**Differential Cytotoxic Effects of 7-Dehydrocholesterol-derived Oxysterols on
Cultured Retina-derived Cells: Dependence on Sterol Structure, Cell Type, and Density**

Bruce A. Pfeffer^{1,2,3}, Libin Xu⁴, Ned A. Porter⁵, Sriganesh Ramachandra Rao^{1,2,3}, and

Steven J. Fliesler^{1,2,3§}

¹ Research Service, VA Western New York Healthcare System, Buffalo, NY (USA); ² SUNY Eye Institute, Buffalo, NY (USA); ³Departments of Ophthalmology and Biochemistry, University at Buffalo, The State University of New York (SUNY), Buffalo, NY (USA); ⁴Department of Medicinal Chemistry, University of Washington, Seattle, WA (USA); and ⁵Department of Chemistry and Vanderbilt Institute of Chemical Biology, Vanderbilt University, Nashville, TN (USA);

§ To whom all correspondence should be addressed at: Research Service, Buffalo VAMC, 3495 Bailey Avenue- Mail Stop 151, Buffalo, NY 14215-1129 (USA). Email: fliesler@buffalo.edu

ABSTRACT

Tissue accumulation of 7-dehydrocholesterol (7DHC) is a hallmark of Smith-Lemli-Opitz Syndrome (SLOS), a human inborn error of the cholesterol (CHOL) synthesis pathway. Retinal 7DHC-derived oxysterol formation occurs in the AY9944-induced rat model of SLOS, which exhibits a retinal degeneration characterized by selective loss of photoreceptors and associated functional deficits, Müller cell hypertrophy, and engorgement of the retinal pigment epithelium (RPE) with phagocytic inclusions. We evaluated the relative effects of four 7DHC-derived oxysterols on three retina-derived cell types in culture, with respect to changes in cellular morphology and viability. 661W (photoreceptor-derived) cells, rMC-1 (Müller glia-derived) cells, and normal diploid monkey RPE (mRPE) cells were incubated for 24 h with dose ranges of either 7-ketcholesterol (7kCHOL), 5,9-endoperoxy-cholest-7-en-3 β ,6 α -diol (EPCD), 3 β ,5 α -dihydroxycholest-7-en-6-one (DHCEO), or 4 β -hydroxy-7-dehydrocholesterol (4HDHC); CHOL served as a negative control (same dose range), along with appropriate vehicle controls, while staurosporine (Stsp) was used as a positive cytotoxic control. For 661W cells, the rank order of oxysterol potency was: EPCD > 7kCHOL >> DHCEO > 4HDHC $\approx$ CHOL. EC₅₀ values were higher for confluent vs. subconfluent cultures. 661W cells exhibited much higher sensitivity to EPCD and 7kCHOL than either rMC-1 or mRPE cells, with the latter being the most robust when challenged, either at confluence or in sub-confluent cultures. When tested on rMC-1 and mRPE cells, EPCD was again an order of magnitude more potent than 7kCHOL in compromising cellular viability. Hence, 7DHC-derived oxysterols elicit differential cytotoxicity that is dose-, cell type-, and cell density-dependent. These results are consistent with the observed progressive, photoreceptor-specific retinal degeneration in the rat SLOS model, and support the hypothesis that 7DHC-derived oxysterols are causally linked to that retinal degeneration as well as to SLOS.

KEY WORDS: photoreceptor; Müller cell; retinal pigment epithelium; oxysterol; cell viability assay; cell culture

1. Introduction

Smith-Lemli-Opitz syndrome (SLOS) is a recessive metabolic disease caused by defects in the gene that encodes 7-dehydrocholesterol reductase (DHCR7; also known as 3 β -hydroxysterol Δ^7 -reductase; EC 1.3.1.21; OMIM #602858), which converts 7-dehydrocholesterol (7DHC) to cholesterol (CHOL) (Kelley and Hennekam, 2000; Smith et al., 1964). In humans afflicted with this disease, 7DHC, the immediate precursor of CHOL, accumulates as the dominant sterol in tissues and body fluids (Tint et al., 1994). Although some of the functional deficits and phenotypic traits associated with SLOS may be ascribed to a deficiency of CHOL, particularly during critical periods of embryonic development (Cooper et al., 2003; Kelley and Hennekam, 2000), there is evidence that abnormally elevated levels of 7DHC, or of oxidized derivatives of 7DHC, make a unique and significant contribution to the pathophysiology of this disease (DeBarber et al., 2011; Kelley and Hennekam, 2000; Xu et al., 2012a). While 7DHC itself can be incorporated into either biological or model membranes with relatively benign effects (Fliesler, 2013; Keller et al., 2004), this compound, by virtue of its Δ^7 unsaturation, is ca. 200-fold more susceptible to oxidation than is CHOL (Xu et al., 2009), and thereby gives rise to a variety of oxysterol products (Xu et al., 2010; Xu et al., 2011b), some of which are extremely cytotoxic (Korade et al., 2010). Systemic treatment of rats with the synthetic (and relatively specific) DHCR7 inhibitor, AY9944 (N-[(2-chlorophenyl)methyl]-1-[4-[(2-chlorophenyl)methylamino] methyl]-cyclohexyl]methanamine; dihydrochloride), beginning prenatally and continuing through early postnatal life, has been exploited successfully to create the “AY rat” model of SLOS (Fliesler et al., 2004; Kolf-Clauw et al., 1996), recapitulating the biochemical and some of the phenotypic characteristics of the human disease. The more severe forms of SLOS

are uniformly fatal, either at or shortly after birth (Fitzky et al., 2001; Salen et al., 1996; Wassif et al., 2001); however, the AY rat model remains viable for up to three postnatal months, during which time progressive photoreceptor death ensues after about six postnatal weeks (Fliesler, 2010; Fliesler et al., 2004). Hallmarks of the AY rat retinal degeneration include: gradual loss of cells exclusively in the outer nuclear layer (ONL), TUNEL-positive cells in the ONL, significantly attenuated rod outer segment length, and deficits in both the a- and b-waves of the electroretinogram (Fliesler, 2010; Fliesler et al., 2004; Xu et al., 2012b). Proteomic, lipidomic, and genomic analyses, comparing neural retinal tissue from the AY rat model vs. age-matched controls, have uncovered differences from control tissues that are emblematic of deranged metabolism that goes well beyond the initial defect in conversion of 7DHC to CHOL, including protein and lipid oxidation and up-regulation of cellular signaling pathways involved in cell death, oxidative stress, and autophagy (Boesze-Battaglia et al., 2008; Fliesler, 2010, 2014; Ford et al., 2008; Tu et al., 2013; Xu et al., 2012b).

While photoreceptor dropout in the AY rat model seems to be associated, either directly or indirectly, with these pathological changes, there is no evidence for parallel mortality of other retinal cell types in this model, including other retinal neurons, Müller glial cells (MCs), and retinal pigment epithelium (RPE) cells. Notably, the RPE—despite being engorged with phagosomes and other membranous inclusions—retains its viability and characteristic polarized morphology as an intact monolayer, while MCs also remain viable, but display cytological changes commonly observed in many other forms of neurodegenerations caused by genetic, biochemical, or mechanical insults, such as hypertrophy, hyperplasia (gliosis), and robust up-regulation of GFAP expression (Tu et al., 2013). Based upon detailed lipidomic analyses, 7DHC has been shown to accumulate in the neural retina of AY rats as they mature postnatally, in stark contrast to the retinas of age-matched control rats (where 7DHC is barely detectable) (Fliesler et al., 2004); as a result, excess *in situ* 7DHC-derived oxysterol formation

also occurs in the retina and other tissues (Xu et al., 2012). The cellular distribution of oxysterols within the retina/RPE complex of AY9944-treated rats has not been ascertained at this point, and it is thus far assumed that all cells within this tissue are exposed to biologically relevant levels of these potentially cytotoxic compounds, raising the question of the mechanisms by which individual retinal cell types may exhibit differential vulnerability to 7DHC-derived oxysterols. Furthermore, there is a wide range of molecules within the “oxysterol” structural framework that emanate not only from initial oxidation of 7DHC, but downstream products as well, resulting from both xenobiotic metabolism and non-enzymatic reductive processes within cells (Korade et al., 2010; Shinkyo et al., 2011; Xu L et al., 2013). While at least one of these oxysterol-derived products, 7-ketocholesterol (7kCHOL) (Shinkyo et al., 2011), possessing well-characterized cell toxicity (Rodriguez et al., 2004), is known to occur in vertebrate (including human) tissues associated with aging and vascular disease (Lyons and Brown, 1999), including age-related macular degeneration (Rodriguez and Larrayoz, 2010b), the great majority of the oxysterol by-products of 7DHC are apparently unique to SLOS (Korade et al., 2010; Xu L et al., 2013). When tested on a mouse neural cell line (Neuro-2a), 7DHC-derived oxysterols exhibited a variable range of cytotoxicity, with some compounds being benign, while others were markedly lethal (with EC_{50} 's in the range of 5-50 μ M) (Korade et al., 2010). The latter includes 5,9-endoperoxy-cholest-7-ene-3 β ,6 α -diol (EPCD), a potent, primary 7DHC-derived oxysterol that is rapidly and efficiently converted to more stable oxysterols detected in cultured cell models of SLOS (Xu et al., 2013). Mouse primary cortical neurons have been shown to be at least 10 times more vulnerable to 7DHC-derived oxysterols than Neuro2a cells under the same conditions (Xu et al., 2012a).

The apparent specificity of the progressive cell death observed in the photoreceptor layer of the AY rat retina suggests a marked differential responsiveness and susceptibility to oxysterols for different retinal cell types. In the present study, we attempted to recapitulate this

scenario *in vitro*, comparing the relative effects of various 7DHC-derived oxysterols on cellular viability, treating two immortalized retinal cell lines (*i.e.*, mouse cone photoreceptor-derived (661W) cells (Tan et al., 2004) and rat Müller cell-derived (rMC-1) cells (Sarthy et al., 1998), as well as normal diploid monkey RPE (mRPE) cells with these compounds. We demonstrate that the *in vivo* AY rat model was, in fact, predictive of the culture model with respect to specificity of loss of cellular viability, *i.e.*, the photoreceptor-derived cell line was far more susceptible to the cytotoxic effects of oxysterols than were the rMC-1 cell line or mRPE cells. Also within this context, oxysterols with different molecular structures demonstrated the expected disparate range of potencies, validating the prior observations of Korade et al. (2010) using a completely different cell type (Neuro-2a cells). These results provide further support for the hypothesis that the observed retinal degeneration in the AY rat model of SLOS is due to the exquisite sensitivity of photoreceptor cells to cytotoxic, primary oxysterols derived from free radical oxidation of 7DHC, as well as their metabolites, including those known to form and accumulate in the retina in this animal model (Fliesler, 2010, 2014; Xu et al., 2012b).

