) studies on benzothiophene based SERMs have been performed (36-39), and the piperidin side chain of raloxifene is described to be essential for activity. Raloxifene derivative Y 134, containing a rigid piperazine side chain can potentially impact the selectivity for ER subtype in comparison with those with a flexible chain (40). Selectivity for the ERα versus ERβ may be accountable for the observed calcium activation-signaling phenomenon. To confirm the presence of target ERs in U2OS cells, we performed RT-PCR and were able to detect the transcript of three classical ERs; ERα (Esr1), ERβ (Esr2) and GPER30 (Gper1) (Supplementary Figure 6). Thus, in our in vitro study we identified several effective candidates with EC$_{50}$ < 30 µM in maintaining calcium homeostasis; clomiphene, toremifene, tamoxifen, afimoxifene, endoxifen, ospemifene, raloxifene and bazedoxifene. Our preliminary in vivo test showed that all tested candidates displayed a variety of protective effects against light-induced photoreceptor degeneration in Abca4$^{-/-}$Rdh8$^{-/-}$ mice at 40 to 100 mg/kg body weight dose range (data not shown). As a result, we decided to select raloxifene as our lead candidate along with its derivative Y 134 acting as internal negative control in vitro and in vivo.

SERMs protective effect against bright light-induced retinal degeneration

The Abca4$^{-/-}$Rdh8$^{-/-}$ mouse model that is susceptible to bright-light induced retinal damage is a well-studied and widely used animal model for mechanistic and pharmacological studies of atRAL toxicity (5). Mice carrying a double knock-out of Rdh8, which encodes an enzyme that reduces atRAL to atROL (41), and Abca4, which encodes a transporter that translocates atRAL from the inside to the outside of photoreceptor disc membranes (42,43), rapidly accumulate atRAL. Then manifest retinal pigmented epithelial (RPE) and photoreceptor dystrophy when they are exposed to a short period of bright light. To evaluate the protective effects of SERMs on atRAL-induced toxicity in acute light-induced photoreceptor degeneration in vivo, we treated Abca4$^{-/-}$Rdh8$^{-/-}$ mice with clomiphene (100 mg/kg), raloxifene (60 mg/kg), Y 134 (60 mg/kg) and vehicle control (DMSO). Mice at 6-week of age were given intraperitoneal (i.p) injections 30 min prior to light exposure at 10,000 lux for 30 min, and the effect of treatment was assessed 7 days later (Figure 6A and B). Optical coherence tomography (OCT) scans revealed a significant reduction in the outer nuclear layer (ONL), demonstrating disrupted photoreceptor structures in DMSO and Y 134-treated mice, whereas clomiphene and raloxifene-treated mice...
exhibited a highly preserved retinal morphology like that of mice unexposed to bright light (Figure 6C and D). The efficacy of these treatments was further confirmed by scanning laser ophthalmoscopy (SLO) showing an increased number of autofluorescent spots likely due to infiltrating microglia and macrophages into the subretinal space in DMSO (44) and Y 134-treated mice, whereas clomiphene and raloxifene-treated mice showed only a few of autofluorescence spots in the superior region of the fundus (Figure 6E). The efficacy profile of these SERMs in the retinal light damage model in vivo coincides with in vitro efficacies in the calcium influx assay.

**Raloxifene treatment preserves retinal function**

To determine the effects of raloxifene and Y 134 in rescuing visual function, we recorded the scotopic electroretinography (ERG) responses of Abca4−/−Rdh8−/− mice preconditioned with raloxifene, Y 134, or DMSO at 10 days after the bright light challenge. Our results showed that raloxifene preserves both a- and b-wave ERG responses with similar amplitude and light sensitivity as unbleached mice. However, Y 134-treated mice showed substantially decreased ERG responses with similar amplitude and light sensitivity compared with unbleached mice. These findings further confirmed that raloxifene but not Y 134 exhibited a highly preserved retinal morphology like that of mice unexposed to bright light (Figure 6C and D). The efficacy of these treatments was further confirmed by scanning laser ophthalmoscopy (SLO) showing an increased number of autofluorescent spots likely due to infiltrating microglia and macrophages into the subretinal space in DMSO (44) and Y 134-treated mice, whereas clomiphene and raloxifene-treated mice showed only a few of autofluorescence spots in the superior region of the fundus (Figure 6E). The efficacy profile of these SERMs in the retinal light damage model in vivo coincides with in vitro efficacies in the calcium influx assay.

