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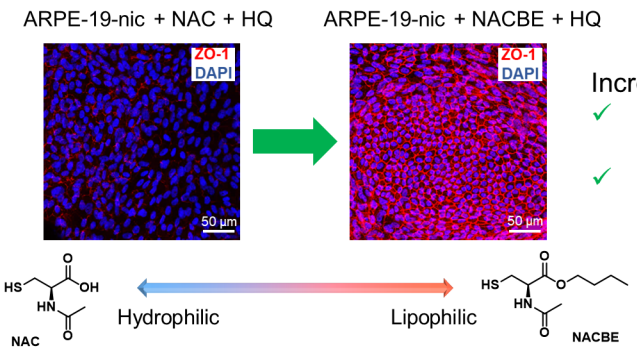

Protection of Human Retinal Pigment Epithelial Cells from Oxidative Damage using Cysteine Prodrugs

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ABSTRACT

Age related macular degeneration (AMD) is one of the major causes of vision loss in the elderly in most developed countries. Among other causes, oxidative stress in the retinal pigment epithelium (RPE) has been hypothesized to be a major driving force of AMD pathology. Oxidative stress could be treated by antioxidant administration into the RPE cells. However, to achieve high *in-vivo* efficacy of an antioxidant, it is imperative that the agent be able to penetrate the tissues and cells. Evidence suggests that lipophilicity governs cellular penetrance. Out of many antioxidant candidates, *N*-acetyl-*L*-cysteine (a prodrug of *L*-cysteine) (NAC) is a potent antioxidant as the bioavailability of the parent drug, *L*-cysteine, determines the production of glutathione; the universal antioxidant that regulates ROS. To increase the lipophilicity, four ester derivatives of *N*-acetylcysteine: *N*-acetylcysteine methyl ester, *N*-acetylcysteine ethyl ester, *N*-acetylcysteine propyl ester, and *N*-acetylcysteine butyl ester were synthesized. To mimic *in vitro* AMD conditions, hydroquinone, a component of cigarette smoke, was used as the oxidative insult. Cytosolic and mitochondrial protection against oxidative stress were tested using cytosolic and mitochondrial specific assays. The results provide evidence that these lipophilic cysteine

prodrugs provide increased protection against oxidative stress in human RPE cells compared with NAC.

Keywords: Age-related macular degeneration, oxidative stress, *N*-acetylcysteine butyl ester (NACBE), glutathione, mitochondria

INTRODUCTION

Reactive oxygen species (ROS) fulfill many vital functions in eukaryotic cells, including communication between nucleus and mitochondria, autophagy of abnormal mitochondria, and protection against pathogens.[1-4] However an excess of ROS can lead to oxidative stress, which damages DNA, proteins and lipids, and ultimately results in dysfunction of organelles and cells.[4] In healthy cells ROS regulation (redox homeostasis) can be achieved via enzymatic antioxidants (glutathione peroxidase, superoxide dismutase, and catalase), and non-enzymatic antioxidants such as glutathione (GSH), α-tocopherol, ascorbic acid and certain amino acids (cysteine, histidine, tryptophan and tyrosine).[1, 3]

Age related macular degeneration (AMD) is a pathological condition that occurs in the elderly.[5-8] It is speculated that oxidative injury in the retinal pigment epithelial (RPE) cells is one of the causes of AMD.[9-11] Recent studies have revealed that there is a strong connection between mitochondrial damage in RPE and AMD.[2] Mitochondrial dysfunction can occur as a result of poor regulation of oxidative stress. As mitochondria produce the majority of energy needed for the cells in the form of ATP, mitochondrial dysfunction leads to an energy crisis and this is believed to be one of the drivers of AMD pathology. In healthy cells GSH, a ubiquitous intracellular antioxidant, protects cells against oxidative injury. GSH is primarily produced in the cytosol and is transported from cytosol into the mitochondria using glutathione specific

transporters. Increasing GSH levels in the cytosol would be a useful way to increase protection against oxidative stress for both cytosolic and mitochondrial damage.

Biosynthesis of GSH involves two steps; L-cysteine and glutamate first combine in the presence of γ-glutamylcysteine synthetase to produce a dipeptide and it is subsequently converted into GSH with glycine in the presence of glutathione synthetase.[12] It has been found that the rate limiting step for the process is the first step and solely governed by the bioavailability of L-cysteine. However, given the high hydrophilicity of L-cysteine, exogenous administration is not anticipated to result in a greater accumulation or retention in the RPE cells. Using a lipophilic prodrug approach to deliver antioxidants into RPE cells could be beneficial in this aspect. It is been shown previously that lipophilic small molecular drugs permeate better into the RPE than hydrophilic small molecular drugs.[13-15] *N*-acetylcysteine (NAC)[16-18] and *N*-acetylcysteine amide (NACA)[19-21] are among some of the lipophilic constructs of cysteine that have been used with ARPE-19-nic cells to show protection against oxidative damage. Hence, in an attempt to further increase the protection given from these small molecular antioxidants, NAC ester prodrugs were synthesized with increasing lipophilicity. We selected NAC as the parent drug as it can be subjected to chemical alterations easily. Upon cell uptake, NAC ester derivatives will undergo de-esterification via endogenous esterases to produce NAC and then to cysteine through the activity of amidases. The produced cysteine will then participate in GSH synthesis, thereby increasing the availability of GSH to the cell.

N-acetylcysteine ethyl ester (NACEE) has been previously demonstrated to increase cellular GSH levels through the conversion to NAC and then to L-cysteine.[22, 23] In this report, three additional ester derivatives of NAC were synthesized; *N*-acetylcysteine methyl ester (NACME), *N*-acetylcysteine propyl ester (NACPE), and *N*-acetylcysteine butyl ester (NACBE), and

evaluated for their ability to provide cellular and mitochondrial protection against oxidative damage. As smoking is a clinically relevant oxidative injury,[11, 24-28] hydroquinone (HQ), a component of cigarette smoke, was used to create oxidative damage in human RPE cells.[9, 29-31]

MATERIALS AND METHODS

All commercially available chemicals/media were purchased from Fisher Scientific, Sigma Aldrich or Cayman Chemicals and were used as received unless otherwise mentioned. Thionyl chloride was distilled fresh prior to use. DMEM:F-12 and FBS were purchased from ATCC. CellTiter-Glo® and GSH/GSSG-Glo™ were purchased from Promega. ^{1}H and ^{13}C NMR were recorded on a 500 MHz Bruker AVANCEIII™ spectrometer using deuterated chloroform as the solvent. All NMR spectra are calibrated to chloroform. Multiplicities are given as: singlet (s), doublet (d), triplet (t), quartet (q), doublet of a doublet (dd), multiplet (m) and broad singlet (br. s). UV-Vis spectra were recorded on a Thermo Scientific NanoDrop™ 2000 spectrophotometer. Fluorescence spectra and plate readings were recorded on SpectraMAX Gemini EM microplate reader and absorption readings for XTT assay were obtained using VersaMax microplate reader. Luminescence readings were recorded on LUMIstar Omega microplate luminometer. Confocal images were obtained using Carl Zeiss LSM 800 system, Axio Observer microscope. Reversed phase high performance liquid chromatographic (RP-HPLC) analysis were performed using Agilent 1100 Series HPLC equipped with an Agilent autosampler (model G1313A) and an Agilent UV detector (model G1365A).