2. Materials and methods

2.1 Sterols and oxysterols

Cholesterol (CHOL) and 7-ketocholesterol (7kCHOL) in solid form were purchased from Sigma-Aldrich Chemical Corporation, St. Louis, MO (stored at -20°C, desiccated, and protected from light until ready for use). Purity and identity of these compounds were evaluated by reverse-phase HPLC prior to use, in comparison with authentic sterol standards, and verified to be >99% pure. EPCD, DHCEO (3 β ,5 α -dihydroxycholest-7-en-6-one), and 4HDHC (4 β -hydroxy-7-dehydrocholesterol) were all custom synthesized and purified by previously described methods (Xu et al., 2010; Xu et al., 2011a,b), and supplied as dried solids (stored at -80°C, under Ar atmosphere, protected from light, and desiccated, until ready for use). Structures of CHOL and 7DHC-derived oxysterols are shown in **Fig. 1**. Preparation of CHOL and oxysterol

stock solutions was performed under dim light conditions as follows: 1) For EPCD, aliquots (ca. 0.5 – 2.5 mg) were proportionately dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to achieve a final stock concentration of 5 or 10 mM, and stored desiccated, under Ar, at -80°C; or 2) For CHOL and all other oxysterols, aliquots (2 μ mol each) were first prepared by drying down volumetrically dispensed solutions in absolute ethanol, followed by storage under Ar and desiccated, protected from light, at -20°C, until ready for reconstitution. These aliquots were then solubilized by the sequential addition of absolute ethanol and a 45% (w/v) aqueous stock solution of hydroxypropyl- β -cyclodextrin (HP β CD; Sigma-Aldrich) (Larrayoz et al., 2010) to give a final volumetric proportion of EtOH:HP β CD stock solution =1:9, and yielding an oxysterol or CHOL stock concentration of 10 mM. After purging with Ar gas and thorough vortexing, the aqueous compound mixtures were incubated at ambient room temperature (22-25°C) for 1 h, with intermittent vortexing, protected from light; the resultant (oxy)sterol/HP β CD stocks appeared clear and were stored at 4°C, under Ar atmosphere, protected from light, for up to two months.

2.2 Cell culture reagents

For basal media, Dulbecco's modified Eagle's medium (DMEM) with HEPES, and Coon's modified Ham's F-12 with HEPES (F12) were obtained from Sigma-Aldrich. DMEM without calcium chloride was obtained from Life Technologies (Carlsbad, CA). KBM-Gold™ (keratinocyte basal medium, calcium ion-free) from Lonza (Walkersville, MD).

Unless otherwise stated, the following reagents were used as purchased from Sigma-Aldrich: Alanyl-glutamine, bovine serum albumin, calcium chloride, cholesterol, ethanolamine, fructose, fucose, galactose, glucose, glucosamine, gluconic acid, glucuronic acid, glutathione (reduced), heparin, hydrocortisone, hypoxanthine, lactose, LONG®^{R3} IGF-1, manganese (II) chloride, non-essential amino acids, oxalacetic acid, progesterone (soluble), putrescine, retinyl acetate (soluble), sodium pyruvate, sodium selenite, TAPSO (N-[Tris(hydroxymethyl)methyl]-3-amino-2-

hydroxypropanesulfonic acid), taurine, thioglycerol, partially saturated human transferrin, triiodothyronine (T3), uridine, and vitamin E acetate. Other reagents and sources were as follows: Ascorbic acid phosphate, Mg salt (Wako Chemicals, Richmond, VA); ammonium molybdate (Fisher Scientific, Suwanee, GA); bovine calf serum (CS; Lonza); carnitine tartrate (LKT Laboratories, St. Paul, MN); linoleic acid (Cayman Chemical, Ann Arbor, MI); thiamine and thymidine (Santa Cruz Biotechnology, Dallas, TX).

2.3 Cells

Given recent concerns raised in regard to the need to validate cell lines used in vision research and other scientific disciplines (Boatright et al., 2013; Clark et al., 2013; Al-Ubaidi, 2014; Freedman et al., 2015), we rigorously characterized the cell lines used in these studies (see **Supplementary Data, Section 1.1**, for molecular authentication and characterization of cultured cells).

2.3.1 661W cells

661W cells were obtained from Dr. Muayyad Al-Ubaidi, under a Material Transfer Agreement with the University of Oklahoma Health Sciences Center (Oklahoma City, OK). This cell line originated from surviving cone photoreceptor precursors that were derived from an explant culture of an IRBP promoter/SV40 T-antigen-expressing retinal tumor induced in an early postnatal mouse (Al-Ubaidi et al., 1992; Tan et al., 2004). Cells were received in passage 24, and were expanded in their original designated growth medium (DMEM-HEPES with 10% fetal bovine serum (FBS), 2 mM glutamine, 0.36 mM putrescine, and 0.57 mM β -mercaptoethanol (Al-Ubaidi et al., 2008), utilizing Accutase (Innovative Cell Technologies, San Diego, CA) (Bajpai et al., 2008) as the passaging enzyme. Subcultures were adapted over a period of two weeks to partly defined, reduced serum-containing growth medium based on Neurobasal medium with B-27 supplements (Brewer et al., 1993; Chen et al., 2008), but whose basal medium in this case was a 1:1 (v/v) combination of DMEM:F12. The final formulation contained 0.2% (v/v) bovine calf serum, plus the media additives enumerated above. Growth medium for 661W cells also

contained an aqueous extract of bovine retinas (Pfeffer, 1991), at a final total protein concentration of 6 µg/ml. Cells adapted to this latter medium were expanded in like manner for storage in liquid nitrogen until ready for use.

2.3.2 rMC-1 cells

rMC-1 cells were a generous gift of Dr. Vijay Sarthy, under a Material Transfer Agreement with Northwestern University (Evanston, IL). This immortalized cell line is descended from a primary culture of adult rat Müller glial cells that underwent transformation using SV40 T-antigen (Sarthy et al., 1998). Cells were received in passage 10, and were expanded and ultimately adapted to reduced serum medium in equivalent manner to 661W cells, with storage of stock cultures in liquid nitrogen as described above for 661W cells.

2.3.3 Monkey RPE cells

Normal diploid primary RPE cells were generated from freshly enucleated rhesus macaque eyes (*Macaca mulatta*; Lonza) using previously published methods (Pfeffer, 1991). All animal procedures had been carried out in accordance with the ARVO *Statement for the Use of Animals in Ophthalmic and Vision Research*

([http://www.arvo.org/About_ARVO/Policies/Statement for the Use of Animals in Ophthalmic and Visual Research/](http://www.arvo.org/About_ARVO/Policies/Statement_for_the_Use_of_Animals_in_Ophthalmic_and_Visual_Research/)). Frozen stocks of passage 2 or 3 cultures were thawed

and plated in T-25 flasks in reduced calcium ($[Ca^{++}] < 0.1$ mM) medium, using 1:1 calcium-free DMEM:KBM-Gold™ as the basal medium. Detached cells were released into the medium during proliferation, not only as the cultures expanded, but also continually when the monolayer reached confluence on the tissue culture plastic substrate (Pfeffer, 1991); the accumulated adherence-independent cells were harvested on a regular schedule over a period of up to two weeks to seed multiwell plates for experiments (see below).

2.4 Cell culture methods

Routine and experimental incubator conditions were 36.5°C and 6.0% CO₂, with humidification. Cells were seeded in 48-well tissue culture plastic plates (JetBiofil, Elgin, IL, or

Falcon-BD, Franklin Lakes, NJ), after wells first had been coated with poly-L-ornithine (Sigma-Aldrich), rinsed, and then conditioned with 1% (w/v) bovine calf serum (CS) in basal medium (Michler et al., 1989) in a cell culture incubator for 1 h. This medium was aspirated completely without subsequent rinsing, before addition of cells. For the experiments using 661W and rMC-1 cells, the following procedures were employed. For sub-confluent cultures, 5,000 cells per well were plated in complete growth medium (above), in a final volume of 400 μ l. Approximately 16 h after seeding (overnight), 200 μ l of medium was withdrawn from each well and replaced with 200 μ l of incubation medium (IM), which consisted of basal medium with only the following supplements added: CS (0.2%), plus transferrin, hydrocortisone, non-essential amino acids, alanyl-glutamine (half of growth medium concentration), sodium pyruvate, T3, glucose, fructose and TAPSO. Experimental treatments commenced 5-6 h after the partial medium change. When confluent cells were used, they were instead seeded at 30,000 per well in 500 μ l growth medium, which was exchanged after two days. By three days after plating, cells had become confluent, and growth medium was fully exchanged for 400 μ l of IM. Treatments were initiated one day later.

For experiments employing primary monkey RPE cells, the following procedures were employed. For sub-confluent RPE, cells were seeded at 25,000 cells per well in 500 μ l of reduced calcium (<0.1 mM) medium. After two days, small colonies of cells were evident, and medium was completely changed to 400 μ l of growth medium with the $[Ca^{++}]$ adjusted to 0.5 mM; this was achieved by partial substitution in the basal medium mixture of an appropriate amount of HEPES-buffered DMEM, containing 1.8 mM $CaCl_2 \cdot 2H_2O$, to bring the $[Ca^{++}]$ to the desired level. In the latter medium, colonies expanded at a much slower rate compared to reduced calcium medium, so that after 2-3 days, a full exchange was made for 400 μ l of IM, and experimental treatments commenced one day later. For confluent RPE cultures, cells were seeded at 50,000 cells per well in 500 μ l reduced calcium medium, and maintained using this growth medium for up to two weeks, during which time confluence was attained. The $[Ca^{++}]$ was

now adjusted to 0.45 mM, and stable confluent monolayers were maintained for at least one week and up to two months. One day before experimental treatments, cells were completely switched to IM.