**Cellular expression patterns of SERM targets**

These neuroprotective effects of SERM compounds in retina raise the question of exactly where and how they act. We addressed this using single-cell RNA-Seq datasets generated from adult mouse and human retinas (45). Somewhat surprisingly, we see essentially undetectable expression of either Esr1, Esr2 or the plasma membrane estrogen receptor Gper1 in mouse rod photoreceptors whereas only Esr2 was detected in human rods (Figure 8). In retina, detectable levels of Esr1 are observed in horizontal cells in both mouse and human, while high levels of Gper1 are detected in endothelial cells in both species. This suggests either that SERM compounds protect photoreceptors from light damage through a cell non-autonomous mechanism, or that they act on a yet unidentified target. While estrogen-related receptors, particularly estrogen-related receptor beta (Esrrb), are strongly and selectively expressed in rod photoreceptors (46) and have been shown to have neuroprotective function (47), these have been reported to be insensitive to raloxifene (48), and are unlikely targets for the observed effect.

**DISCUSSION**

AtRAL is a critical player in the pathogenesis of retinal degeneration through its association with photoreceptor cell degeneration (9,49). In this study, we pharmacologically characterized the cytotoxicity of atRAL in cultured cells with various concentrations and incubation time. Our real-time measurements of calcium intensity revealed that atRAL rapidly induces calcium influx prior to cell death in a dose-dependent manner. We have shown that all visual cycle-essential retinals, including atRAL, 11-cisRAL and 9-cisRAL, induce calcium influx that eventually causes cell apoptosis. Pharmacologically scavenging the aldehyde moiety of these Vitamin A derivatives effectively abolishes their cytotoxicity from disrupting calcium homeostasis, confirming that the aldehyde moiety is the cytotoxic source of atRAL. Herein, we developed a robust and sensitive image-based assay that detected changes in calcium homeostasis, using a calcium fluorescent indicator fluo-3 AM, as a means of identifying compounds that can inhibit atRAL toxicity. Previous in vitro studies indicate that the concentration of atRAL rapidly released from 100% bleached opsin is roughly 3 mM (50). Assuming that a 1% bleach represents an exposure to bright sunlight that produces about 30 µM atRAL (51,52), the concentration of atRAL used in our HTS assay was equivalent to that achievable in the retina under normal conditions. To ensure the highest sensitivity possible in our HTS assay, we carefully optimized our protocol and method of data analysis to minimize systemic errors and determine a stringent hit selection cutoff value with rigorous statistical power.

From a small-scale screen of 2,400 pharmacologically active compounds, we identified twelve hits that protect cells from atRAL-induced calcium influx, among which included antimalarial and antipsychotic drugs. We identified two SERMs from these hits and examined multiple derivatives of SERMs in vitro and in vivo. Our medicinal chemistry analysis showed the calcium-associated signal transduction of SERMs is not restricted to a single chemical scaffold triggering rapid rises in intracellular calcium. Previous studies demonstrated that plasma membrane-associated ERs respond to estrogen administration with
cAMP generation and calcium changes (53). mER signaling has been reported to mediate calcium influx via the L-type calcium channels activated by the Src/ERK/cAMP signaling cascade that is involved in the rapid intracellular calcium responses to estrogen (22). AtRAL is known to activate the PLC/IP3-mediated calcium signaling via modulating the Gq and Gs cascades (6). Thus, antagonizing ER activity may restrain calcium influx directly or indirectly against atRAL mediated pathways. Expression of ERs in the retina already has been shown by others (54) and was again confirmed in our RT-PCR and single-cell RNAseq analysis. However, it is important to mention that the low expression level of ERs in both U2OS cells and mouse retina are not detectable on the western blot to confirm presence of ERs in a protein level. For this reason, we performed RT-PCR to support the presence of ER transcripts in U2OS cells and included single-cell RNA-seq analysis. Tamoxifen, a member of SERM, was previously reported to protect the retina from light damage in vitro (33). Our in vivo results further validated that other SERMs, such as clomiphene and raloxifene effectively protect the retina from light-induced atRAL toxicity, and the efficacies of tested SERMs are consistent with their in vitro activities in blocking atRAL induced calcium flux.

To our knowledge, this is the first study demonstrating that the retinal protective effect of benzothiophenes family of SERMs may be attributed to the inhibition of atRAL-induced calcium elevation in the retina. In this examination, we developed a model that pharmacologically targeting mER inhibits atRAL-induced calcium elevation that protects the retina from light-induced toxicity. We previously showed that pharmacologically targeting G protein-coupled receptors (GPCRs) activating the Gi cascade or inactivating the Gq and Gs cascades effectively protect retina from light damage as well (55). In this study, we revealed that mER is a valid and novel target for treating retinal degeneration associated with atRAL toxicity, which adds to our current system pharmacology inventory for synergistic treatments of retinal degenerative diseases. However, the absence of expression in photoreceptors of the known components of mER -- Esr1, Esr2 and Gper1 -- suggests that the neuroprotective effects of these compounds on photoreceptors may reflect either non-cell autonomous effects of SERMs or their action on currently unknown molecular targets. The consistency of our in vitro and in vivo results validated that our HTS assay is a highly unbiased, robust and sensitive assay for identifying potent drug candidates and novel molecular targets protecting the retina from atRAL toxicity via large-scale small molecule libraries or RNAi screens.