Synthesis of N-acetylcysteine esters and dansyl tagged N-acetylcysteine esters

Details of the synthesis for the chemical compounds used in this study are contained in the supplementary method section.

Cell culture of ARPE-19-nic

ARPE-19-nic cells were grown in DMEM:F-12 supplemented with 10% fetal bovine serum (FBS). For all experiments these cells were split and grown in 6-well plates using MEM-Nic supplemented with 1% FBS according to a previously published procedure.[32] Cells used for all the experiments were between passages 25-30. For all experiments, the 96-well plates and 8-well slides were coated with 0.039 mg/ mL collagen I at 6 µg/ cm^2. The cells were seeded at a cell density of 70,000 cells/ well and 150,000 cells/well using MEM-Nic media for 96-well plates and 8-well slides, respectively. Once the cells are confluent, media was replaced with MEM α, GlutaMAX™, supplemented with 1% FBS for 24 hours before carrying out assay protocols. Treatment with drugs were carried out in MEM α, GlutaMAX™, supplemented with 1% FBS and treatment with HQ was carried out in serum free DMEM:F-12. All assays were carried out in triplicate wells unless otherwise mentioned.

XTT cell viability assay

The ARPE-19-nic cells were grown in clear flat bottom 96-well plates. Various concentrations of HQ (100 – 1000 µM) were introduced to the cells and cells were incubated at 37 °C, 5% CO_2 for 16 hours. For pretreatment assays, the drugs were introduced at a concentration of 0.05 mM for 2, 24 and 48 hours and then treated with 500 µM of HQ for 16 hours. Before the addition of XTT+PMS (XTT:2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide, PMS: phenazine methyl sulfate) reagent, the HQ solutions were removed and replaced with DMEM:F-12 supplemented with 1% FBS.

Cell viability assay was carried out using XTT/PMS reagent mixture as previously described[33] and the absorbance readings were obtained using a plate reader at 450 and 660 nm.

ZO-1 Staining

ARPE-19-nic cells were grown on an 8-well slide until confluent. The cells were exposed to 1 mM NAC and NACBE 24 hours, followed by treatment with 500 µM of HQ for 2 hours. The cells were washed with 3 cycles of PBS, and then fixed with 4% paraformaldehyde at 4 °C for 30 mins. After fixation the cells were blocked in PBST (0.2% Triton X-100) + 1% BSA for 60 mins. Primary antibody (rabbit anti-ZO-1, Invitrogen) was diluted 1/100 in PBST + 1% BSA and added overnight at 4 °C. Cells were washed x3 with PBS and secondary antibody (Donkey anti-Rabbit AF-555, Abcam) was added for 4 hours at RT. Cells were washed x3 with PBS and mounted with Prolong Diamond Mountant with DAPI (Invitrogen).

HPLC Analysis

ARPE-19-nic cells were seeded onto a 60 cm^2 dish and was allowed to grow to confluency. The cells were treated with 1 mM NAC and NACBE for 1 hour in HBSS. The drug solution was aspirated and washed twice with HBSS. The cells were scraped with the aid of methanol (~ 1 mL) and collected into 2 mL centrifuge tubes. The cell suspension in methanol was sonicated in a water bath for 30 seconds and was centrifuged at 16,000 g for 15 minutes. The supernatant was transferred to a HPLC vial and methanol was evaporated under a stream of nitrogen. The sample was resuspended in 50 µL of methanol before injecting into the HPLC system. Samples and standard (20 µL) were injected with an autosampler. Separation was conducted by 0.8 mL/min gradient elution with a water/0.1% formic acid and acetonitrile mobile phase on a 250 × 4.6-mm

(5-mm) C18 column (Restek, Pinnacle II) maintained at 25 °C. The samples were monitored at 205 nm with a UV detector and analyzed with Agilent Chemstation software.

GSH assay

ARPE-19-nic cells were grown in white 96-well plates. The cells were exposed to 1 mM solutions of NAC-NACBE, NACA, and glutathione ethyl ester (GSH-EE) for 24 hours. The solutions were removed and washed with PBS once. Afterwards, GSH assay was carried out according to manufacturer recommended protocol (Promega GSH/GSSG-Glo™) and luminescence readings were obtained.

Confocal microscopy with dansyl tagged N-acetylcysteine esters

ARPE-19-nic cells were grown in 8-well slides before exposing to 1 mM solutions of Dan-NACME, Dan-NACEE, Dan-NACPE and Dan-NACBE for 1 and 24 hours. The cells were washed twice with PBS followed by mounting using PBS.

JC-1 assay

ARPE-19-nic cells were grown in black, clear bottom 96-well plates. Dose and time dependent assay for HQ was carried out using 25, 50 and 100 µM for 1, 2, 4, 6, 8 and 16 hours. For pretreatment assays, 1 mM solution of all drugs were incubated for 24 hours before treating with 50 µM HQ for 4 hours. The HQ solutions were removed and washed once with PBS before the addition of JC-1 reagent.

10 µM solution of JC-1 reagent in serum free DMEM:F-12 was prepared by diluting 1 mM JC-1 solution in DMSO. The 10 µM solution was centrifuged at 7,200 g for 5 minutes before the addition to the cells followed by incubating at 37 °C, 5% CO_2 for 30 minutes. The JC-1 solution

was removed and washed once with PBS and fluorescence measurements were obtained in PBS at 485 nm excitation and emission at 535 nm and 590 nm.