2.5 Treatment of cultured cells with sterols, oxysterols and other agents

For all studies, treatments were performed in triplicate (N=3). Concentrated CHOL and oxysterol stock solutions were serially diluted in IM, initially preparing a series of 10X strength working stock solutions encompassing the desired final dose range for each study. IM for dilution also contained DMSO or HP β CD vehicle at a level matching that used to deliver the highest concentration of experimental compound in an individual study, including vehicle control treatments. In selected experiments, staurosporine (Stsp; Calbiochem; La Jolla, CA) treatments in the range of 40 – 50 nM also were included as a cytotoxic positive control. Finally, cells incubated in IM without added vehicle as well as cell-free blank wells (IM alone) were included as negative controls in each plate. Unless otherwise noted, all incubations of cultured cells with experimental compounds were for 24 h, at which time end point viability assays were carried out (see below).

2.6 Viability assays

2.6.1 Individual assays

After completion of incubation with test substances (or under control conditions), cellular viability was determined by two different and complementary types of assays. The first (resazurin reduction (RR) assay) was adapted from methods published previously (Lor et al., 2007; O'Brien et al., 2000). Cells were rinsed with modified Hanks' balanced salt solution (MHBSS) containing Ca⁺⁺ and Mg⁺⁺, at room temperature, after which 0.125% (w/v) resazurin (Sigma-Aldrich) in modified Earle's BSS (MEBSS), containing 0.01% Pluronic-F68 (Sigma-Aldrich), was added to each well. The plate was returned to the cell culture incubator for 2 h, after which aliquots of the supernatant were transferred to 96-well, black-walled, clear-bottom plates, and the fluorescence of the samples was measured (530 nm excitation, 590 nm

emission) in a Synergy-HT™ plate reader (Biotek, Winooski, VT). Here, as with all subsequent assays, cell-free wells were used as treatment blanks. The protocol for the second assay, crystal violet staining of cells and the subsequent quantitative (CV) assay was based on previously published methods (Bonnekoh et al., 1989; Scragg and Ferreira, 1991). The remaining RR assay medium was first aspirated from the 48-well plates containing the cultured cells; this step (and the previous removal of incubation medium) removed any cells that had detached previously during the incubation with experimental test substances. After a brief, gentle wash with cold (0-4°C) MHBSS, the cells were “fixed” with dry ice-chilled methanol (MeOH) for 10 min, followed by decanting and air-drying of the plate. Cells were then stained with crystal violet (Acros, Belgium; final concentration 0.1% (w/v), from 1% (w/v) stock in MeOH, diluted 1:9 (v/v) with 89 mM Tris-borate buffer, pH 8.0) for 10 min at ambient room temperature. After thorough washout of unretained dye with cold tap water, plates were fully dried overnight at 37°C. Digital images of stained cells were then captured (see below), and dye was solubilized with a solution of 0.5% sodium dodecyl sulfate (SDS) in 1:1 (v/v) 95% ethanol:Tris-borate buffer for 1 h at 37°C. Aliquots of the extract were transferred to 96-well plates, and the absorbance of the samples at 590 nm was determined using a Biotek Synergy-HT™ plate reader.

2.6.2 Duplex Live-Dead assay

In the second assay (Calcein AM/Sytox Orange assay), Calcein acetoxymethyl ester (CaAM) was obtained as a solution in DMSO from Anaspec (Fremont, CA). A working stock solution of 50 µM CaAM was prepared by dilution in a solution of 400 µM probenecid (4-(dipropylsulfamoyl)benzoic acid; Sigma-Aldrich) in MEBSS that was first pH-neutralized and then warmed and CO₂-equilibrated in the cell culture incubator. Viable cells internalize CaAM and hydrolyze it enzymatically to the fluorescent product calcein (Bozyczko-Coyne et al., 1993).

Probenecid has been shown to be a competitive inhibitor of cell membrane organic ion (multidrug) efflux systems (Di Virgilio et al., 1990), and in our hands inclusion of this agent improved the dynamic range and reproducibility of the CaAM assay (results not shown). CaAM was added as a 10X concentrated stock directly to the plate wells, without removing the culture medium at the end of the 24-h test substance exposure period. Incubations with CaAM proceeded for 30 min in the cell culture incubator, and then fluorescence measurements were taken with the Biotek plate reader, using 485 nm/528 nm excitation/emission filters, respectively. Raw data were recorded as relative fluorescence units (RFU). All CaAM procedures were carried out with minimal exposure to fluorescent room lights. Following CaAM assay measurements, plates were temporarily stored in the cell culture incubator awaiting the succeeding steps.

Sytox Orange (SO) was supplied as a DMSO solution (Invitrogen, Eugene, OR), and was diluted to a working concentration of 10 μ M in incubation medium at room temperature immediately before use. Plasma membranes of viable cells are not permeable to SO; however, in the course of events leading to cell death, the membrane integrity of cells is compromised, allowing entry of SO into the cell and, subsequently, access to nuclear DNA, with which it intercalates to form a fluorescent complex (Pierce et al., 2003; Yan et al., 2000). Hence, increased fluorescent signal is indicative of increased cell death. SO-labeled cells included cells that had visibly detached from the substrate, since much of the signal was lost if medium was exchanged before the assay was performed (not shown). SO was added to cells as a 10X concentrated stock, proportional to well volume, without medium exchange, *i.e.*, directly to the previously CaAM-treated wells, and plates were again incubated for 30 min in the cell culture incubator. Bound SO fluorescence was determined using 530 nm/590 nm excitation/emission filters, respectively, without interference from calcein. Since neither CaAM (or intracellular calcein) nor SO are intrinsically cytotoxic, plates could be returned briefly to the incubator pending final assessment of correlative morphology of the cultured cells.

2.7 Digital imaging

Phase-contrast images of the center portions of at least one replicate well from each experimental treatment were recorded, to show either fixed crystal violet-stained cells, or live cells (adherent plus detached), and recorded as .tif files, using an Axiovert 25 CFL inverted photomicroscope (Carl Zeiss Microscopy LLC, Thornwood, NY), equipped with an Evolution™ MP digital CCD 5.0 MP camera (MediaCybernetics, Rockville, MD)

2.8 Statistical analysis of data

Background fluorescence from blank wells in each plate was averaged and subtracted from the values for all associated treatment wells. Graphs were prepared using Microsoft® Excel software. For inter-plate comparisons, cytotoxic agent and vehicle control values were normalized using the means of control treatments without vehicle for each plate. For comparisons of treatments with cytotoxic agents, and comparisons of CaAM assays with and without multidrug efflux inhibitors (Probenecid), one-way analysis of variance (ANOVA), with Tukey's *post hoc* test, was computed using Systat® V7.0 for Windows® (SYSTAT, Chicago, IL) software. Statistical significance was set at a *p*-value ≤ 0.05 .

3. Results

3.1 Effect of oxysterols on 661W cells

We first assessed the relative cytotoxicity of oxysterols on sub-confluent cultures of 661W cells, an immortalized photoreceptor-derived cell line used as a surrogate for photoreceptor cells. For each compound tested, we correlated qualitative data obtained by morphological assessment (phase contrast microscopy) with quantitative data obtained using complementary spectrophotometric or fluorescent cellular viability assays. The viability assays employed included: a) crystal violet (CV), correlating with attached cells; b) resazurin reduction (RR) and Calcein AM (CaAM), both of which measured viable cell metabolism; and c) Sytox Orange (SO),

which labeled dead or dying cells by virtue of the loss of integrity of their plasma membrane permeability barriers. The appearance of CV -stained, sub-confluent 661W cells following treatments with test substances was examined in comparison with cells incubated under control conditions, including both negative controls (vehicle-alone, and no vehicle, with CHOL serving as a negative sterol control) and a positive control (medium containing Stsp at 25 - 50 nM, a concentration range expected to show efficacy in cell lines displaying a neuronal phenotype (Seo and Seo, 2009)). The results are shown in **Fig. 1**. Following a 24-h incubation with increasing concentrations of 7DHC-derived oxysterols, a proportional loss of cells remaining attached to the substrate, and available to take up stain, was observed, with the order of cytotoxicity being EPCD >> 7kCHOL > DHCEO >> 4HDHC $\approx$ CHOL. By visual evaluation, the threshold doses for complete release of cells from the culture well substrate were above 0.6 mM and at or below 1 μ M for EPCD, and close to 23 μ M for 7kCHOL. Using this qualitative criterion alone, some loss of viability was demonstrated for DHCEO at the highest dose tested (30 μ M), while no appreciable effect on cellular viability was observed for 4HDHC or CHOL under the conditions employed. From phase-contrast images of unprocessed 661W cultures following the 24-h treatment period it was possible to discern cells whose morphology indicated a stepwise progression to complete cell death (**Fig. 2**). At lower doses within the efficacy range of the test substances, the most potent oxysterols (7kCHOL and EPCD) caused the 661W cells generally to retract their neuritic-like processes, become bipolar, and round up (*e.g.*, thick arrow in **Fig. 2D**. Note: not to be confused with cells rounded up at mitosis (arrowheads in **Figs. 2A** and **2C**)); however, judging from their phase-refractile appearance, they apparently retained their cellular integrity (Longo-Sorbello et al., 2005). Eventually, at higher doses, this phase-refractile appearance was lost, and either fragments of cells and cell processes remained attached to the cell culture substrate, or else detached cells drifted in the medium, above the substratal focal plane (thin arrows in **Figs. 2D** and **2E**). These latter cells tended to lose their phase-refractile properties, appearing “ghost-like,” at times exhibiting an inflated, spherical profile. However, for

assays requiring media changes and washes, the detached cells (which included a SO-positive population; results not shown) were not retained in the wells and their properties could not be assessed for comparison with any remaining attached cells.

As expected, the results of the CV colorimetric assay yielded quantitative values commensurate with the density of cells remaining attached and visualized by CV staining in each well (**Figs. 3A-C**). For example, there were no statistically significant changes in cell viability across the range of CHOL concentrations tested, 3 – 45 μ M (**Fig. 3A**, white bars); in contrast, an abrupt decrease in CV absorbance was observed as the 7kCHOL concentration increased from 13 to 20 μ M, with virtually no signal detected at concentrations of this oxysterol at or above 23 μ M (**Fig. 3A**, black bars). Statistically significant differences in measured absorbance were observed for cells treated with 7kCHOL beginning at a concentration of 13 μ M, with a mean decrease in absorbance relative to the vehicle control (VC) being 13.6%. Using this same assay, in like manner to CHOL, 4HDHC treatment did not affect viability over the entire dose range tested, while DHCEO did display some apparent cytotoxicity at 30 and 45 μ M, with decreases in viability compared to VC of 34.1% and 67.4%, respectively (**Fig. 3B**). The most potent compound investigated was EPCD, whose efficacy range was an order of magnitude less than that of 7kCHOL (**Fig. 3C**), showing statistically significant effects in the CV assay (gray bars) at concentrations as low as 0.2 μ M, with a resulting reduction in viability of 19.6% compared to VC, and full efficacy at 1 μ M and above, and with a loss of $\geq$ 95.7% of signal compared to the VC values (gray bar in **Fig. 3C**). It should be noted that whenever values for VC were compared to those obtained for control incubation medium *without* addition of vehicle (CnoV; normalized value always =1), in all cases there were no statistically significant differences between these two control treatment conditions, except where noted below. In addition, the positive cytotoxicity control Stsp at 50 nM markedly and significantly decreased 661W cell viability in the crystal violet assay (gray bar in **Fig. 3C**).