**EXPERIMENTAL PROCEDURES**

**Chemicals:** AtRAL, atROL, 9-cisRAL, Nilvadipine, triflupromazine, toremifene, micanazole, ebselein, clomiphene, gam bogic acid, plumbagin, zinc pyrithione, sanguinarine, melfloquine, sulconazole, hexylresorcinol, triphenylethylene (1), 1-Chloro-triphenylethylene (2), 10-(4-(Dimethylamino)-alpha-phenylbenzylidene)-9-anthrone (3), 4-(4-(Dimethylamino)- alpha - phenylbenzyli dene)-1(4H)-naphthalenone (4), tamoxifen, afimoxifene, ospemifene and endoxifen were all purchased from Sigma-Aldrich (St. Louis, MO). A2E was synthesized as described previously (56) and 11-cisRAL was isolated as described previously (57). Fluo-3 AM was purchased from AAT Bioquest Inc. (Sunnyvale, CA). Non-cellular fluo-3 was obtained from Thermo Fisher Scientific (Waltham, MA). MB-001, MB-002 and Emixustat were synthesized as previously described (26,58). The Spectrum Collection containing 2,400 pharmacologically active compounds was purchased from MicroScource (Gaylordsville, CT). Raloxifene, bazedoxifene and Y 134 were obtained from Tocris Bioscience (Bristol, UK).

**Nuclease morphology measurements in U2OS cells after atRAL treatment:** On day 1, U2OS cells were detached from their tissue culture flasks with 0.25% trypsin (Thermo Fisher Scientific) and suspended in DMEM, high glucose medium containing 10% FBS and 1% penicillin-streptomycin 10,000 U/mL (Thermo Fisher Scientific). Cells were counted and then diluted to 2 x 10⁴ cells/mL. The cell diluent was dispensed into a black 384-ViewPlate with an optically clear bottom (PerkinElmer, Waltham, MA) at 20 μL/well. On day 2, 5 μL of atRAL (Sigma-Aldrich) was added to each well to achieve a final concentration of 90, 45, 30, 15 or 7.5 μM in triplicates. Hoechst 33342 (Sigma-Aldrich) nuclease acid stain was used as a cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA. After an incubation for 15 min at 3 μM final concentration, fluorescent intensity of nuclease morphology was detected with an Operetta high-content imaging System (PerkinElmer) using bright-field and ultraviolet
light source along with an analysis implemented by Harmony® high-content imaging and analysis software (59). Cells were subsequently cultured and monitored for three days. Nuclease morphology was color coded by Red = Dead cells, nuclear area [px2] < 200, Green = Live cells, nuclear area [px2] > 200.

**Cell viability assay after atRAL exposure:** U2OS cells were detached from the tissue culture flask with 0.25% trypsin (Thermo Fisher Scientific) and suspended in DMEM, high glucose medium containing 10% FBS and 1% penicillin-streptomycin 10,000 U/mL (Thermo Fisher Scientific). Cells were counted with a hemocytometer and then diluted to about 3 x 10⁴ cells/mL. The cell diluent was dispensed into a black 384-ViewPlate, with an optically clear bottom (PerkinElmer) at 40 µL/well using an EL406 plate dispenser (BioTek, Winooski, VT). Column 1 contained only medium to serve as a control with no ATP. Next day, 5 µL of atRAL (Sigma-Aldrich) was added to each well to achieve a 3 μM final concentration. After incubating cells were treated with 30 µM of atRAL (Sigma-Aldrich) for 15 min followed by 30 min incubation with 3 μM cell permeable fluorescent calcium indicator fluo-3 AM (AAT Bioquest, Inc.). Cells were then washed three times with PBS and then incubated with 3 µM Hoechst 33342 (Sigma-Aldrich) for another 3 h at 37 °C with 5% CO₂. Fluorescence measurements using an Operetta high-content imaging system (PerkinElmer) were performed as described previously (11).

