The GPCR Antagonistic Drug CM-20 Stimulates Mitochondrial Activity in Human RPE Cells

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Abstract:

Background:

Age-related macular degeneration (AMD) is the leading cause of low vision and vision loss in the elderly population. Mitochondrial dysfunction deteriorating bioenergetics in retinal pigment epithelial (RPE) cells is etiologically associated with the onset and progression of AMD. Improvement of mitochondrial function may have therapeutic potential to protect RPE cells from degenerative loss.

Objective:

Test the mitochondrial targeting activity of FDA-approved G-protein coupled receptor (GPCR) antagonist (CM-20), a neuroprotective lead compound we previously identified, in RPE cells.

Methods:

Human-derived RPE cell line (ARPE-19) was differentiated to improve RPE morphology and related function. Dose response experiments were performed in differentiated ARPE-19 (dRPE) cells to test the bioactivity of CM-20 on mitochondrial membrane potential (MMP), denoting mitochondrial activity. Secondary assays with multiplexed live-cell mitochondrial imaging were applied for additional evaluation of CM-20. Protection of CM-20 to mitochondria against the retina-generated endogenous oxidative stressor (hydrogen peroxide) was examined.

Results:

Treatment with CM-20 elicited a dose-dependent increase of MMP. Multiplexed live-cell mitochondrial imaging showed a consistent increase in MMP under treatment with CM-20 at an optimal concentration (12.5 μM). MMP was significantly decreased under oxidative stress, and pre-treatment with CM-20 showed a rescue effect to increase MMP.

Conclusion:

The GPCR antagonist (CM-20) exhibited novel bioactivity to stimulate mitochondrial activity, indicating a dual targeting drug to both GPCR and mitochondria. Since both GPCRs and mitochondria are considered as retinal drug targets, in vivo testing of CM-20 in retinal protection is warranted for poly pharmacological therapy.

Keywords: GPCR, Mitochondria, Poly pharmacology, Multi-target drug, RPE, Oxidative stress, Age-related macular degeneration (AMD)

1. INTRODUCTION

Age-related macular degeneration (AMD) is a leading cause of irreversible vision impairment and loss in the elderly population [1]. AMD occurs in two forms: the neovascular (wet) form and the atrophic (dry) form. Anti-vascular endothelial growth factor therapy is a standard care for the treatment of wet AMD [2]. There is no approved therapy for dry AMD to date. AMD is typically started with degeneration

of retinal pigment epithelial (RPE) cells, followed by degeneration of their supporting cells-the retinal photoreceptors [3]. There is evidence to suggest that RPE cells and photoreceptors are maintained in a symbiotic relationship [4]. Stressed RPE cells not only cause dysfunction and degeneration of themselves per se but also lead to collateral damage of photoreceptors [5]. In etiology, oxidative stress is suggested to play key pathogenic roles at the early stage of AMD [6]. There is an observation of greater loss of both mitochondrial number and content in the RPE layer of AMD eyes than that of normal aging eyes [7]. Increased mitochondrial DNA damage is also found in the RPE cells of AMD eyes, that is correlated with the disease stage [8]. Primary RPE cell cultures derived from AMD eyes exhibited more loss in the capacity of mitochondrial respiration than those derived from normal aging eyes [9].

Our previous work using an unbiased phenotype-based high-throughput chemical screen identified neuroprotective lead compounds in photoreceptors against environmental risk factor (light)-induced oxidative damage and mitochondria-initiated apoptosis [10, 11]. Given the symbiotic relationship between RPE cells and photoreceptors, pharmaceutical drugs that protect both RPE cells and photoreceptors would be more desirable from a therapeutic standpoint. To test this idea and integrate the strategy of polypharmacology using multi target drugs for more effective treatment of complex diseases including neurodegenerative disorders in the central nervous system [12], we rescreened the photoreceptor neuroprotective lead compounds in cultured RPE cells to see whether positive hits targeting mitochondria can emerge. The GPCR antagonist (CM-20) was identified as a positive hit. CM-20 is FDA-approved for the treatment of hypertension and heart disease. The mechanism of action of CM-20 may involve inhibition of G-protein coupled adrenoreceptors and stimulation of estrogen receptors (ERs). G-protein coupled adrenoceptors are suggested as therapeutic targets for inherited retinal diseases [13]. ERs play important regulatory roles in mitochondrial function and biogenesis [14] and are expressed in human RPE cells [15].