The results for the RR assay on sub-confluent 661W cells paralleled those observed using the CV colorimetric assay for all compounds tested. For 7kCHOL (black bars in **Fig. 3D**), there was a significant reduction in viability noted at the threshold concentration between 13 and 20 μM , with virtually complete loss of signal (and, hence, viability) at 23 μM . EPCD was again the most potent compound tested by this assay (**Fig. 3C**, black bars), causing intermediate and nearly complete losses of viability at 0.6 and 1 μM , respectively. These comparatively high efficacies for 7kCHOL and EPCD were contrasted with both CHOL (**Fig. 3D**, gray bars) and 4HDHC (**Fig. 3E**, gray bars), neither of which demonstrated toxicity in this assay throughout the concentration ranges tested. Finally, in good accordance with the CV assay results, DHCEO evoked only low to modest cytotoxicity in the RR assay at the two highest doses used (**Fig. 3E**, black bars). Compared to the CV assay, some apparent slight decreases in compound efficacy may have been observed with the RR assay (e.g., compare **Figs. 3A** and **3D** for 20 μM 7kCHOL). This can be explained readily by the fact that the former assay involves more changes of solution and washes compared to the other assays, and cells that became rounded up during incipient changes in viability effected by active compounds likely were more easily dislodged mechanically than under the assay conditions that did not include washes.

3.2 Sytox Orange (SO) assay performed alone on sub-confluent 661W cells

When displayed graphically, the results of the SO assays were comparable inversely to those obtained with the other viability assays, in that the relative fluorescence values increased in a dose-dependent fashion as cellular viability was lost. For example, as the concentration of 7kCHOL was increased from 13 to 20 μM (**Fig. 4A**, black bars), the measured efficacy jumped from a background level— not statistically significantly different from vehicle control (VC) values— to a statistically significant 6.2-fold change relative to the VC values. The SO values increased with dose dependence up to 26 μM , at which point higher doses evinced a slight decline to levels that were still significantly greater than those obtained with vehicle alone.

Since digital images of cells treated with $\geq 26 \mu\text{M}$ 7kCHOL did not indicate the presence of more live cells (*cf.* **Fig. 1**), the observed drop-off in SO-specific fluorescence suggests a decay of signal, possibly related to progressive post-mortem changes in cellular nuclear integrity (*i.e.*, loss of intact DNA, the binding target of SO (Frankfurt et al., 1996; Oshige et al., 2011; Yan et al., 2000)) and may reflect a later stage in a sequential cell death program in cells treated with these oxysterol compounds. This scenario was further borne out in a time course study using a single toxic dose of 7kCHOL (20 μM), for which the CaAM assay indicated a continuous and sustained loss of viability over a 24-h period (see below), while the corresponding fluorescence signals in the SO assay rose and fell (see **Supplementary Figs. 8 and 9**). The expected, statistically significant increases in SO assay measurements *vs.* vehicle control at the 24-h endpoint were attained with toxic doses of Stsp (**Fig. 4A**).

As with the assays mentioned above, treatments across the dose range with either CHOL or 4HDHC failed to cause significant changes in the SO assay readouts compared to vehicle alone (**Fig. 4B**), indicating little or no cytotoxicity. However, while DHCEO caused a significant loss of viability (relative to vehicle alone) at 30 and 45 μM in the CV and RR assays (**Figs. 3B and 3E**), it did not show efficacy even at 45 μM in the SO assay; a side-by-side comparison of the results obtained with DHCEO *vs.* 7kCHOL highlights the significant difference in cytotoxic potency between these two oxysterols (**Fig. 4A**, gray bars).

Again, as expected from the other cytotoxicity assays, EPCD treatment demonstrated high potency in the SO assay, with concentrations of 0.6 μM and 1 μM yielding intermediate and nearly full efficacy, respectively, compared with the vehicle alone baseline results (**Fig. 4C**). An EC_{50} of between 0.4 and 1 μM for EPCD effects on viability of subconfluent 661W cells was therefore demonstrated consistently through all the assays performed (*cf.* **Figs. 1 and 3C**). As in the context of the other assays utilized, Stsp served as an effective positive control for the SO assay (**Fig. 4C**). It should be noted that, as opposed to all of the other assays in these studies (including the CaAM assay), the full efficacy window as determined by the SO assay can be

visualized immediately to be expressed in positive multiples, with maximal values attaining a ca. 8- to 9-fold change over control values for the two most potent compounds, EPCD and 7kCHOL, when tested on sub-confluent 661W cells.

Compilation of results obtained with each of the above cytotoxicity assays, for each of the oxysterols and CHOL are depicted in **Supplementary Figs. 10A-C**. Using a purely graphical approach, it can be seen that EC_{50} values for EPCD and 7kCHOL were ca. 0.5 μ M and 15 μ M, respectively, for all three assays presented thus far.

3.3 Effects of oxysterols on confluent 661W cells

Our original rationale was to assess the effects of 7DHC-derived oxysterols on sub-confluent cultures of 661W and rMC-1 cells, because their morphology— particularly that of 661W cells, which was more neuronal at lower cell densities— changed when the cells became confluent, and because post-confluent cultures of transformed cells continued to proliferate, releasing some dead, non-phase-refractile cells into the medium, even under control conditions (not shown). Furthermore, it was reasoned that *confluent* monkey RPE (mRPE) cultures would be more representative of their native counterparts in the eye, since it has been demonstrated that these non-transformed, normal diploid cells can be maintained as stable, non-dividing, polarized monolayers at confluence (Pfeffer, 1991). We also considered the possibility that the potency of the cytotoxic oxysterols could be proportional to cell density, *e.g.*, it might *decrease* as *higher* cell numbers were treated in 48-well plates. It was considered important to control for this variable parameter of cell density when comparing different ocular cell types for their responses to experimental compounds. Therefore, in follow-on studies to those reported above, we tested 7kCHOL, EPCD, and CHOL for their effects on the viability of *confluent* 661W cells, and comparatively taking into account both sub-confluent and confluent cultures of rMC-1 and mRPE cells. For all succeeding experiments described here, a sequential, duplex, single-well CaAM/SO assay was utilized at the 24-h experimental endpoint (see *Methods*).

Newly confluent 661W cells formed directly apposing cell borders without overlapping processes, with a cobblestone-like morphology (**Fig. 5A**). When a dose range of 7kCHOL similar to that tested on sub-confluent 661W cells was applied to confluent cells, the threshold for loss of viability was shifted markedly to a significantly higher concentration (26 μ M), as determined by both the CaAM and SO assays (black bars, **Figs. 5B and C**). For confluent 661W cells, the potency of EPCD was an order of magnitude greater vs. 7kCHOL, much like the findings obtained with *sub*-confluent 661W cells, except that, as for 7kCHOL, the threshold for statistically significant loss of cell viability was again shifted to a higher concentration (black bars in **Figs. 5D and E**). CaAM assay results yielded a presumed EC₅₀ of between 1 and 3 μ M for confluent 661W cells, compared to *ca.* 0.6 μ M for *sub*-confluent 661W cells exposed to EPCD (*cf.* **Figs. 4C and 5D**, black bars); this was manifested as a steep drop-off between these two tested doses. The data obtained with the SO assay for confluent 661W cells was indicative of a more gradual effect on viability (or, more precisely, on plasma membrane permeability and integrity), with significant changes observed, compared to incubations with vehicle alone, beginning at 3 μ M EPCD and reaching maximal cytotoxic efficacy at the highest concentration tested, 20 μ M (**Fig. 5E**, black bars). This is in stark contrast to the value of 3 μ M for full efficacy of EPCD as determined with the SO assay when using sub-confluent cells (*cf.* **Fig. 4C**). The EPCD experiment was carried out separately from the full dose-range study using 7kCHOL, but included a treatment of the 661W cells with 26 μ M 7kCHOL as a positive control; the closely corresponding values obtained in both sets of CaAM/SO assays using 26 μ M 7kCHOL (black bars in **Figs. 5D and E**) as the test substance confirmed the equivalence of the cell preparations used to obtain these data, performed independently. Hence, these results effectively served as one facet of assay validation.

3.4 Effects of oxysterols and CHOL on viability of sub-confluent and confluent rMC-1 cells

In similar manner to the results described above for 661W cells, sub-confluent rMC-1 cells (a Müller glial cell surrogate) exposed to a dose range of 7kCHOL exhibited a lower concentration threshold for loss of viability, compared to *confluent* rMC-1, as measured in the CaAM assay (black bars, **Fig. 6**, and white bars, **Fig. 5B**, respectively), with the highest fully tolerated concentrations occurring at 16 and 26 μM , respectively. At 30 μM (the highest concentration tested), confluent rMC-1 cells demonstrated only a partial, but statistically significant, decline in viability (to 34.7% of the vehicle control value in **Fig. 5B**, white bars). These results correlate well with the phase-contrast microscopic appearance of these cultures (**Figs. 7 and 8**); although a partial rounding-up of cells in sub-confluent cultures was observed at 16 μM 7kCHOL, this apparently had no correlation in the CaAM assay results. However, at 20 μM 7kCHOL, the majority of sub-confluent rMC-1 cells had detached from the culture well substrate (polyornithine-coated tissue culture plastic), and this was manifested in a nearly complete abrogation of CaAM signal (**Fig. 6**, black bars). In confluent rMC-1 cultures, 30 μM 7kCHOL caused only slight denudation of the monolayer, which was otherwise uniformly present at lower concentrations and in vehicle control wells (**Fig. 8**).