**HTS calcium influx assay:** On day 1, cells were detached from the tissue culture flask with 0.05% trypsin (Thermo Fisher Scientific) and suspended in DMEM, high glucose medium containing 10% FBS and 1% penicillin-streptomycin 10,000 U/mL (Thermo Fisher Scientific). Cells were diluted to 26,250 cells/mL. The cell diluent was dispensed into a black ViewPlate-384 plate, with an optically clear bottom (PerkinElmer) at 40 µL/well using an EL406 plate dispenser (BioTek), followed by three washes with 1.05 mM KH₂PO₄, 155.1 mM NaCl and 2.96 mM Na₂HPO₄·7H₂O, pH 7.4 (PBS). Cells were then incubated with 30 µL of 10 µM Hoechst 33342 (Sigma-Aldrich) in PBS to achieve a 3 µM final concentration. Subsequently, plates were incubated at 37 °C with 5% CO₂ for 30 min, using an EL406 plate washer/dispenser (BioTek), followed by three washes with 1.05 mM KH₂PO₄, 155.1 mM NaCl and 2.96 mM Na₂HPO₄·7H₂O, pH 7.4 (PBS). Cells were then incubated with 30 µL of 10 µM Hoechst 33342 (Sigma-Aldrich) in PBS to achieve a 3 µM final concentration. After incubation for 3 h, the fluorescent intensity of cells was detected with an Operetta high-content imaging system (PerkinElmer) (8-10,62). U2OS cells were treated with 10 µL of 180 µM 11-cisRAL, 9-cisRAL, atRAL, atROL or A2E similarly as atRAL in culture medium to achieve a 30 µM final concentration for 15 min as described above. After incubation for 3 h, fluorescent intensity of cells was imaged as described above. The assessment was performed in various cell types, including U2OS, NIH3T3, ARPE19, CHO, HEK293 and MCF-7 cells.

**atRAL quenchers:** U2OS cells were cultured in 384-well plates (1050 cells/well), at 37 °C overnight and then further incubated with MB-001, MB-002 and emixustat in a dose-dependent manner with the concentrations ranging from 0.8 to 200 µM in triplicates. The following day, the cells were treated with 30 µM of atRAL (Sigma-Aldrich) for 15 min followed by 30 min incubation with 3 µM cell permeable fluorescent calcium indicator fluo-3 AM (AAT Bioquest, Inc.). Cells were then washed three times with PBS and then incubated with 3 µM Hoechst 33342 (Sigma-Aldrich) for another 3 h at 37 °C with 5% CO₂. Fluorescence measurements using an Operetta high-content imaging system (PerkinElmer) were performed as described previously (11).

**Intracellular calcium influx assessment after atRAL exposure:** On day 1, cells were seeded in 384-well plates as described above. On day 2, cells were treated with 10 µL of 180, 120 and 90 µM atRAL (Sigma-Aldrich) in culture medium to achieve a 30, 20 and 15 µM final concentration, incubated for 15 min, followed by treatment with 10 µL of cell permeable fluo-3 AM (AAT Bioquest, Inc.), to achieve a 3 µM final concentration. Subsequently, plates were incubated at 37 °C with 5% CO₂ for 30 min, using an EL406 plate dispenser (BioTek), followed by three washes with 1.05 mM KH₂PO₄, 155.1 mM NaCl and 2.96 mM Na₂HPO₄·7H₂O, pH 7.4 (PBS). Cells were then incubated with 30 µL of 10 µM Hoechst 33342 (Sigma-Aldrich) in PBS to achieve a 3 µM final concentration. After incubation for 3 h, the fluorescent intensity of cells was detected with an Operetta high-content imaging system (PerkinElmer) (8-10,62). U2OS cells were treated with 10 µL of 180 µM 11-cisRAL, 9-cisRAL, atRAL, atROL or A2E similarly as atRAL in culture medium to achieve a 30 µM final concentration for 15 min as described above. After incubation for 3 h, fluorescent intensity of cells was imaged as described above. The assessment was performed in various cell types, including U2OS, NIH3T3, ARPE19, CHO, HEK293 and MCF-7 cells.
treated with 3 μM cell permeable calcium indicator fluo-3 AM (AAT Bioquest, Inc.) and incubation for 30 min. Cells were then washed with PBS and treated with 3 μM Hoechst 33342 (Sigma-Aldrich) using the EL406 plate washer/dispenser (BioTek). After a 3 h incubation at 37 °C with 5% CO₂, the fluorescence intensity of cells were used to image the spatial dynamics of calcium signaling with identified HTS hits with concentrations ranging from 0.2 to 400 µM in triplicates using an EL406 plate washer/dispenser (BioTek). After a 3 h incubation at 37 °C with 5% CO₂, the fluorescence intensity of cells was used to image the spatial dynamics of calcium signaling with identified HTS hits with concentrations ranging from 0.2 to 400 µM.