JC-1 staining

Confluent ARPE-19-nic cells were pretreated with the drugs for 24 hours before exposing to 50 µM HQ for 4 hours. Following HQ treatment, a 10 µM solution of JC-1 was added to the cells for 30 minutes, the cells were washed with PBS x 3 and then mounted in Antifade Mountant (Invitrogen). The cells were imaged on the confocal (Zeiss LSM 800) by excitation with the 488 nm laser and emission imaged at 530 nm (green channel) and 590 nm (red channel).

Mitochondrial GSH assay

ARPE-19-nic cells were grown in 6-well plates until 100% confluent in MEM-NIC media. The cells were exposed to 3 mL of 1 mM solutions of NAC and NACBE for 24 hours. The solutions were removed and washed with 3 mL of HBSS before adding 1 mL of 0.25% Trypsin-EDTA and incubating for 10 minutes. 2 mL of DMEM:F-12 supplemented with 10% FBS was added to each well, harvested and centrifuged at 300 rcf for 5 minutes. The supernatant was removed and cell pellet was resuspended in 2 mL of isolation buffer (0.25 M sucrose and 10 mM HEPES). Cells were disrupted using a probe sonicator (Misonix S-3000) for 10 seconds in ice. Subsequently, intact cells and debris were removed by centrifuging at 1000g for 10 mins. Supernatant was collected, and centrifuged at 20,000g for 25 minutes. Pellet containing mitochondria were saved and washed using 0.5 mL of isolation buffer. After centrifuging at 20,000g for 25 minutes the mitochondrial pellet was resuspended in 50 µL of HBSS. 25 µL was used to determine total GSH and GSSG and 25 µL was used to determine GSSG levels. GSH

assay was then carried out according to manufacturer recommended protocol (Promega GSH/GSSG-Glo™) and luminescence readings were obtained.

CellTiter-Glo assay

ARPE-19-nic cells were grown in white 96-well plates. The cells were first exposed to 500 µM of HQ for 3, 6 and 8 hours to obtain a time dependent response. For pretreatment experiments, first, the cells were pretreated with 1 mM solutions of NAC and NACBE for 24 hours followed by exposure to 500 µM of HQ for 3, 6 and 8 hours. Manufacturer recommended CellTiter-Glo assay protocol (Promega CellTiter-Glo®) was followed and luminescence readings were obtained.

DNA fragmentation assay

The mitochondrial DNA damage assay was performed according to the protocol outlined in a previous publication.[34] Briefly, ARPE-19-nic cells were grown to confluency in 6-well plates. The cells were treated with NAC compounds for 24 hours, washed and then treated with 500uM HQ for an additional 24 hours. DNA was isolated from the treated cells with a QIAamp DNA mini kit (Qiagen). The DNA samples were diluted to 3 ng/µL for use in PCR reactions. Primers 5'-TCT AAG CCT CCT TAT TCG AGC CGA-3' and 5'-TTT CAT CAT GCG GAG ATG TTG GAT GG-3' were used to amplify a large mitochondrial target (8.9kb). Primers 5'-CCC CAC AAA CCC CAT TAC TAA ACC CA-3' and 5'-TTT CAT CAT GCG GAG ATG TTG GAT GG-3' were used to amplify a small mitochondrial band. PCR products were quantified using the Quant-iT Picogreen dsDNA Assay kit (Invitrogen). The relative amplification of the large band was normalized to untreated cells. The amplification of the small mitochondrial band was used to

normalize the date obtained from the large band to account for mitochondrial DNA copy number.

RESULTS

Synthesis of N-acetylcysteine esters

All ester derivatives of NAC were synthesized by conversion of the carboxylic acid group in NAC to acyl chloride and the subsequent esterification with the appropriate alcohol (Figure 1). All compounds were purified with column chromatography using silica gel. Pure compounds were obtained in moderate yields and were characterized with ^{1}H and ^{13}C NMR spectroscopy (see Supplemental Data for details. Figures S1-S8). The synthesized compounds have increasing lipophilicity from NACME to NACBE with the increase in the number of carbon atoms.

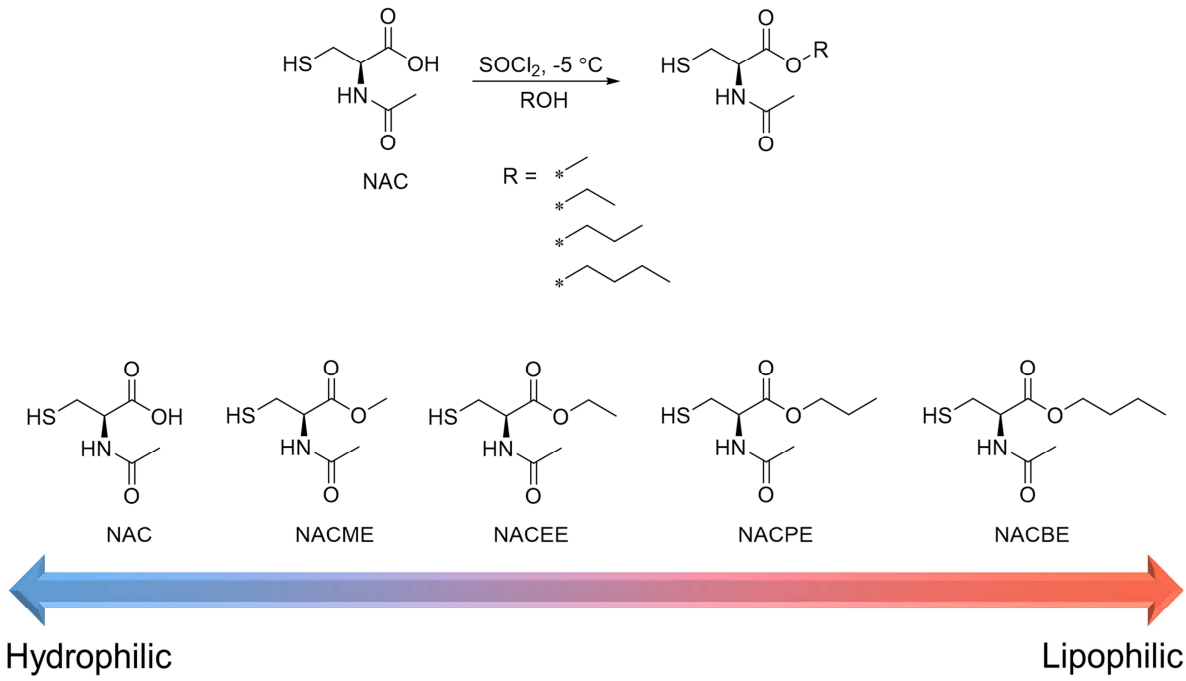

Figure 1. Synthesis of the NAC ester derivatives (top) and a schematic representation of increasing lipophilicity from NAC to NACBE (bottom).