In clear contrast to the congruence between the CaAM and SO viability assays for 661W cells described above, however, neither sub-confluent nor confluent rMC-1 cells displayed significant loss of viability, as documented by the SO assay, across the full concentration range of 7kCHOL when compared to vehicle controls (white bars in **Figs. 6 and 5C**, respectively). Both 10 and 13 μM 7kCHOL actually lowered (with statistical significance) the fluorescence signal magnitude for SO, compared to vehicle controls, initially suggesting a slight *increase* in cell viability. There also was an elevated SO signal at 23 μM 7kCHOL, which was statistically significantly different compared to the two higher 7kCHOL doses, but not different from the vehicle control values. However, these discontinuities were not reflected in cellular morphology as assessed by phase-contrast microscopy (**Fig. 7**), and may represent an

artifact possibly due to variability of initial seeding densities of cells in replicate wells (e.g., compare images in **Fig. 7** corresponding to vehicle control cultures, vs. those following treatment with 13, 20, 23, and 26 μM 7kCHOL).

The relatively modest effect of 7kCHOL on rMC-1 cells, per the SO assay, was not universal for oxysterols, however; EPCD-treated, confluent rMC-1 cells did display vulnerability that could be documented using either assay. The CaAM assay (**Fig. 5D**, white bars) revealed a statistically significant, 29.2% decrease in viability at 10 μM , and full cytotoxic efficacy at 13 μM , relative to vehicle control. A somewhat greater sensitivity was evident using the SO assay (**Fig. 5E**, white bars): approximately 34.6% of maximal efficacy (using the mean values for vehicle control and 10 μM as 0 and 100%, respectively) was attained for rMC-1 cells with 3 μM EPCD. Beyond 10 μM EPCD, which brought about a 2.2-fold increase in fluorescence signal (relative to that obtained for vehicle controls), there was finally a slight drop-off in the SO values at 13 and 20 μM EPCD, both of which were statistically significantly different from the maximum value observed at 10 μM EPCD; this attenuation resembled the previously described results obtained for 661W cells treated with 7kCHOL (see **Fig. 4A**). Phase-contrast images (**Fig. 8**) of initially confluent rMC-1 cultures revealed areas of both cell detachment and some contraction of remaining portions of the cell layer subsequent to treatments with, e.g., 10 μM EPCD, while cells exposed to 3 μM EPCD were indistinguishable from vehicle controls (images not shown). As found for 661W cells, no viability changes were exhibited by rMC-1 cells incubated with CHOL, with doses ranging as high as 30 μM (**Table I**; the apparent outlying data point observed at 10 μM CHOL in the CaAM assay was not reflected in the results obtained using the SO assay). As expected, Stsp treatment of rMC-1 cells also led to significant loss of viability, as documented utilizing both elements of the duplex assay (**Table I**).

3.5 Effects of oxysterols and CHOL on confluent and sub-confluent primary monkey RPE cells

When cultured in medium containing < 0.1 mM Ca^{++} concentration, mRPE cells generate viable cell suspensions that can be collected and replated, in effect passing cells without the use of proteolytic enzymes (Pfeffer, 1991). However, if the medium is switched to a more standard Ca^{++} concentration (e.g., 0.5 mM) a few days after initial plating, and well before the cells reach high cell density or confluence, the resulting mRPE cells can be maintained as subconfluent colonies briefly before experimental use (Pfeffer, 1991). The central portions of these colonies contain non-proliferating cells bearing a differentiated epithelial morphology (**Fig. 9A**). Such cultures established in 48-well plates were used to yield sub-confluent, yet representative, mRPE cells for testing the hypotheses that: a) in the presence of active, potent oxysterols, sub-confluent RPE cells, like 661W and rMC-1 cells, should display decreased viability, with lower dose-response thresholds, compared to what was demonstrated for the matching *confluent* cell type; and b) sub-confluent and confluent mRPE cells, nevertheless, should be less sensitive to these oxysterols than are 661W at matching cell densities and equivalent concentrations of test substances.

As suggested from results with the AY rat model (Tu et al., 2013), mRPE cells—documented first at sub-confluence—were found to be far more resistant *in vitro* to the cytotoxic effects of the oxysterols 7kCHOL and EPCD than were 661W cells, as demonstrated by both the CaAM and SO assays (**Figs. 9B and C; Supplemental Figs. 12B and 12C; cf. Figs. 4A and 4C**). The threshold dose of 7kCHOL necessary to cause statistically significant lowering of calcein fluorescence was 23 μM in both assays, slightly higher than the 16 to 20 μM observed for sub-confluent 661W cells in all the assays described (see above). Moreover, unlike 661W cells, sub-confluent mRPE did not exhibit dose-dependent cytotoxicity in the CaAM assay, nor did the dose range effects culminate with full toxicity; a maximal effect of only 40.8% reduction, compared to vehicle control values, was realized at the highest concentration tested, 45 μM (**Fig. 9B**). These findings correlated well with the morphological appearance of the cultured

mRPE cells (**Fig. 10**): 23 μ M 7kCHOL caused occasional cell contraction without widespread detachment (**Fig. 10E**), whereas at 26 μ M, the majority of cells had rounded up (**Fig. 10F**), again with minimal detachment under the incubation and assay conditions preceding digital photographic documentation. However, a dose-response was observed with sub-confluent mRPE treated with 7kCHOL using the SO assay, for which the maximal effect (ca. 14-fold increase in signal magnitude, relative to vehicle control) was reached at 45 μ M 7kCHOL (**Fig. 9C**), notably almost twice the concentration required for full efficacy— accompanied by global loss of viability— with sub-confluent 661W cells. In like manner, EPCD was far less potent with regard to its cytotoxicity when applied to sub-confluent mRPE than was the case for 661W cells. For the former, a concentration of 16 μ M EPCD was required before a statistically significant, and only partial, reduction in viability (to 50.1% of VC) was attained, as measured in the CaAM assay (**Supplemental Figure 12B**), while mean reductions to 87.0% and 61.0% of VC from incubations with 10 μ M and 13 μ M EPCD were not statistically significant. EPCD did elicit a dose-responsive increase in toxicity with sub-confluent mRPE when assessed by the SO assay, with an apparent threshold concentration of 10 μ M (**Supplemental Figure 12C**), a value more than an order of magnitude greater than that for sub-confluent 661W (**Figure 4C**). The morphological changes induced by EPCD on sub-confluent mRPE cells largely paralleled the assay results (**Supplemental Fig. 13**); although some initial rounding-up of cells was observed at 6 μ M EPCD (**Supplemental Fig. 13C**), this was not reflected in either the CaAM or SO assay values.

The distinction between mRPE and 661W cells in terms of their susceptibility to oxysterols also was pronounced when *confluent* cultures were challenged with the two most potent oxysterols, 7kCHOL and EPCD. Results obtained with both the CV and the RR assays suggested that stable, confluent monolayers of mRPE were resistant to 7kCHOL (and unresponsive in these assays to CHOL, as a negative control, as well) throughout a dose range from 1 to 30 μ M (see **Supplementary Figs. 11A,B**). There was no discernable dose-

dependence observed in the CaAM assays for confluent mRPE treated with either 7kCHOL or EPCD (gray bars, **Fig 5B** and **Supplementary Fig. 12A**), applying either oxysterol at concentrations as high as 45 and 20 μ M, respectively. [In **Fig. 5B**, note that 16 μ M 7kCHOL provoked a statistically significant, relatively modest (17.9%), apparent decrease in viability vs. vehicle control, but no obvious correlation with compound dosage was observed in the study depicted.] The resistance of confluent mRPE cells to cellular damage and loss of viability was evident from the microscopic appearance of the cells following the treatments, which nonetheless differed slightly for the two oxysterols used (**Fig. 10**). At the highest dose of 7kCHOL (45 μ M), a very small proportion of the monolayer was denuded, while the remaining cells maintained their integrity and the cobblestone morphology characteristic of RPE *in vivo* (**Fig. 10C**). The most visible manifestations of abnormal morphology when mRPE was challenged with EPCD at a concentration of 20 μ M were irregularities in cell shape and increased vacuolization (**Fig. 10I**). However, the SO assay did reveal some apparent dose-related toxicity for both oxysterols: for 7kCHOL (**Fig. 5C**, gray bars), the cytotoxicity concentration threshold for mRPE cells was 26 μ M, at which dose 19.8% of the maximum efficacy (for 45 μ M) was realized (the latter a 2.4-fold increase, relative to vehicle control), while for EPCD (**Fig. 5E**, gray bars), the corresponding values were 10 μ M for threshold, with 22.5% of the maximum efficacy that was exhibited at 20 μ M (a 15.6-fold increase, relative to vehicle control). When comparing the quantitative results for confluent 661W and mRPE cells in the SO assay in **Figs. 5C** and **5E**, at first glance there would seem to have been a disparity between the efficacy window in the SO assay and the viability of the cultures recorded with microscopic images. This could be due to the combination of the extremely high sensitivity of the SO assay, and the actual differences between baseline cell death in control and treated cultures of, on the one hand, immortalized, continuously proliferating 661W cells, and the relatively stable mRPE cultures on the other. In comparing and contrasting these SO assay results, we also noted that the baseline (*i.e.*, vehicle control) fluorescence values were much lower for confluent mRPE

than for 661W cells, typically in the range of 400 – 600 relative fluorescence units (RFU) for the former, vs. 1000 – 2000 RFU for the latter. Again, the resilience of the mRPE cultures suggested by these assay results was clearly reflected in the microscopic appearance of the cells (**Fig. 10**). The contrast between confluent 661W and mRPE cultures was apparent even when incubation of the latter with EPCD was extended to 48 h (**Supplementary Fig. 14**).