**Counter screen measuring the non-cellular fluo-3 effect on identified hits:** Fluo-3, pentapotassium salt (Thermo Fisher Scientific) was used to image the spatial dynamics of calcium signaling with identified HTS hits with concentrations ranging from 0.8 to 200 μM in triplicates. DMEM, high glucose medium was dispensed into a black ViewPlate-384 plate, with an optically clear bottom (PerkinElmer) at 40 μL/well using an EL406 plate dispenser (BioTek). Columns 3 to 22 of the 384-well plates were treated with all identified hits in a dose dependent manner using a JANUS workstation (PerkinElmer). The following day, columns 2-23 were incubated for 15 min with 30 μM atRAL followed by fluo-3 treatment for 30 min. After a 3 h incubation, the fluorescent intensity was measured using an EnSpire Multimode Plate Reader (PerkinElmer) with excitation and emission at 506 and 526 nm wavelengths, respectively.

**Animals and treatments:** Abca4<sup>−/−</sup>Rdh8<sup>−/−</sup> double knockout mice (5) were used to evaluate the protective effect of SERMs in light-induced retinal degeneration. Clomiphene, raloxifene and Y 134 were dissolved in DMSO and administered to mice by intraperitoneal (i.p) injection at 100, 60 and 60 mg/kg body weight, respectively. Both male and female mice were used in all experiments and no significant sexual differences were seen in responses to treatments. All mice were housed in the University Laboratory Animal Resources (ULAR) facilities at the University of California, Irvine (UCI) and maintained in a 12 h light (~300 lux) / dark cycle. Adaptations in the dark were performed under dim red light. All experiments were conducted according to protocols approved by a local Institutional Animal Care and Use Committee (IACUC # AUP-18-124) at the University of California, Irvine.

**Inducing light damage:** Abca4<sup>−/−</sup>Rdh8<sup>−/−</sup> double knockout mice were dark-adapted for 24 h before exposure to bright light. Retinal degeneration was initiated by exposing dark-adapted mice to 10,000 lux of diffuse white fluorescent light (150 W spiral lamp) for 30 min as previously described (12). Before light exposure, pupils of mice were dilated with 1% tropicamide (Akorn, Inc., Lake Forest, IL). After exposure, animals were kept in the dark until evaluation. Clomiphene, raloxifene, Y 134 or DMSO was administered i.p. 30 min before exposure to bright light. Effects of clomiphene, raloxifene, Y 134 and DMSO were tested at 100 to 60 mg/kg of body weight. Each injection volume was 50 μL. Retinal morphology and function were analyzed in vivo by spectral domain optical coherence tomography (SD-OCT), SLO and ERG.

**SLO imaging and SD-OCT:** SLO (HRAII, Heidelberg Engineering) and SD-OCT (EnvisuTM C-Class SDOIS) were employed for in vivo imaging of mouse retinas as implemented in previous publications (12,64). Briefly, mice were anesthetized by i.p. injection of ketamine (20 mg/mL) and xylazine (1.8 mg/mL) at a dose of 100 μL/20 g body weight. Pupils of...
Abca4−/−Rdh8−/− double knockout mice were diluted with 1% tropicamide. The accumulation of autofluorescent spots (particles) can be observed in the retina by SLO imaging in the autofluorescence mode (65). Twenty OCT images acquired in the B-scan mode were averaged to five at 0° (showing temporal and nasal retina) and 90° (showing superior and inferior retina) and saved for ONL thickness measurements. To evaluate changes in the retinas of mice exposed to bright light, the outer nuclear layer (ONL) thickness was measured 500 μm from the optic nerve head (5 to 6 mice/group).  

Electroretinography (ERG): The ERG responses were measured 10 days after light-induced damage in raloxifene, Y 134 and DMSO-treated groups along with the unbleached group as previously described (66). Each group consisted of four to five 6-week of age Abca4−/−Rdh8−/− mice. Prior to recording, mice were dark adapted for 24 h. Under dim red light, mice were anesthetized by i.p. injection of ketamine (20 mg/mL) and xylazine (1.8 mg/mL) at a dose of 100 μL/20 g body weight. Pupils were dilated with 1% tropicamide, and 2.5% hypromellose was applied to keep the corneas hydrated. Contact lens electrodes were placed onto the corneas, and the reference and ground electrode needles were placed on the forehead and tail, respectively. The a-wave and b-wave responses were measured followed by a light stimulus of different flash intensities (-3.3 to 2.7 log cd·s/m²). For each intensity, 3 to 20 recordings were taken and averaged with the resting intervals for recovery from photobleaching effects. All ERGs were recorded with the Celeris ophthalmic electrophysiology system (Diagnosys LLC, Lowell, MA) and analyzed with Espion V6 software (Diagnosys LLC).  