NAC prodrugs protect against cell viability loss with HQ treatment

A dose dependent study was first carried out for ARPE-19-nic cells using HQ concentrations varying from 100-1000 µM for 16 hours (Figure 2a). HQ doses of less than 400 µM were non-lethal and using 500 µM HQ for 16 hours gave a cell viability of ~60%. To evaluate the ability of the ester derivatives to provide cellular protection against oxidative stress, cells were treated with 0.05 mM of NAC, NACME, NACEE, NACPE and NACBE for 2, 24 and 48 hours prior to exposing to 500 µM HQ for 16 hours (Figure 2b). With a pretreatment time of 2 hours, no effect on cell viability was observed with any of the drugs tested. With a pretreatment time of 24 and 48 hours, NAC did not have an effect on cell viability upon exposure to HQ. However, the ester derivatives of NAC significantly raised the cell viability levels compared to both control and NAC treated cells. A Krusakl-Wallis' test showed that drug type (NAC, NACME, NACEE, NACPE and NACBE) had an effect on cell resistance to HQ, $H(5) = 29.587$, $p < 0.001$, but the amount of time that they were exposed to it did not, $H(2) = 3.475$, $p = 0.176$. Dunn's post hoc test of groups show that there is a significant difference between NAC and the esterified NACPE and NACBE ($p < 0.001$, $p < 0.001$).

To compare the effectiveness of NACBE and NAC in protecting against oxidative damage, a dose dependent study was carried out. The pretreatment concentration of NAC and NACBE ranged from 0.001 to 1.0 mM. As seen in Figure 2c, when the concentration of NACBE reached 0.05 mM, cell viability reached 100% and was significantly higher than the response seen with NAC. For pretreatment with NAC, 100% cell viability response was observed at a concentration of 0.5 mM. Kruskal-Wallis test showed that drug type had an effect on cell viability, $H(1) = 5.771$, $p < 0.01$, and that the concentration also had an effect, $H(6) = 29.537$, $p < 0.001$.