4. Discussion

Our results demonstrate that retina-derived cultured cells representing morphologically and functionally disparate retinal cell types display marked differences in their response to an array of 7DHC-derived oxysterols, as determined by both qualitative and quantitative cell viability assays. Moreover, within the range of known structures for the oxysterols, generated by oxidation of 7DHC and considered to be biomarkers of SLOS (Xu et al., 2010, 2011a,b, 2012a,b), there are differences in both potency and efficacy with respect to oxysterol-induced toxicity for each of the three retinal cell exemplars that we tested at both sub-confluent and confluent densities in culture: two retina-derived immortal cell lines (661W and rMC-1 cells, as surrogates for photoreceptor and Müller cells, respectively), and normal diploid monkey RPE cells originating from primary tissue explants. The much greater relative sensitivity of 661W cells to the cytotoxic effects of such oxysterols, compared to either rMC-1 or mRPE cells, is consistent with the apparently selective degeneration and death of photoreceptors that occurs in the progressive and irreversible retinal degeneration observed in the AY9944-induced rat model of SLOS (Fliesler, 2010, 2014; Fliesler et al., 2004). In that rat model, although MCs exhibit gliotic changes (both in terms of morphology as well as massive up-regulation of GFAP) and RPE cells express certain abnormal cytological features (*e.g.*, congestion with phagosomes), neither of these cell types seems to undergo cell death or dropout (Fliesler et al., 2004; Tu et al., 2013). Our findings also are very consistent with those reported by Korade et al. (2010), who tested the same oxysterols— as well as several others derived via oxidation of 7DHC— on

cultured Neuro-2a cells, and found that certain oxysterols were extremely toxic to this murine, neuronal cell line, while others had only a moderate or no effect on cellular viability. In particular, the endoperoxide EPCD (See **Fig. 1**, and also structure [2a] in Korade et al., 2010) was at least an order of magnitude more potent in terms of toxicity than the “benchmark” oxysterol, 7kCHOL, a finding that was replicated in the present study. Importantly, the choice of oxysterols employed in both studies is relevant to the AY rat model of SLOS, since these same oxysterols— or, in the case of EPCD, by inference from previous studies that showed the formation of more stable, downstream metabolites of primary oxysterol endoperoxides (Xu et al., 2013; see also **Supplemental Figure 16D**)— were formed in the retina and other bodily tissues and fluids (*i.e.*, liver, brain, and serum) in AY9944-treated rats, but not in tissues from untreated normal age-matched control rats (Xu et al., 2011b, 2012b).

That 7kCHOL and other oxysterols exert toxic effects on a variety of cells in culture is well-documented (Li et al., 2011; Pedruzzi et al., 2004; Rodriguez et al., 2004). However, our findings represent the first report on the effects of such oxysterols using pure cultures of photoreceptor cell-derived cell lines. The inclusion of 7kCHOL in the present study was, in part, rationalized by the recent report that this molecule can be generated intracellularly via direct enzymatic conversion of 7DHC by cytochrome P450 7A1 (Shinkyo et al., 2011). 7kCHOL is also useful as a positive control against which to compare our novel oxysterol candidates, as it is the most widely studied oxysterol, usually considered within the context of CHOL oxidation. R28 cells, a heterogeneous rat cell line capable of expressing multiple retinal cell phenotypes, including neuronal and glial (Seigel, 2014), showed a statistically significant reduction in overall cell viability when challenged at confluence with 25 μ M 7kCHOL as a lower threshold concentration (Ong et al., 2003), but universal cell death was not manifested even at 100 μ M. This resistance to oxysterol cytotoxicity, plus the fact that R28 cell cultures potentially can express phenotypes corresponding to ganglion cells and other non-photoreceptor retinal

neuronal cell types, is fully consistent with the observation that cell death in the SLOS rat model retinal degeneration is essentially photoreceptor-specific (Fliesler, 2010, 2014; Fliesler et al., 2004).

As measured by our assays, the responses of rMC-1 cells to the damaging effects of potent oxysterols were intermediate between those observed with 661W and mRPE cells. This ranking is also consistent with the results comparing oxysterol treatments of embryonic mouse cortical neurons vs. early postnatal brain astrocytes (Xu et al., 2012a). At confluence, rMC-1 cells clearly appeared more to resemble mRPE cells in their resistance to oxysterol toxicity. Although these initial studies have been limited to illustrating the toxic effects and loss of viability when the rMC-1 cell line was exposed to oxysterols, previous observations on native MC in the AY rat model, most notably the increased expression of GFAP (Tu et al., 2013) suggest that this cell line could also be used to assess potential effects of these compounds as they relate to glial survival and hypertrophy, rather than cell death.

Our results obtained with cultured mRPE cells also correlate well with previous indications that the RPE in the SLOS rat model is much more resistant, compared to photoreceptors, to potentially toxic effects of oxysterols generated within the retina as a by-product of 7DHC accumulation (Fliesler, 2010, 2014; Fliesler et al., 2004). Notably, no TUNEL-positive RPE cells were observed in the retinas of AY9944-treated rats, while the outer nuclear layer (containing the nuclei of photoreceptors) exhibited extensive TUNEL staining (Tu et al., 2013). Numerous published investigations have shown that 7kCHOL as well as other oxysterols (exclusive of the 7DHC-derived ones tested here) are toxic to ARPE-19 cells—a human RPE-derived immortalized cell line—*in vitro* (Dasari et al., 2010; Dugas et al., 2010; Ong et al., 2003; Rodriguez et al., 2004). These previous studies have indicated that levels of 7kCHOL ranging from 10 to 25 μ M can cause a statistically significant loss of viability of the cells, leading to the conclusion that this cell line is much more vulnerable to 7kCHOL than the non-transformed,

diploid, comparatively low-passage-number mRPE cells used in the present study. On the other hand, doses of 7kCHOL that achieved maximum efficacy, reaching as high as 125 μ M, did not appear to cause complete loss of viability of ARPE-19 in some of the earlier reports (e.g., Ong, et al., 2003), as determined by the qualitative and quantitative assays employed by the investigators. Cultured mRPE cells at confluence exhibited a similar tolerance for the oxysterols we tested, in that the reduction in viability, as measured by the metabolically-based CaAM assay, never exceeded 10%, for the maximal doses of either 7kCHOL or EPCD. [However, in some cases, significant effects at lower doses were revealed using the more sensitive SO assay.] Taken together with our findings reported here, these results suggest that RPE cells are extraordinarily resistant to the potentially damaging effects of oxysterols, especially when compared to neuronal cells.

There are numerous mitigating factors likely to be responsible for at least some of the apparent differences observed between our results and those of other laboratories for related cell types (661W vs. Neuro2a and R28 cells; ARPE-19 vs. mRPE cells) treated with oxysterols, which preclude perfectly balanced point-for-point comparisons. Our experimental design using three retina-derived cell types— all tested under equivalent incubation conditions— differed from previous protocols with respect to several parameters, including: *in vitro* phenotype, the cell number/density that were implemented, growth area/substrate, media formulations, vehicles employed for test substance delivery, exposure time, and assay methods.

Some additional facts regarding our cell culture procedures are worth mentioning. First, we reduced the proportion of serum used in both the growth and incubation media, from the conventional 10% (v/v), to 1% or less, with the balance largely replaced by defined media supplements. This was done for all of our *in vitro* cell cultures, including 661W cells, as opposed to the use of 10% (v/v) delipidized fetal bovine serum for culturing Neuro2a cells (although results similar to those reported by Korade et al. (2010) also were obtained in supplemented,

serum-free medium (Z. Korade, personal communication)). We also eliminated from our oxysterol incubation medium the routine supplementation with proteins and small molecules that conceivably could have acutely modified the cells' antioxidant status or other cytoprotective mechanisms, such as supplements modulating the glutathione pathway (e.g., selenite, ascorbate, and vitamin E). Therefore, in this respect, our nominal baseline results can be utilized in the future to examine the effects of antioxidants and other potentially cytoprotective treatments on the effects of oxysterols. The further rationale for any of the abovementioned adjustments was to isolate the effects of a given individual oxysterol, while also avoiding competition from excess medium sources of CHOL, such as serum lipoproteins, that could interfere with the uptake, biological activity, and metabolism of the test substances. In this regard, at the low serum concentrations we employed, we also did not detect any differences in the results of our viability assays when bovine calf serum (CS) delipidized by incubation with silica (Stephan and Róka, 1968) was substituted for unmodified CS in the incubation media (see **Supplementary Figs. 15A,B**). With these considerations in mind, our routine use of low-serum media significantly reduced the need for accommodation of our cultures to abrupt changes in media formulation, most notably avoiding any confounding effects of serum withdrawal (Asada et al., 2008; Voccoli et al., 2007).

Previous studies by others using R28 cells have suggested that exogenously supplied LDL could have a protective effect in the face of challenge by 7kCHOL (Neekhra et al., 2007), thereby providing an additional reason to limit exposure to serum in our studies, so as not to mask the full biological effects of the oxysterol being evaluated. However, the presence of lipoproteins in culture media containing serum does tend to facilitate solubilization of oxysterol molecules added to the media as concentrated stock solutions (Larrayoz et al., 2010) for *in vitro* incubations. It has been suggested, in fact, that oxysterols bound to lipoproteins are taken up by cultured cells via CD36 scavenger lipoprotein receptors, which is physiologically relevant for

an RPE *in vitro* model, since RPE cells express such lipoprotein receptors (Gordiyenko et al., 2004; Hayes et al., 1989). However, such a capability has not been directly documented, to our knowledge, for retinal neurons. Our approach was influenced, in part, by evidence that *de novo* cholesterol synthesis proceeds autonomously within the neural retina, supplementing the ability of the mammalian retina *in vivo* (including the RPE) to take up CHOL from blood-borne lipoproteins via MCs and RPE as intermediaries (Fliesler, 2015; Fliesler and Bretillon, 2010; Fliesler and Keller, 1997; Lin et al., 2015; Rodriguez and Larrayoz, 2010a; Zheng et al., 2015). The relative contributions of these two CHOL supply pathways to the overall CHOL content of the retina, or their activities in the different individual retinal cell types and cell layers, is currently not fully elucidated; however, it may be assumed that the synthetic pathway is nominally active in cells in all layers of the retina depending on their particular, individual homeostatic needs (Fliesler, 2015; Fliesler and Bretillon, 2010; Fliesler and Keller, 1997; Lin et al., 2015; Rodriguez and Larrayoz, 2010a; Zheng et al., 2015). For the homogeneous and defined cells we utilized in the present study, then, the upregulation of cholesterol synthesis in response to the low levels of serum, and therefore potentially limiting amounts of exogenous cholesterol, in our culture media must also be considered as a possibility. If the recent immunohistochemical results of Zheng et al. (2015) are indicative of the status of 661W cells, as surrogate cone photoreceptors, to synthesize cholesterol *de novo*, it might be expected that this cell line was not autonomous in this respect; however, as immortalized cells whose replicative potential has not been diminished in our hands— even without cholesterol supplementation (not shown)— then, by definition, they in fact must have an active cholesterol biosynthetic capacity (Silber et al., 1992). Taken together, our main consideration in incubating cultured cells with 7DHC-derived oxysterols was to effect their internalization, leading to the presence, accumulation to potentially toxic levels, metabolic and oxidative conversions, and subsequent biochemical activity of these molecules and their downstream products. In this manner we intended to approximate to the best of our ability the *in situ* cell-mediated formation of such molecules,

emblematic of retinal tissue elements, to meet the criteria for an *in vitro* model with relevance to SLOS patients and in SLOS animal models such as the AY9944-treated rat model. In the case of our pure cell cultures, which lack the complex organizational context of their native counterparts *in vivo* in the intact retina, we chose to solubilize the test substances (which have limited aqueous solubility) using either HP β CD (Larrayoz et al., 2010), or the common organic solvent, DMSO, as excipients. This technically non-physiological, yet practical, approach did accomplish uptake of oxysterols by the cultured cells (**Supplemental Figure 16**), yielding dose-dependent viability results that at least qualitatively paralleled those for oxysterols tested using Neuro2a and ARPE-19 cells (Korade et al., 2010; Larrayoz et al., 2010; Rodriguez et al., 2004). (As further demonstrated in **Supplemental Figures 16B and 16C**, EPCD was rapidly converted by 661W cells to the 6 α -tetraol, a potential precursor of THCEO (3 β ,5 α ,9 α -trihydroxycholest-7-ene-6-one); the latter molecule, a more long-lived end-product, may be considered by analogy with DHCEO to be a stable biomarker for the accumulation and primary oxidation of 7DHC in the tissues of the AY rat and of a genetic mouse model of SLOS (Xu et al., 2013, 2011b).)