RT-PCR, gel electrophoresis and sequencing for detection of estrogen receptors  
Total RNA from MCF-7 and U2OS cells and mouse retinas were isolated by using the RNeasy Plus Kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. Total mouse brain RNA was purchased from Takara (Kusatsu, Japan). One μg of purified RNA from each sample was reverse-transcribed to cDNA using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA), and the cDNA was used for performing PCR reactions using the primers in Supplementary Table 1. PCR reactions were set up using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) following the manufacturer’s instructions. The cycling protocol used was 95 °C for 1 s, 60°C for 30 s for 40 cycles. The final PCR products were separated in 1.5% agarose gel stained with SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA) and visualized using an Odyssey Fc imaging system (Li-Cor, Lincoln, NE). The remaining volume of PCR products was purified using a QIAquick PCR Purification Kit (Qiagen) and were subjected to Sanger sequencing to ensure the amplification from correct target.  

Single-cell RNA sequencing (ScRNA-Seq): Tissue dissection and cell dissociation: A human globe from an 86-year old Caucasian female who died of a myocardial infarction and had no known ocular disease other than cataracts, was obtained from the Alabama Eye Bank (Birmingham, AL) and processed within 3.3 hours after death. The study was approved by Johns Hopkins Institutional Review Board. The neural retina and RPE/choroid were dissected from the globe in ice-cold PBS. First, a circular incision was made on the sclera, behinds the limbus, to remove the anterior parts, lens and vitreous body. The neural retina was then peeled off from the eyecup and dissociated using Papain Dissociation System (Worthington Biochemical, Lakewood, NJ) following the manufacturer’s instructions. RPE cells were dissociated from the eyecup by incubating with 2 mL of 0.05% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific) for 20 min at 37 °C. Dissociated cells were re-suspended in ice-cold PBS, 0.04% BSA and 0.5 U/UL of RNase inhibitors. CD1 mice between 7 to 9-weeks of age were purchased from Charles River Laboratories (Wilmington, MA). All experimental procedures were pre-approved by the Institutional Animal Care and Use Committee (IACUC) of the Johns Hopkins University School of Medicine. Mice were euthanized, and eye globes were removed and incubated in ice-cold PBS. Retinas were dissected and cells were dissociated using Papain Dissociation System. Totally, 4 biological replicates were used for the mouse scRNA study. Each replicate contained 4 retinas from each of 2 male and 2 female mice. Dissociated cells were re-suspended in ice-cold PBS, 0.04% BSA and 0.5 U/UL of RNase inhibitors. Cell count and viability were assessed by Trypan blue staining.  
ScRNA-Seq: Dissociated cells (~10k) were loaded into a 10x Genomics Chromium Single Cell system (10x Genomics, CA) using v2 chemistry following the manufacturer’s
instructions (67). Libraries were pooled and sequenced on Illumina NextSeq with ~200 million reads per library. Sequencing results were processed through the Cell Ranger 2.1.1 pipeline (10x Genomics) with default parameters. Seurat v2.3.1 (68) was used to perform downstream analysis following the standard pipeline using cells with more than 200 genes and 1000 UMI counts, resulting in 16,659 mouse cells and 14,286 human cells. Samples were aggregated, and cell clusters were annotated based on previous literature (45). A t-distributed stochastic neighbor embedding (t-SNE) dimension reduction was performed on the top principal components learned from high variance genes. Mclust version 5.4 was used to cluster cells in t-SNE space, at which point cell type identity of clusters was assigned based on expression of known marker genes for either retinal or nonretinal tissue.

**Statistical analyses**: HTS image analysis was performed with Harmony® high-content image analysis software and average intensities of the FITC channel were exported in a spreadsheet software. Z'-factor was calculated for each parameter to evaluate the assay quality as described above. A Z'-factor higher than 0 indicates a moderate assay sufficient for a high-content screen, and a Z' factor between 0.5 and 1 suggests an outstanding assay required for a high-throughput screen (69). Controls used in the screen included 16 replicates, whereas compounds were tested in triplicate at six to ten concentrations for dose dependence evaluation as means ± SDs.

For animal studies, results were collected from at least four mice from each experimental group determined by a power analysis to ensure sufficient statistical power with α ≤0.05 (Type I error) and 1-β≥0.90 (Power) (70). Statistical analyses were performed with ANOVA on ranks with Dunn’s post hoc test. Data are presented as means ± SEM. P ≤ 0.05 is considered statistically significant.

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**Conflict of interest**

K.P. is Chief Scientific Officer at Polgenix, Inc. ZD and XM are employees of Polgenix, Inc.

**Author contributions**

Participated in research design: TG, SS, TH, JH, ZD, YC, SB and KP. Conducted experiments: TG, SS, TH, JH, ZD, XM. Contributed new reagents or analytical tools: TG, SS, TH, JH, ZD, YC and SB. Performed data analysis: TG, SS, TH, JH and ZD. Contributed to manuscript writing: TG, SS, YC, SB and KP.