Herein, we presented findings showing that treatment with CM20 in RPE cell cultures stimulated mitochondrial activity and protected mitochondria from functional damage under oxidative stress.

2. MATERIALS AND METHODS

2.1. Cell Culture and Differentiation

Human RPE cell line (ARPE-19) was obtained from Fisher Scientific (Pittsburgh, PA). Frozen cell stock was thawed in 37^0C water bath. Cultures were established in T75 flasks in medium containing DMEM + GlutaMAX with 10% FBS and 1% penicillin/streptomycin at 37^0 C supplied with 5% CO_2. At confluence, cells were trypsinized with TrypLE Express Enzyme phenol red and harvested for differentiation for two weeks [16].

2.2. Compound Treatment

CM-20 stock solution was made in methanol. To reduce potential confounding effects caused by growth factors, cellular treatment was performed under a low serum condition (1% FBS). The cells were treated with a serially increased concentration of CM20 (0-85 μM) or a fixed concentration (12.5 μM) for a period of 24-72 h.

2.3. Multiplexed Live-cell Mitochondrial Imaging

Mitochondrial membrane potential (MMP) was detected in microplate cultures of dRPE cells using the mitochondrial specific Image-iT TMRM fluorescent reagent (Thermo Fisher Scientific, Waltham, MA). Essentially, dRPE cells were incubated with a non-quenching concentration (10 nM) of TMRM at 37^0 C. Fluorescent signals were monitored in real-time with a Sartorius IncuCyte S3 microplate reader for 4 h. Controls included cells without TMRM and blanks with or without TMRM. Fluorescent signals including total fluorescent object area (μm^2/image), total fluorescent integrated intensity (RCU × μm^2/image), intensity raw value, total fluorescent cell counts, and mean fluorescent intensity/cell were calculated using IncuCyte Base software.

2.4. Oxidative Stress and Detection of Cell Viability

Microplate cultures of dRPE cells were exposed to hydrogen peroxide (250 μM-1 mM) in serum-free DMEM for 5 hours at 37^0C. After that, the supernatant was removed with a liquid handler and the cells were subject to TMRM staining. Cell viability was measured using CellTiter-Glo luminescent reagent (Promega, Madison, WI) following the manufacturer's instructional protocol.

2.5. Statistical Analysis

Two-tailed *t*-test was used to analyze the difference between two sample groups. To analyze

the difference from more than two sample groups, one-way ANOVA was applied. A *P* value of less than 0.05 was considered significant.

3. RESULTS

3.1. CM-20 Stimulated Mitochondrial Activity

To test the bioactivity of CM-20 on mitochondria, we first carried out dose response experiments in differentiated ARPE-19 (dRPE) cells. dRPE cells exhibited the cobblestone morphology of primary RPE cells compared to undifferentiated ARPE-19 cells (Fig. 1A). Independent microplate cultures of confluent dRPE cells were then subjected to one-time treatment with serially increased concentrations of CM-20 for 24-72 h. Mitochondrial membrane potential (MMP), denoting mitochondrial activity, was detected with the mitochondria-specific fluorescent indicator TMRM. There was a dose-dependent increase of TMRM fluorescence in significant correlation with CM-20 treatment ($R^2 > 0.7$) (Fig. 1B). The effect of CM-20 on increasing TMRM fluorescence was more pronounced at 48 h than at 24 h time and sustained at 72 h. Saturation of TMRM fluorescence was observed at 25 μM of CM20.