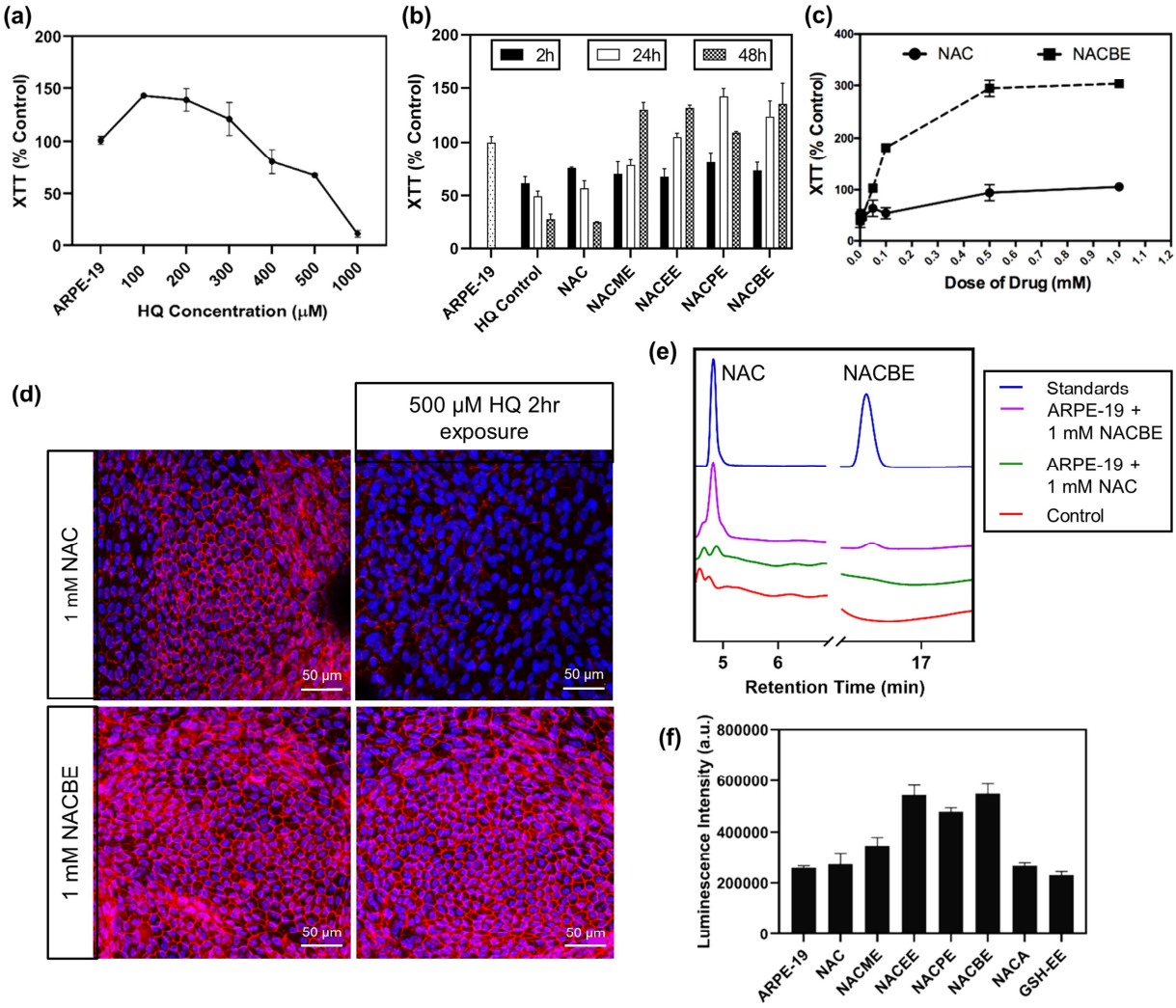

Figure 2. (a) Dose responsive XTT assay for HQ. ARPE-19-nic cells were exposed to 100-1000 µM HQ for 16 hours. Readings were obtained in triplicate wells. **(b)** Time dependent XTT assay for NAC and NAC ester derivatives with a 16-hour exposure to 500 µM HQ. ARPE-19-nic cells were pretreated with NAC and NAC ester derivatives for 2, 24 and 48 hours followed by the exposure to 500 µM HQ for 16 hours. Readings were obtained in triplicate wells (Kruskal-Wallis, H (5) = 29.587, p< 0.001). **(c)** Dose dependent XTT assay for NAC and NACBE. ARPE-19-nic cells were pretreated with NAC and NACBE at 0.001 – 1.0 mM for 24 hours followed by exposure to 500 µM HQ for 16 hours. Readings were obtained in triplicate wells (Kruskal-Wallis, H (1) = 5.771, p <0.01, H (6) = 29.537, p <0.001). **(d)** Confocal images of ARPE-19-nic cells with ZO-1 staining (red) expressing cellular junctions. ARPE-19-nic cells were pretreated with 1 mM NAC and NACBE followed by 2-hour exposure to 500 µM HQ. **(e)** HPLC chromatograms of ARPE-19-nic cells with and without treatment with 1 mM NAC and NACBE. **(f)** GSH assay for NAC, NAC ester derivatives, NACA and GSH-EE. ARPE-19-nic cells were exposed to 1 mM drug concentration for 24 hours before measuring cytoplasmic GSH levels Readings were obtained in triplicate wells (Kruskal-Wallis, H(7) = 20.787, p<0.004).

NAC prodrugs protect against cell-cell junction disruption

ARPE-19-nic cells were treated with 1 mM NAC or NACBE for 24 hours and were exposed to 500 µM HQ for 2 hours before immunostaining for ZO-1. Cells treated with NAC or NACBE exhibit proper cell-cell junctions as indicative of the ZO-1 staining (Figure 2d). After exposure to HQ, ZO-1 staining that was present in cells treated with NAC diminished or was completely absent. For cells pretreated with NACBE, the cellular junctions were intact even after exposure to HQ.

Intracellular NAC levels rise with NAC prodrug treatment

Ester pro-drugs are predicted to undergo hydrolysis. Therefore, NACBE should increase the intracellular levels of NAC. To confirm this, ARPE-19-nic cells were treated with 1 mM NAC/NACBE for 1 hour followed by cells lysis and methanol extraction. When ARPE-19-nic cells were treated with 1 mM NAC, no NAC was detected and the HPLC chromatogram was very similar to ARPE-19-nic cells only (Figure 2e). In contrast, after treating ARPE-19-nic cells with 1 mM NACBE for 1 hour, HPLC analysis demonstrated both NACBE (prodrug) and NAC (metabolite) (Figure 2e).

Intracellular GSH levels rise with NAC prodrug treatment

The presence of necessary enzymes to produce GSH in these ARPE-19-nic cells were first evaluated using semi-quantitative RT-PCR (Figure S20). The ARPE-19-nic cells contain the enzymes glutamylcysteinelygase catalytic and the modulatory units, (GCLC and GCLM respectively), and glutathione synthase (GSS), all of which is necessary to produce GSH in the cell. The internal levels of GSH in ARPE-19-nic cells after exposure to drugs were measured using a GSH assay. ARPE-19-nic cells were exposed to 1 mM NAC, NACME, NACEE,

NACPE, NACBE, NACA and GSH-EE for 24 hours. Afterwards, the cellular GSH levels were measured with a luminescent assay. The Kruskal- Wallis test shows that there was a significant difference of the type of drug that they were exposed to, $H(7) = 20.787$, $p<0.004$. Post hoc testing using Bonferonni's correction revealed that NAC, NACA, and GSH-EE did not raise intracellular GSH level compared to untreated cells ($p <0.05$, Figure 2f). In comparison, all of the ester derivatives raised the levels of GSH.

Increasing NAC prodrug lipophilicity leads to higher intracellular levels

Dansyl chloride was reacted with ammonium hydroxide to yield dansyl amide. Then the dansyl probe: *N*-((5-(dimethylamino)-1-naphthalen-1-yl)sulfonyl)acrylamide was synthesized by reacting dansyl amide and acryloyl chloride. Both dansyl amide and N-((5-(dimethylamino)-1-naphthalen-1-yl)sulfonyl)acrylamide were obtained in good yields and were characterized with ^{1}H NMR spectroscopy (Figure S9, S10). NACME, NACEE, NACPE and NACBE were then reacted with *N*-((5-(dimethylamino)-1-naphthalen-1-yl)sulfonyl)acrylamide in the presence of triethylamine to give Dan-NACME, Dan-NACEE, Dan-NACPE and Dan-NACBE respectively (Figure 3a). All dansyl tagged compounds were characterized using ^{1}H and ^{13}C NMR spectroscopy (Figure S11-S18) and all of them possess similar absorption and fluorescence profiles with λ_{ex} ~ 320 nm and λ_{em} ~ 520 nm (Figure 3b and 3c).

Confocal images were obtained after exposing ARPE-19-nic cells to 1 mM solutions of NACBE, Dan-NACME, Dan-NACEE, Dan-NACPE and Dan-NACBE for 1 and 24 hours (Figure 3d). NACBE was used as the control. After 1 hour incubation time with the dansyl tagged drugs, the fluorescence intensity increases with increasing lipophilicity. The intensities further improved upon extending the incubation time to 24 hours, and Dan-NACBE had the highest fluorescence intensity.