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All-trans-retinal toxicity

Ophthalmology at UCI, the Canadian Institute for Advanced Research (CIFAR), and the Alcon Research Institute (ARI). JH is the Robert Bond Welch Professor. KP is the Leopold Chair of Ophthalmology.

The abbreviations used are: AM; acetoxymethyl, AMD; age-related macular degeneration, atRAL; all-trans-retinal, atROL; all-trans-retinol, AC; amacrine cell, BC; bipolar cell, CCB; calcium channel blocker, 9-cisRAL; 9-cis-retinal, 11-cisRAL; 11-cis-retinal, A2E; diaretinoid-pyridinium-ethanolamine, ERG; electroretinography, EC; endothelial cells, ERα; estrogen receptor α (gene Esr1), ERβ; estrogen receptor β (gene Esr2), ERs; estrogen receptors, ERRβ; estrogen-related receptor beta (gene Esrrb), EDTA; ethylenediaminetetraacetic acid, ERK; extracellular signal-regulated kinase, FDA; food and drug administration, GPCRs; G protein-coupled receptor, GPER30; G protein-coupled estrogen receptor 1 (gene Gper1), HTS; high-throughput screen, HC; horizontal cell, IP3; inositol trisphosphate, INT; intensity, i.p; intraperitoneal, mERs; membrane estrogen receptors, OCT; optical coherence tomography, ONL; outer nuclear layer, PLC; phospholipase C, QC; quality control, RGC; retinal ganglion cell, RPE; retinal pigmented epithelial, RP; retinitis pigmentosa, ROS; rod outer segment, SLO; scanning laser ophthalmoscopy, SERMs; selective estrogen receptor modulators, SD-OCT; spectral domain optical coherence tomography, SAR; structure activity relationship, t-SNE; t-distributed stochastic neighbor embedding.
FIGURES AND FIGURE LEGENDS

Figure 1. Image-based evaluation of nuclear morphology in U2OS cells after atRAL exposure. (A) Apoptotic changes in nuclear morphology observed by Hoechst 33342 staining. Representative images of the nuclear morphology counterstained with Hoechst 33342 after an 8 h incubation with atRAL at 7.5, 15, 30, 45 and 90 µM or with DMSO. Nuclear morphology was assessed with Harmony® high-content imaging and analysis software. Dead cells (red), nuclear area [px²] < 200, live cells (green), nuclear area [px²] > 200. (B) Nuclear morphology quantification monitored through 3 days post atRAL at 7.5, 15, 30, 45 and 90 µM, or with DMSO.
Figure 2. Exposure to atRAL rapidly increases intracellular calcium. (A) Chemical structure of fluorescent fluo-3-Ca\textsuperscript{2+} complex with an excitation at 506 nm and emission at 526 nm. (B) Quantification of fluo-3-Ca\textsuperscript{2+} complex fluorescence intensity (INT) ratio responses in U2OS cells caused by 15, 20 and 30 µM of atRAL exposure for 15 min. Ca\textsuperscript{2+} INT was obtained by a subtraction of Fluo-3 intensity in the background (mean) from Fluo-3 intensity within cells (mean). (C) Representative fluorescence images of fluo-3 AM (green, calcium indicator) and Hoechst 33342 (blue, nuclear marker) in U2OS cells treated with 15, 20 and 30 µM atRAL.