All of the end-point assays reported here were conducted at 24 h, as compared to 48 h for exposure of Neuro2a cells to the same oxysterols in the studies previously reported by Korade et al. (2010); hence, in this respect 661W cells appear to be more sensitive than Neuro2a cells to the cytotoxic effects of these oxysterols. With 661W cells cultured both at sub-confluence and confluence, and also with sub-confluent rMC-1 and mRPE cells, the 24-h time frame with dose-ranging accommodated a suitable maximum efficacy window for the two most toxic oxysterols (EPCD and 7kCHOL), ranging from no significant difference to complete and global loss of viability, relative to vehicle controls. The resulting data in some instances displayed extremely abrupt transitions from zero to virtually 100% efficacy between small incremental increases in dosage (e.g., see Fig. 4c, 661W cells treated with EPCD). For confluent cultures of rMC-1 and mRPE cells, we did observe progressive increases in efficacy,

but still not approaching 100% loss of viability, for EPCD at 48 h compared to 24 h (see **Supplementary Fig. 14**), similar to results obtained with ARPE-19 cells challenged with 7kCHOL (Rodriguez et al., 2004).

The magnitude of the signal in the CaAM assay is proportional to viable cell number; hence, as with the RR and similar viability assays, when sub-confluent cells were used, the assay in part logically assessed the effects of oxysterols on proliferation, which has been considered one facet of viability (Cook and Mitchell, 1989). In contrast, especially in regard to the stable, confluent mRPE cultures, both the CaAM assay and the SO assay provided a more accurate and representative measure of cell survival. Our sequential duplex viability assay (CaAM/SO protocol), which eliminated rinses or transfers, was advantageous in that it measured signals from both live and dead cells, assessing both metabolic capacity (*i.e.*, metabolic enzyme (esterase) activity in the CaAM assay; Bozyczko-Coyne et al., 1993), and loss of membrane integrity (with uptake and subsequent binding of the SO reagent to nuclear DNA; Pierce et al., 2003). Both these facets of cellular deterioration could be associated with multiple forms and/or stages of cell death, not necessarily restricted to, *e.g.*, classical apoptosis (Kepp et al., 2011). Indeed, our correlative biochemical and immunohistochemical analyses of retinas in the SLOS rat model thus far suggest that photoreceptor cell death in this animal model does not involve caspase-3 activation and downstream activity (Tu et al., 2013; Xu et al., 2012b). Further experiments will be required to delineate both primary and secondary cell death mechanisms induced by 7DHC-derived oxysterols, both in our surrogate cell culture systems as well as in the SLOS rat model.

Regarding mechanisms of both cell death and cell survival, and what may be reasonably inferred and applied back to the SLOS rat model, both the 661W and rMC-1 cell lines are admittedly less than perfect models of *in situ*, morphologically differentiated photoreceptors and Müller glia, respectively, if only because they are immortalized and do not enter the G₀ phase of

mitotic quiescence associated with fully differentiated neural cells (including unreactive glial cells) (Myers et al., 2008; Zhu et al., 1999). Unlike retinal photoreceptor cells, 661W cells lack the key hallmark of such cells, *i.e.*, an outer segment; 661W cells also lack the exquisite, highly polarized and differentiated cytological architecture of retinal photoreceptors. Yet, they possess key molecular signatures (*e.g.*, visual pigment apoprotein, etc.) only found in photoreceptors, but not other, cells (See **Supplementary Data, Section 1.1**). In contrast, if the homogeneous, stable epithelial morphology alone of the monkey RPE cultures—especially when confluent (Hsiung et al., 2015)—is any indication of how representative of native RPE they are, we suggest that the response of mRPE cells to oxysterol challenge is more physiologically relevant than what information has been obtained to date using ARPE-19 cells. The case may be made that, for the latter, the extended culture time after plating determined to be necessary for morphological homogeneity and phenotypic maturation may not have been achieved (Luo et al., 2006).

Even within the unique subset of chemical structures described thus far and delineated as oxysterols derived from 7DHC (of which there are at least 15 known), there are a variety of molecular pathways responsible for their generation, and also multiple modes of metabolism and ultimate utilization of these compounds by cells (Xu et al., 2011a; Xu L et al., 2013). It therefore would be expected that different molecular structures within this class of oxysterols would not only have a range of efficacies with regard to toxicity, but also that the mechanisms of action leading to loss of viability for the most potent compounds would be diverse, and that this last consideration might distinguish 7kCHOL from EPCD. It has been postulated, for example, that the endoperoxide moiety of EPCD is subject to internal modification via intracellular reducing equivalents to form an intermediate, transient 6 α -tetraol derivative, with eventual metabolism to THCEO, a compound that is structurally similar to DHCEO (Xu et al., 2011a; Xu L et al., 2013), and from these modifications may reasonably be expected also to exert toxic

effects. By analogy to the proposed pathways employed to detoxify bisretinoid endoperoxides (Yoon et al., 2011), such as those associated with and implicated in the etiology of age-related macular degeneration and juvenile (Stargardt's) macular degeneration (Delori et al., 1995; Sparrow et al., 2010), EPCD may be a "sink" for glutathione consumption, possibly adversely affecting intracellular redox status. In the case of 7kCHOL, although both photoreceptors and RPE express the 7kCHOL-metabolizing enzyme cytochrome P450 (CYP) 27A1 (Lee et al., 2006), there may be differences in this enzymatic activity and subsequent cellular efflux of detoxification by-product(s) between these two retinal cell types (Heo et al., 2011), which conceivably could impact their differential sensitivity to this toxic oxysterol. In light of the *in vitro* results reported here, it would be informative to determine the relative expression levels of CYP27A1 in 661W, rMC-1, and mRPE cells incubated with and without 7kCHOL, as well as a determination of the existence and accumulation of less toxic metabolites of 7kCHOL using mass spectrometric techniques. Specific initial molecular pathways involving 7kCHOL in cell death have not yet been elucidated and proven, but considerable evidence obtained from a number of *in vitro* and *in vivo* systems implicates this and other 7DHC-derived oxysterols in increased oxidative stress, ER stress, autophagy, inflammatory cytokine production, and compromised mitochondrial function (Chang et al., 2014; Du M et al., 2013; Jane and Lee, 2011; Larrayoz et al., 2010; Lizard et al., 2000; Rodriguez and Larrayoz, 2010a; Xu M et al., 2013). The viability assays we performed are not informative with regard to specific mechanisms or pathways, but some differences between the effects of EPCD and 7kCHOL were apparent, aside from their having significantly different potencies. In particular, 7kCHOL did not exhibit dose-dependent efficacy in either sub-confluent or confluent cultures of rMC-1 cells by the SO assay, whereas EPCD gave a more typical SO assay readout in the context of these studies (*cf.* **Figs. 5C and 5E**, and **Fig. 6**).

One paradigm for cell death in the face of various forms of stress is that cells retain a healthy status by responding to modest challenges that normally do not force them beyond a certain functional threshold (*i.e.*, hormesis, the cellular equivalent of the old saying, "That which does not kill you makes you stronger") (Calabrese, 2008). Formation and progressive steady-state accumulation of oxysterols initially may cause the cell to up-regulate endogenous protective mechanisms to help maintain cellular integrity and viability; however, beyond a certain stress threshold, these protective mechanisms become exhausted, at which point programmed death pathways are invoked (Chen and Brandizzi, 2013). Photoreceptors, Müller glia, and RPE cells, on the other hand, are believed to differ from one another with respect to the breadth of their armamentarium of antioxidant protective pathways and survival factors; photoreceptors, in particular, and especially cones, are thought to be more vulnerable, and RPE less so, in this respect (Jarrett et al., 2006; Punzo et al., 2012; Rogers et al., 2007). Furthermore, photoreceptors are dependent on both Müller glia and RPE cells for metabolic and trophic support, lessening the intrinsic requirement for photoreceptors to confront in isolation even normal challenges, such as oxidative stress generated constitutively by the high activity of mitochondria in their inner segments (Du Y et al., 2013; Perkins et al., 2003). As a consequence of the formation and steady-state accumulation of 7DHC— which, as mentioned above, is extraordinarily sensitive to oxidation— elevated levels of oxysterols within the outer neural retina occurring in disease states could kill photoreceptors outright, and also might preferentially evolve within the RPE, moderately compromising its homeostatic capabilities while indirectly affecting the viability of the less-endowed and both anatomically- and physiologically-dependent photoreceptors. The latter scenario would then mimic some heritable blinding conditions, as well as aging and toxicological responses involving deficits expressed in, but not causing the immediate demise of, the RPE (Jarrett et al., 2008; Kay et al., 2012), that secondarily but profoundly affect photoreceptor function.

It is expected that the studies reported here will give rise to further experiments delineating oxysterol-induced mechanisms of retinal— and, more specifically, neuronal— loss of function and viability, in both SLOS as well as other diseases that impact the health and function of the retina, and also revealing molecular aspects of neuroprotection by, and sustained survival of, glia and RPE. The insights gained are expected to lead to the development of novel therapies for SLOS, and for retinal and other neurodegenerative diseases.