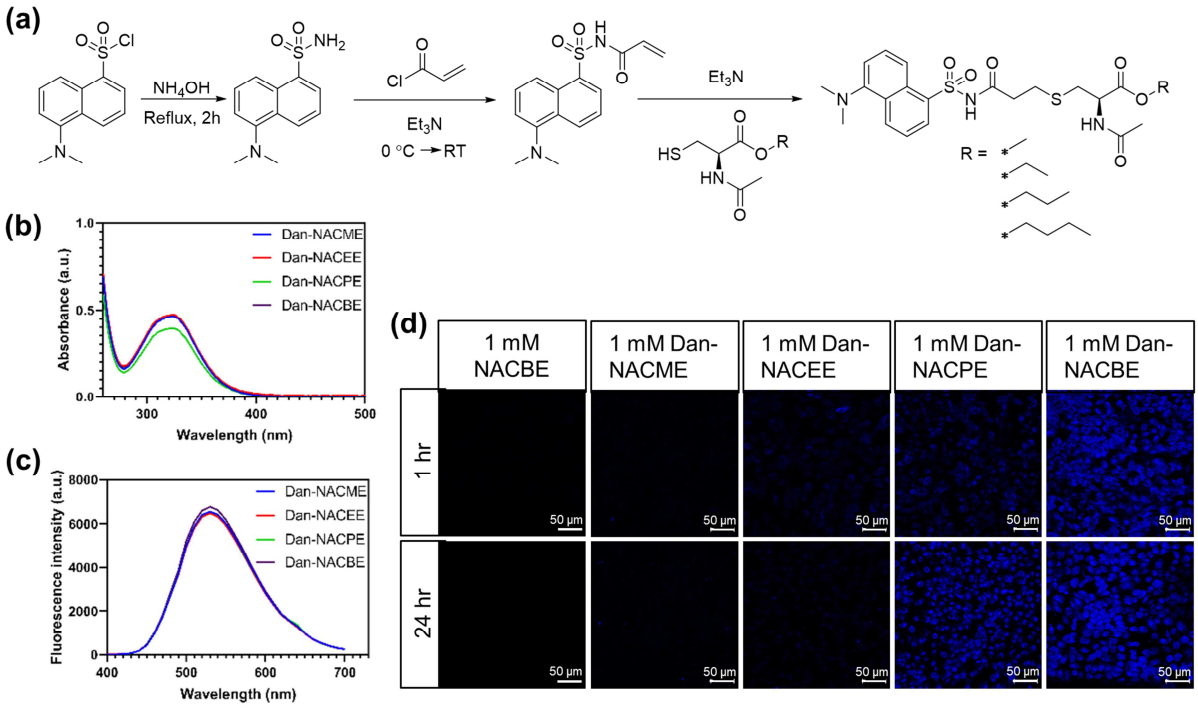

Figure 3. (a) Synthesis scheme for dansyl tagged NAC ester derivatives. (b) UV-Vis absorbance spectra for Dan-NACME, Dan-NACEE, Dan-NACPE and Dan-NACBE in PBS. (c) Fluorescence spectra of Dan-NACME, Dan-NACEE, Dan-NACPE and Dan-NACBE in PBS. (d) Confocal images of ARPE-19-nic cells exposed to NACBE, Dan-NACME, Dan-NACEE, Dan-NACPE and Dan-NACBE at 1 mM for 1 and 24 hours.

NAC prodrugs protect against mitochondrial membrane depolarization with HQ treatment

To evaluate the protection of the synthesized ester derivatives towards mitochondrial damage, we carried out the JC-1 assay. This assay evaluates the change in mitochondrial membrane potential. Typically, JC-1 dye has an inherent green fluorescence at 530 nm. Upon reaching the cell, due to the structural properties of the dye, it will accumulate in the mitochondria making aggregates known as J-aggregates. These J-aggregates consists of a red shifted fluorescence (590 nm). Damaged or unhealthy mitochondria, due to their depolarized membrane potential, will have lesser amounts of aggregates and lower intensities of red emission.

Since this assay is more sensitive than the XTT assay, lower doses of the insult were used. A dose dependent study was carried out using 25, 50 and 100 µM HQ at 1, 2, 4, 6, 8 and 16 hour time points to determine the dose and time of the insult (Figure 4a). With increasing time and dose of HQ, a reduction in 590/530 nm fluorescence is seen due to the depolarization of the mitochondria. For the assay 50 µM HQ for 4 hours was used as the dose and time for the insult, since it reduces in 590/530 nm fluorescence by approximately 50%.

To assess the synthesized drugs ability to protect against oxidative damage in the mitochondria, we pretreated ARPE-19-nic cells with 1 mM NAC, NAC ester derivatives, NACA, GSH-EE and 1 µM MitoQ for 1 and 24 hours. Following pretreatment, the cells were exposed to 50 µM HQ for 4 hours and JC-1 was used to measure mitochondrial depolarization. NACA and GSH-EE were used as the positive controls. As these molecules are not targeted towards mitochondria, we selected MitoQ, a well-known mitochondrial targeted antioxidant, as an additional positive control. We found out that using higher concentrations of MitoQ ($\geq$ 5 µM) reduces the cell viability drastically. Therefore, we were restricted in using a low dose of MitoQ (1 µM) in this assay. With a pretreatment time of 1hr, only NACBE protected against mitochondrial depolarization caused by HQ (Supplemental Figure S19). The Kruskal-Wallis test shows that there is a significant effect of drug type, $H(7) = 24.151$, $p = 0.001$, and time, $H(1) = 18.218$, $p<0.001$. Post hoc comparisons showed that when the pretreatment time was increased to 24 hours, all drugs with the exception of NACA and MitoQ protected against mitochondrial depolarization, (Figure 4b, $p< 0.01$).