Figure 3. Retinoid and primary amine effects on intracellular calcium. (A) Cellular calcium imaging after treatment with DMSO or with 30 µM 11-cisRAL, 9-cisRAL, atRAL, atROL and A2E. (B) Condensation reaction of atRAL with primary amines resulting in N-retinylidene Schiff base formation. (C) Dose-response effects of emixustat, MB-001 and MB-002 on calcium influx due to atRAL quenching. The dose response curves of primary amine compounds emixustat and MB-001 along with the hydroxyl conjugated MB-002 compound plotted by intensity (INT) ratio of fluo-3-Ca\textsuperscript{2+} complex. Concentrations tested were in the range of 0.8 to 400 µM. Error bars, SDs of triplicate readings.
Figure 4. HTS calcium influx assay. (A) High-content images of calcium intensity (green) controls: non-treated cells (DMSO), dye only treated cells (flu-3 AM), atRAL treated cells and drug (CCB) pre-treated cells. (B) Dose-response effect of nilvadipine (CCB) tested in 0.8 to 200 µM concentrations. Error bars, SDs of triplicate readings. (C) Average fluorescence intensities quantification of U2OS cells exposed to atRAL with and without nilvadipine pre-treatment. Error bars, SDs of 16 replicate determinations. The HTS quality control parameters S/B ratio and Z’ (inset). (D) Activity score plot of 2,400 compound library screen. The screen identified 37 drugs (red vs grey) that block the rise in intracellular calcium due to atRAL exposure (activity score ≥ 60%). Cells exposed to atRAL were used to set the 0% score and pre-nilvadipine treated cells were used to establish the 100% score. Activity score = (F_{comp}-F_{all})/(F_{all}-F_{CCB}) x100. (F_{comp} = fluorescent intensity of the tested compound; F_{all} = fluorescent intensity of atRAL only; and F_{CCB} = fluorescent intensity of CCB). Identifies lead hits toremifene (1) and clomiphene (2).
Figure 5. SERMs effect on intracellular calcium influx. (A) Chemical structures of identified SERM hits toremifene (1) and clomiphene (2) with the corresponding EC50 values. (B) Non-active substructure modifications of triphenylethylene parent compounds (SAR-1) to (SAR-4). (C) Dose-response effects and corresponding EC50 values of triphenylethylene parent compound tamoxifen on intracellular calcium signaling after atRAL treatment. (D) Tamoxifen parent metabolites afimoxifene, endoxifen and ospemifene featuring structural changes in their aminoethoxy and aliphatic side chain (red, purple) along with 4-positionhydroxyl group (blue) showed a dose-dependence effect on intracellular calcium concentrations after exposure to atRAL. (E) Indole and benzothiophene scaffold SERMs: bazedoxifene, raloxifene and Y 134 effects on intracellular calcium. Raloxifene and alkylated piperazine substituted Y 134, demonstrated differing effects on intracellular calcium homeostasis in a dose dependent manner. Concentrations ranged from 0.8 µM to 400 µM. Error bars are SDs from experiments performed in triplicate.
Figure 6. Protective effects of SERMs against light-induced retinal degeneration in \( \text{Abca}^{-/-}\text{Rdh}^{-/-} \) mice. (A) Schematic of the experimental protocol. \( \text{Abca}^{-/-}\text{Rdh}^{-/-} \) mice were dark adapted 24 h prior to clomiphene, raloxifene, Y 134 or DMSO administration to 6-week of age male and female mice. Mice were i.p. injected 30 min before exposure to bright light at 10,000 lux for 30 min. Mice were kept in the dark for 7 days before optical coherence tomography (OCT) and scanning laser ophthalmoscopy (SLO) were performed. (B) Chemical structures of clomiphene, raloxifene and Y 134. (C) Representative retinal OCT images obtained 7 days after \( \text{Abca}^{-/-}\text{Rdh}^{-/-} \) mice were either unexposed to bright light (no light) or exposed to bright light after pre-treatment with DMSO or the indicated compounds (clomiphene, raloxifene and Y 134) at the doses of 100, 60 and 60 mg/kg respectively. Asterisk indicates completely damaged ONL with severe retinal detachment in DMSO and Y 134 treated mice. (D) Quantification of the ONL thickness was determined from OCT images from five to six mice 7 days after light exposure. Changes in the ONL thickness observed after treatment with clomiphene and raloxifene compounds compared to DMSO and Y 134 treated mice. (E) Representative SLO images show the retinas of \( \text{Abca}^{-/-}\text{Rdh}^{-/-} \) mice unexposed to bright light or exposed to light after the indicated pretreatment. Mice unexposed to bright light or exposed to bright light after pretreatment with clomiphene and raloxifene exhibited fewer autofluorescent spots indicating less damage of the retinas.
Figure 7. Retinal function in Abca4<sup>−/−</sup>Rdh8<sup>−/−</sup> mice is substantially preserved by SERM raloxifene. ERG recordings of scotopic a-wave (left) and b-wave (right) were plotted to evaluate the retinal function of drug-treated Abca4<sup>−/−</sup>Rdh8<sup>−/−</sup> mice 10 days after light-induced damage. Both a-wave amplitude, which reflects the response from photoreceptors, and b-wave amplitude, which reflects the response of downstream bipolar cells, were significantly higher in the raloxifene-treated group as compared to the DMSO and Y 134-treated groups (P < 0.05). In comparison with the unbleached control group (no light), raloxifene-treated group showed no significant difference in a- and b-wave amplitudes, suggesting that raloxifene provides nearly full protection of the retina against the light-induced damage. N=4-5. Data are shown as means ± SEM.

Figure 8. Single-cell RNAseq analysis of ER expression in mouse and human retinas. Heatmaps showing the correlation between the expressions of ER genes in different cell clusters from adult (A) mouse and (B) human whole retina at the single-cell level. AC, amacrine cell; BC, bipolar cell; HC, horizontal cell; RGC, retinal ganglion cell; RPE, retinal pigment epithelium cells; EC, endothelial cells. The color intensity in the heatmap is based on the expression levels of ER genes in the retina.
The selective estrogen receptor modulator raloxifene mitigates the effect of all-trans-retinal toxicity in photoreceptor degeneration
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