3.2. Secondary Assays with Multiplexed Live-cell Mitochondrial Imaging

To further evaluate CM-20 on mitochondrial targeting, we conducted multiplexed live-cell mitochondrial imaging of MMP in dRPE cells. The multiplexed assay format significantly increased the robustness, reliability, data output, and power of statistical analysis. Microplate cultures of dRPE cells were treated with CM-20 (12.5 μM) for 24 h to 72 h, followed by TMRM staining to detect MMP. Heat map of TMRM fluorescence in individual plates showed stronger signals under treatment with CM-20 than nontreated control at 48 h and 72 h. Mean TMRM fluorescence/cell was also significantly higher under treatment with CM-20 than nontreated controls. We also took a direct microscopic examination of TMRM fluorescence. Fluorescent intensity of TMRM under treatment with CM20 was much higher and denser in individual dRPE cells (Fig. 3). In summary, the live-cell mitochondrial imaging data are consistent with the findings from dose response experiments supporting the effect of CM-20 on stimulating mitochondrial activity.

3.3. Oxidative Stress Damaged Mitochondria but did not Cause Cell Death

Given the effect of CM-20 on stimulating mitochondrial activity, we next examined whether CM-20 could protect mitochondria from damage under oxidative stress. We first tested the sensitivity of mitochondria under oxidative stress. Exposure of dRPE cells to the retina-generated endogenous oxidative stressor (hydrogen peroxide) caused a significant loss of MMP (Fig. 4A). To know whether oxidative stress-induced mitochondrial damage is associated with cell death, we detected cell viability in the same microplate samples. Surprisingly, there is no significant loss of cell viability (Fig. 4B).

3.4. CM-20 Protected Mitochondria under Oxidative Stress

Next, we examined the protective effect of CM-20 on mitochondrial activity under oxidative stress. Under exposure to hydrogen peroxide, the heat map of TMRM fluorescence in microplate dRPE cells pretreated with CM-20 showed stronger signals than the non-treated controls (Fig. 5). Further analysis of mean TMRM fluorescence/cell between CM-20 pretreated and nontreated controls also showed a significant difference. The protective effect of CM-20 on mitochondrial activity was related to the level of oxidative stress. At 250 μM (or lower) of hydrogen peroxide, mitochondrial activity was protected. At higher concentration of hydrogen peroxide (> 500 μM), no obvious protective effect of CM-20 was observed (data not shown).

4. DISCUSSION

In this report, we found a novel bioactivity of a FDA-approved GPCR antagonist that could function as a mitochondrial modulator in RPE cells. GPCRs are a major class of drug targets for neurodegenerative diseases in the central nervous system [17, 18]. Mitochondria have been considered as a therapeutic target for many prevalent diseases including AMD [19, 20]. Treatment with CM-20 stimulated mitochondrial membrane potential/mitochondrial activity in a dose-dependent manner. The measured readout of mitochondrial membrane potential is the most reliable indicator for mitochondrial function in bioenergy production during the process of oxidative phosphorylation [21, 22]. Using a multiplexed live-cell mitochondrial imaging technique, we provided additional supporting experimental evidence to validate CM-20 on stimulating mitochondrial activity. We did not present the data of TMRM-EC50 for CM-20 as the calculated value was affected by the amount of TMRM used in the assays and by the treatment duration with CM-20. In fact, different

conditions in cell-based assays can affect the EC50 value of the testing drug [23]. The intensity of TMRM fluorescence could be used as an indicator of mitochondrial abundance [24]. An increase in TMRM fluorescence under CM-20 treatment suggests that the protective mechanism of this drug compound may involve boosting mitochondrial biogenesis.