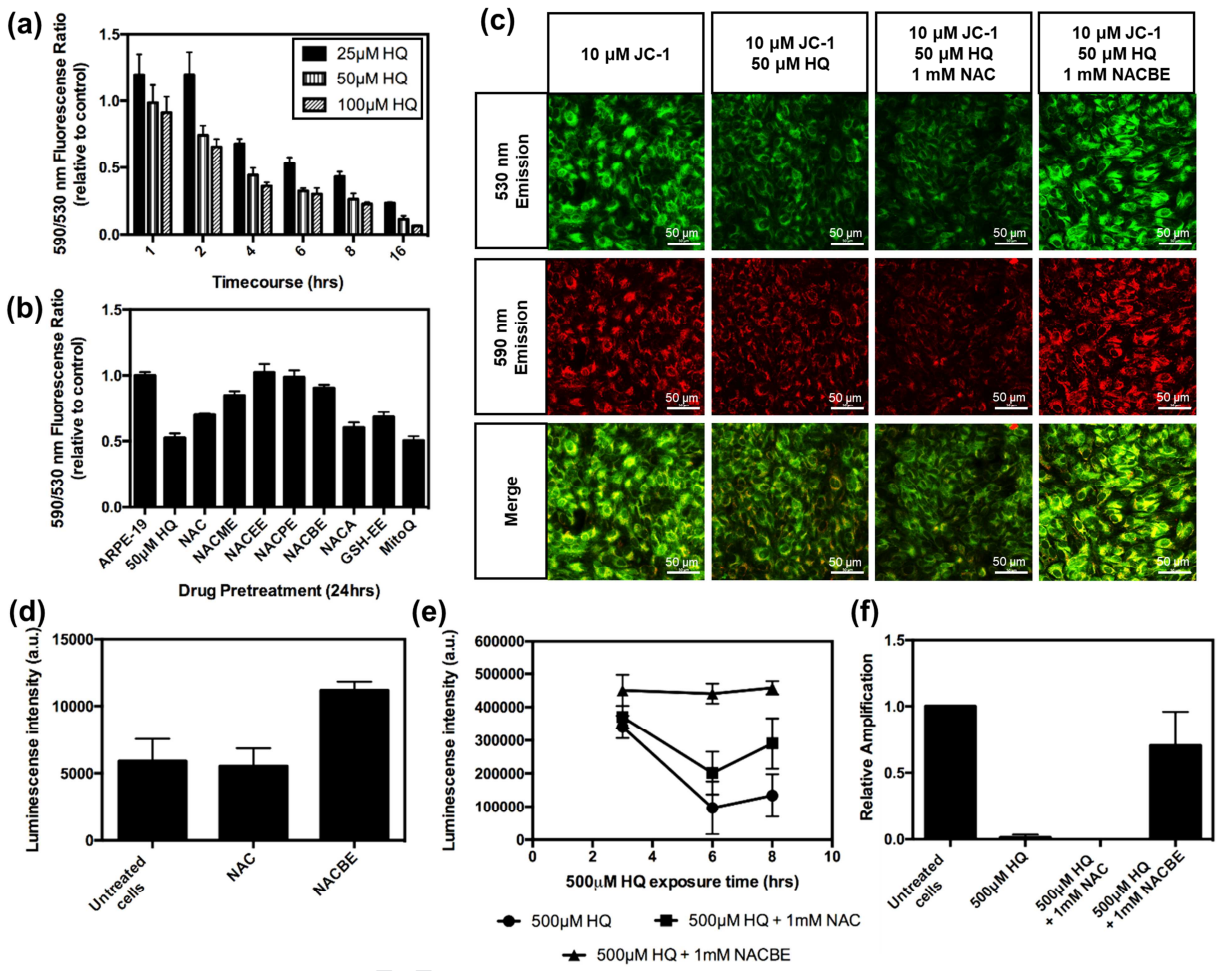

Figure 4. (a) JC-1 assay for ARPE-19-nic cells exposed to 25, 50 and 100 µM HQ at 1, 2, 4, 6, 8 and 16 hours. Readings were obtained in triplicate wells. **(b)** JC-1 assay for ARPE-19-nic cells pretreated with 1 mM NAC, NAC ester derivatives, NACA, GSH-EE and 1 µM MitoQ for 24 hours before exposing to 50 µM HQ for 4 hours. Readings were obtained in triplicate wells (Kruskal-Wallis, H(7) = 24.151, p= 0.001, and, H(1) = 18.218, p<0.001, for drug type and time respectively). **(c)** Confocal images of ARPE-19-nic cells treated with 10 µM JC-1, 10 µM JC-1 + 50 µM HQ, 10 µM JC-1 + 50 µM HQ pretreated with 1 mM NAC and JC-1 + 50 µM HQ pretreated with 1 mM NACBE. The cells were pretreated with the drugs for 24 hours before exposing to 50 µM HQ for 4 hours. **(d)** Mitochondrial GSH assay after treating with 1 mM NAC and NACBE for 24 hours. Readings were obtained in triplicate wells (Kruskal-Wallis H(2) = 5.600, p<0.05). **(e)** CellTiter-Glo assay for ARPE-19-nic cells for 500 µM HQ, 1 mM NAC + 500 µM HQ and 1 mM NACBE + 500 µM HQ for 3, 6 and 8 hours. Readings were obtained at three independent experiment in triplicate (Kruskal-Wallis, H(2)= 55.900, p <0.001 and H(2)= 9.320, p< 0.01 for treatment type and time, respectively). **(f)** Relative amplification of a large band of mitochondrial DNA from ARPE-19-nic cells treated with 500 µM HQ, 500 µM HQ pretreated with 1 mM NAC, and 500 µM HQ pretreated with 1 mM NACBE. Readings were obtained in three independent experiments (Kruskal-Wallis, H(7) = 20.787, p< 0.01).

As additional evidence, these results were visualized with the aid of confocal microscopy (Figure 4c). As seen in Figure 4c, when the ARPE-19-nic cells were treated with 50 µM HQ, the emission intensity at 590 nm decreased compared to that of untreated ARPE-19-nic cells. Pretreatment with 1 mM NAC did not help to retain mitochondrial depolarization with the introduction of the insult, therefore a reduction in the fluorescence intensity can be seen. On the other hand, pretreatment with 1 mM NACBE preserved the mitochondrial membrane potential and thus a similar fluorescence intensity as the untreated ARPE-19-nic cells is observed.

Mitochondrial GSH levels rise with NAC prodrug treatment

To determine the mechanism of action of these ester derivatives in protecting mitochondria, a GSH assay was carried out for isolated mitochondria. The cells were treated with 1 mM NAC and NACBE for 24 hours followed by mitochondrial extraction and a subsequent GSH assay was performed. Isolation of mitochondria was carried out according to a previously published procedure.[35] As seen in Figure 4d, mitochondria isolated from cells treated with NACBE show an increase in luminescence compared to NAC and ARPE-19-nic cells. The Kruskal-Wallis test shows that there was an effect of drug type on luminescence, $H(2) = 5.600$, $p<0.05$. Post hoc test revealed that mitochondria isolated from cells treated with NACBE show an increase in luminescence compared to NAC and control cells ($d = 5636.667 \pm 1040.373$, $p<0.01$, $d = 5258.33 \pm 1040.373$, $p <0.01$).

NAC prodrug treatment protects against ATP loss with HQ treatment

ARPE-19-nic cells were exposed to 500 µM HQ for 3, 6 and 8 hours and the amount of ATP produced was measured using the CellTiter-Glo assay kit (Figure 4e). Pretreatment with NACBE blocked the reduction in ATP, and luminescence was significantly higher at all time points

observed. Kruskal- Wallis test was utilized for these data and demonstrated that there is an effect of treatment, $H(2)= 55.900$, $p <0.001$, and of time, $H(2)= 9.320$, $p< 0.01$. With increasing incubation time, the luminescence intensity decreased, indicative of reduced ATP levels. A reduction in ATP levels was still observed when cells were pretreated with NAC, although the luminescence levels were higher at 6 and 8hrs of HQ treatment (all p values <0.05).