Acknowledgements

We thank Priyanka Patel, Matthew Behringer, Rohini Narayanaswamy, and Meerim Choi for technical assistance during the course of these studies, and Lisa Prince-Baker for clerical assistance in the preparation of this manuscript. Dr. Zeljka Korade, Vanderbilt University, provided invaluable comments on the manuscript. Drs. Sarah Zhang and Jacey Ma provided immunofluorescence data for cultured monkey RPE cells included in the ***Supplemental Results***. This work was supported, in part, by U.S.P.H.S. (NIH) grants EY007361 (SJF), R00 HD073270-01 (LX), ES013125 and HD064727 (NAP and LX), NSF grant CHE-1057500 (NAP), by an Unrestricted Grant to the Department of Ophthalmology, University at Buffalo-The State University of New York (SJF), and by facilities and resources provided by the VA Western New York Healthcare System (SJF). The opinions expressed herein do not necessarily reflect those of the Veterans Administration or the U.S. Government.

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Figure legends

Figure 1. Crystal violet (CV)-stained cultures of sub-confluent 661W cells after 24-h treatments with dose-ranges of cholesterol (CHOL) and four 7-dehydrocholesterol (7DHC)-derived oxysterols, with accompanying structures of the compounds tested. Also shown are CV-stained cultures following treatments with hydroxypropyl- β -cyclodextrin (HP β CD) vehicle control (used for CHOL and all the oxysterols except EPCD), with control medium without vehicle, and with 40 nM staurosporine (cytotoxic positive control). Loss of cell viability as the concentration of the cytotoxic oxysterols increased (EPCD, 7kCHOL, and DHCEO) was reflected in fewer cells remaining attached to the tissue culture plastic substrate and decreased uptake of stain. The observed fields represent a central 7 mm² area in the center of the 95-mm² total area per well in a 48-well plate. The qualitative results with respect to dose-response and relative potency of oxysterols depicted here show close correlation with the quantitative CV assay results in Figures 3(A-C), (below).

Figure 2. Phase-contrast images of 661W cells treated with a dose-range of 7-ketocholesterol (7kCHOL). The lowest concentrations tested induced no obvious changes in individual cell or colony morphology compared to vehicle control-treated cells, which displayed phase-refractile cell boundaries and exhibited a relatively homogeneous, spread morphology bearing multipolar, short neuritic processes. Nominally viable cells that rounded up during mitosis were especially phase-bright (arrowheads in (A) and (C)). In (D), thick arrow indicates a cell with retracted neurites. Detached cells became numerous at higher 7kCHOL concentrations, and drifted out of the plane of focus (thin arrows in (D) and (E)). (A), Vehicle control treatment; (B) 3 μ M 7kCHOL; (C) 12 μ M 7kCHOL; (D) 16 μ M 7kCHOL; (E) 20 μ M 7kCHOL. All images are at the same magnification; bar in (A) = 100 μ m.

Figure 3. (A, B) Crystal violet (CV); (C) Resazurin reduction (RR) and CV; and (D, E) RR viability assay results for subconfluent 661W cells incubated for 24 h with cholesterol (CHOL) or oxysterols. (A) CHOL treatment (white bars) encompassing a dose range of 3 – 45 μ M did not significantly affect cell viability, compared with 7-ketocholesterol (7kCHOL; black bars), which resulted in statistically significantly lowered cell viability at as low as 13 μ M. All values in (A and B) were normalized to the optical density (O.D.) of the sample treated with control (incubation) medium without vehicle (CnoV; = 1.0). * P <0.05 vs. vehicle control (VC), by ANOVA (Tukey's *post hoc* test); here and in all succeeding assay results presented, statistical analysis was

carried out within the sample set for each individual compound tested. **(B)** Graph comparing CV results for DHCEO and 4DHCHC. DHCEO showed statistically significant effects on cell viability at doses of 30 and 45 μ M, but less than 100% efficacy at these concentrations. Toxicity of 4HDHC was not demonstrated at any dose, similar to CHOL **(A)**. $*P<0.05$ vs. VC. **(C)** Results for EPCD (and positive control staurosporine (Stsp) at 40 μ M) in either the RR (black bars) or the CV (white bars) assays showed good correlation, especially at higher doses of EPCD effecting complete loss of viability. Slightly lower potency and efficacy of EPCD in the RR assay, compared to results for the CV assay at equivalent doses, is attributable to rinsing wells in the latter leading to some loss of cells, while some non-specific background staining of wells led to slightly higher values in the CV assay at higher doses of EPCD. $*P<0.05$ vs. VC. **(D, E)** RR assay comparing results for 7kCHOL, CHOL, DHCEO, and 4HCHC. RFU = Relative fluorescence units; values were normalized to those for CnoV. Results correlated well with the CV assays for each compound shown in **(A)** and **(B)**, respectively. $*P<0.05$ vs. VC. All error bars here and in succeeding charts represent $+ [1.96 \times \text{Standard Error}]$; lower error bars of equal magnitude are not shown.

Figure 4. (A-C) Sytox Orange (SO) assays for each compound tested for 24 h on subconfluent 661W cells. **(A)** 7kCHOL displayed cytotoxicity at 20 μ M and above, whereas DHCEO did not demonstrate statistically significant reductions in cell viability for 661W cells. Positive control Stsp was cytotoxic at 50 nM in both sets of experiments. $*P<0.05$ vs. VC. RFU, Relative fluorescence units, normalized to CnoV (=1.0). **(B)** Neither CHOL nor 4HDHC caused loss of cell viability throughout the concentration range tested. **(C)** Results for EPCD shows increasing toxicity with statistical significance as low as 0.6 μ M, achieving maximal efficacy at 3 μ M. Stsp was tested at 50 nM. $*P<0.05$ vs. VC.

Figure 5. (A) Confluent culture of 661W cells. Arrowhead: mitotic doublet. Bar = 100 μ m. **(B)** and **(C)** duplex Calcein AM (CaAM) and SO assays, respectively, carried out on identical wells of confluent monkey RPE (mRPE), 661W, and rMC-1 cells following 24 h exposure to a dose range of 7kCHOL. $*P<0.05$ vs. VC, for each separate assay performed on an individual cell type. Confluent 661W and rMC-1 cells were not tested with 7kCHOL at 45 μ M (black and white X's). **(D)** and **(E)** CaAM and SO duplex assays on the three cell types, incubated at confluence for 24 h with a dose range of EPCD; also included were samples treated with 26 μ M 7kCHOL (7k) as a positive control with 661W based on results in **(B)** and **(C)**. $*P<0.05$ vs. VC, within each experiment performed on an individual cell type. (mRPE was not tested with 1 μ M EPCD

(gray X in (E).) Results for confluent mRPE tested with EPCD were not included for the CaAM assay data in (D); see **Supplemental Fig. 12 (A)**. Results for *subconfluent* mRPE incubated with EPCD are shown in **Supplemental Figs. 12 (B, C)**

Figure 6. Duplex CaAM (black bars) and SO (white bars) assay carried out on subconfluent rMC-1 cells treated for 24 h with 7kCHOL. * $P < 0.05$ vs. VC in the CaAM assay. # $P < 0.05$ vs. VC for the decreased values in the SO assay. § $P > 0.05$ (not significant) vs. 26 or 30 μM 7kCHOL in the SO assay.

Figure 7. Subconfluent rMC-1 cells incubated with (A) VC, or (B-F) with a dose range of 7kCHOL; (B) 13 μM ; (C) 16 μM ; (D) 20 μM ; (E) 23 μM ; (F) 26 μM). The threshold for loss of cells (between 16 and 20 μM) shows good correlation with the results of the CaAM assay in **Fig. 6**. Bar = 100 μm .

Figure 8. Confluent rMC-1 cells incubated with either (A) VC or (B) negative control (30 μM CHOL) retained integrity with no apparent differences in morphology. (C) Exposure to 30 μM 7kCHOL induced only minor changes in the monolayer; cf. with CaAM and SO assay results shown in **Figs. 5(B) and 5(C)**. (D) 10 μM EPCD treatment caused disruption of the confluent monolayer, although most cells remained attached, showing good apparent correlation with the assay results in **Figs. 5(D) and 5(E)**.

Figure 9. (A) Subconfluent culture of monkey RPE (mRPE) cells. Suspended RPE cells generated in low calcium medium were seeded in 48-well plates, and after allowing time for cell attachment, medium was switched to that containing $[\text{Ca}^{++}]$ at 0.5 mM. Colonies formed displaying epithelial morphology at subconfluence and were used for oxysterol incubation experiments. (B, C) CaAM and SO assays, respectively, of subconfluent mRPE exposed to a dose range of 7kCHOL. * $P < 0.05$ vs. VC. § $P < 0.05$ vs. VC, indicating a small percentage decrease in cell viability as measured in both assays for VC (containing HP β CD), compared with control medium without vehicle (gray bar). 100 nM Stsp (white bars in (B) and (C)) did not affect cell viability in the CaAM assay.

Figure 10. (A-C; G-I) Phase-contrast images of confluent mRPE, and (D-F) subconfluent mRPE. (A) and (D) after HP β CD, or (G) DMSO vehicle control incubations, or 24-h treatments with (B) 30 μM ; (C) 45 μM ; (E) 23 μM ; (F) 26 μM 7kCHOL; or (H) 13 μM ; (I) 20 μM EPCD. All

images at same magnification (bar in **(G)** = 100 μm). Note: **(D)** is a detail of **Fig. 9(A)**. (See **Supplemental Figure 13** for images of *subconfluent* mRPE incubated with EPCD.)

Table I. Quantitative assessment of cellular viability of confluent rMC-1 cells incubated with exogenous cholesterol.

Assay	Treatment					
	23 mM CHOL	26 mM CHOL	30 mM CHOL	Vehicle Control	Control (no Vehicle)	125 nM Stsp
Calcein AM	6608 ± 630	7219 ± 587	6295 ± 806	6981 ± 997	8818 ± 689	1002 ± 371*
Sytox Orange	10251 ± 1079	10184 ± 1294	11293 ± 2743	8757 ± 1443	13093 ± 5127	36375 ± 408*

Values in RFU, ± 1.96xSE. Stsp, staurosporine. *P<0.05 vs. assay vehicle control.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10