Findings from our work indicate that mitochondria in human RPE cells are vulnerable to functional damage under oxidative stress (Fig. 4). Hydrogen peroxide we used in the induction of RPE oxidative stress is an endogenous oxidative stressor in the retina [25]. In our study, we observed a significant loss of mitochondrial activity even under low to moderate levels of oxidative stress. However, the cells are resistant to oxidative stress-induced cell death. This finding is consistent with a previous report showing that more differentiated ARPE-19 cells become more resistant to oxidative stress and have high expression of heat shock proteins [26]. Another report showed that short time exposure of primary RPE cells to an oxidative stressor increases autophagy [27]. It is appropriate to state that while mitochondrial function is impaired under oxidative stress, increased expression of molecular chaperons and elevated autophagy prevent activation of mitochondria-initiated apoptotic cell death.

CONCLUSION

We provided experimental evidence showing that a marketed drug of GPCR antagonist used to treat hypertension and heart disease modulates mitochondrial function and protects mitochondria from functional damage under oxidative stress in RPE cells. This drug compound possesses previously unappreciated dual targeting activities to both GPCR and mitochondria. It offers the potential in polypharmacological therapy for retinal degenerative diseases such as AMD via repurposed applications.

LIST OF ABBREVIATIONS

RPE = retinal pigment epithelial

dRPE = differentiated retinal pigment epithelial

MMP = mitochondrial membrane potential

TMRM = tetramethylrhodamine methyl ester

GPCR = G-protein coupled receptor

ER = estrogen receptor

FBS = fetal bovine serum

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIALS

Not applicable

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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A patent on method-of-use of CM-20 and structurally similar compounds or drugs for retinal therapeutic use has been filed.

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FIGURE LEGENDS:

Fig. (1). CM-20 treatment elicited dose-dependent increase of mitochondrial membrane potential (MMP). (**A**) Cultured ARPE-19 cells were differentiated for 2 weeks. Differentiated ARPE-19 cells (dRPE cells, right panel) displayed the cobblestone morphology of native RPE cells. (**B**) Independent microplate cultures of dRPE cells were treated with CM-20 (0-25 µM, n=3) for 24, 48, or 72 h, after which MMP was detected with the mitochondrial specific fluorescent indicator TMRM. There was a significant correlation between MMP and treatment ($R^2 > 0.7$).

Fig. (2). Multiplexed live-cell mitochondrial imaging. Microplate cultures of dRPE cells (n=12) with equal cell density were treated with CM-20 (12.5 µM) or solvent control in low serum (1% FBS) medium for 48 or 72 h. MMP was detected with TMRM. Shown was the heat map of TMRM fluorescence/well. Mean TMRM fluorescence/cell was significantly higher under treatment with CM-20 at 48 h ($P = 1.72E$-05) and 72 h ($P = 0.00044$) compared to solvent control.

Fig. (3). CM-20 treatment increased MMP fluorescence. dRPE cells were treated with CM-20 (12.5 µM) for 48 h, stained with TMRM, and imaged. Treatment with CM20 showed a stronger and denser fluorescent signal of TMRM in virtually all individual cells in the visual field (white arrows) than the solvent control.

Fig. (4). Oxidative stress caused loss of MMP but not cell death. (**A)** dRPE cells were pretreated with CM-20 (12.5 µM, n=4) for 48 h, followed by exposure to hydrogen peroxide (250 µM or 500 µM) for 5 h. There was a significant loss of MMP following exposure to hydrogen peroxide. (**B**) The same cell samples were subject to detection of cell viability. There was no significant difference in cell viability with or without exposure to hydrogen peroxide.

Fig. (5). CM-20 rescued the loss of MMP under oxidative stress. Microplate cultures of dRPE cells were treated with CM-20 (12.5 µM, n=4) for 48 h or 72 h, followed by exposure to hydrogen peroxide (250 µM) for 5 h. Shown was the heat map of TMRM fluorescence/well. Mean TMRM fluorescence/cell was significantly higher with treatment of CM-20 at 48 h ($P = 0.0096$) and 72 h ($P = 0.0021$).