NAC prodrug treatment protects against mitochondrial DNA damage

Mitochondrial DNA damage has been linked to pathogenic diseases, including AMD.[36] To determine if pretreatment of RPE cells with NAC compounds could protect mitochondrial DNA against oxidative damage, we utilized a long-extension PCR based assay to measure amplification of a large stretch of mitochondrial DNA. Treatment with HQ reduced the amplification of mitochondrial DNA (Figure 4f), and pretreatment with NAC did not appear to protect against mitochondrial DNA damage. The Kruskal-Wallis test showed that there was an effect of drug type, $H(7) = 20.787$, $p< 0.01$ and post hoc test showed that NACBE raised the levels of mitochondrial DNA amplification compared to HQ and NAC+HQ treated cells.

DISCUSSION

The pathogenesis for AMD is currently obscure. However, there is strong evidence that suggests oxidative stress in the RPE plays a major role in AMD. Newer studies also show the correlation of mitochondrial damage in the RPE and AMD. In the present study, the use of lipophilic ester derivatives of NAC is being tested to relieve oxidative stress in ARPE-19-nic cells. The effect of these drugs in mitochondrial damage is also investigated. The expression of multiple proteins in the glutathione pathway (Figure S20), oxidative response related proteins and mitochondrial involved proteins in ARPE-19-nic cells (personal communication - Jim Handa, Johns Hopkins

Medical School) indicate this may be a suitable cell line for purposes of studying oxidative related processes impacting mitochondrial related pathways.

To assess the cellular protection provided by the ester derivatives against HQ induced oxidative stress, several XTT studies were carried out. As seen from Figure 2b, a low pretreatment time was ineffective for all the drugs as these drugs need more incubation time in order to undergo hydrolysis and eventually to synthesize GSH. Therefore, an immediate protection was not observed on pretreating for 2 hours. As such, it is necessary to incubate the cells for a sufficient amount of time in order to obtain proper pharmacokinetics of the drugs. Increasing the incubation time from 2 hours to 24 and 48 hours, showed a significant improvement in cell viability. Overall, NACBE showed comparatively a higher cell viability thereby providing the most protection against the introduced insult. The dose responsive behavior of NAC and NACBE as seen in Figure 2c, shows the effectiveness of NACBE towards protecting cells from oxidative damage compared to NAC. It shows that these lipophilic constructs penetrate into the cells more effectively than the parent compound, providing better protection against oxidative damage. To further demonstrate the protection given by NACBE compared to NAC, ZO-1 staining was used. Exposing ARPE-19-nic cells to HQ disrupt the cellular junctions due to the production of ROS. Introduction of antioxidants such as NACBE, provide protection from the excess ROS produced by the insult. As a result, the cellular junctions will be intact and can be visualized by the ZO-1 staining.

These ester derivatives upon cellular internalization, will undergo ester hydrolysis to yield NAC and cysteine. The endogenously produced L-cysteine will then be converted to GSH. These phenomena were confirmed using several experiments. HPLC analysis were used to confirm the conversion of NACBE to NAC as well as the cellular uptake. According to Figure 2e, it is

evident that NACBE is taken up by the cells more effectively than NAC. Even though we showed the intracellular conversion of NACBE to NAC, we were unable to show the conversion of NAC to L-cysteine directly using HPLC since L-cysteine is too hydrophilic that it would elute closer to the solvent front. An indirect way to demonstrate this would be via investigating GSH levels. The lipophilic ester derivatives, at a concentration of 1 mM, has the ability to generate sufficient GSH concentration in the cytosol (Figure 2f). It is believed that cytosolic GSH is transported into the mitochondria via GSH transporters.[37-39] Within 24 hours, the ester derivatives outperform the controls due to their increased lipophilicity, enhancing the cellular penetration, and thus generating higher levels of GSH. This provides evidence that these ester derivatives undergo hydrolysis and increase the bioavailability of L-cysteine to produce GSH. Also, this observation suggests NAC esters provide protection against oxidative stress via GSH but not by the prodrug form or NAC or L-cysteine metabolite forms.

A higher level of GSH was produced due to an increased accumulation of the drugs in the cells as a result of increased lipophilicity. Even though it is shown indirectly using GSH assay, it would be comprehensive if the accumulation is shown visually. Therefore, we tagged the thiol group of the ester derivatives with a dansyl probe to obtain the fluorescently labeled drugs. Figure 3d shows the accumulation of the dansyl tagged dyes with increasing lipophilicity and time suggesting again time dependent accumulation of these pro-drugs. In addition, these images can be correlated with the XTT data obtained earlier. Where an improved protection with increasing incubation time was observed, which can now be attributed to the increased accumulation of the drugs. Whereas at a lower incubation time a significant protection was not observed due to insufficient accumulation.

Due to the association of mitochondrial damage and AMD, we explored the ability of the ester derivatives to provide protection to mitochondria from oxidative damage. The health of the mitochondria can be evaluated using the JC-1 assay, which is based on mitochondrial membrane potential. Oxidative damage depolarizes the mitochondria and as seen in Figure 4b and 4c, the ester derivatives can retain the mitochondrial membrane potential. The endogenously produced GSH, through de-esterification of the ester derivatives and subsequent conversion to cysteine, transported into the mitochondria to provide protection against the ROS that were produced with the introduction of the damaging agent. This was confirmed through mitochondrial GSH assay (Figure 4d) where mitochondrial GSH levels were higher in cells treated with NACBE than that with NAC. The same phenomena can be attributed to the fact that NACBE retained the production of ATP and provided protection to mitochondrial DNA (Figure 4e and f, respectively). All these assays in conjunction, elaborates the ability of these synthesized NAC ester derivatives to provide protection against oxidative damage that occurs in the mitochondria through the production of GSH.

CONCLUSION

The NAC ester derivatives exhibit enhanced cellular penetration and accumulation than the parent drug, NAC, owing to their increased lipophilicity. We have shown indirectly that these ester derivatives undergo hydrolysis and increases the levels of L-cysteine in human retinal cells, thereby increasing cellular GSH levels than any of the positive controls we have used in this study. As a result of increased GSH levels, the cells have more resistance towards the incoming oxidative damage. Not only these ester derivatives provide cellular protection, but also, they have the ability to protect mitochondria from oxidative damage even though these drugs are not targeted towards mitochondria. In conclusion, cysteine ester prodrugs are more effective on

protecting human RPE cells from oxidative stress compared to commercially available antioxidants.

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Highlights

1. Oxidative stress is a major cause of age-related macular degeneration
2. Anti-oxidants have the ability to relieve oxidative stress
3. Increasing lipophilicity of drugs increases cellular penetration and accumulation
4. Higher accumulation leads to better therapeutic effects of the drug
5. NACBE provides protection against oxidative stress in cytosol and in mitochondria