Delineating Mechanisms of Sigma-2 Receptor Modulators in Regulating Retinal Pigment Epithelial Lipid Uptake

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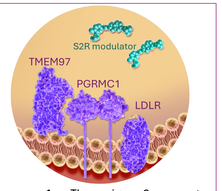
BACKGROUND

RPE cell maintenance of lipid homeostasis is necessary for effective photoreceptor outer segment (POS) trafficking and clearance[1], an essential function that is disrupted in dry age-related macular degeneration (AMD). RPE cells internalize lipids via low-density lipoprotein receptor (LDLR) and related receptors to support retinal lipid homeostasis. Loss of LDL trafficking contributes to RPE degeneration *in vitro*[2] and *in vivo*[2,3]. which point to maintenance of LDL levels as an essential aspect of RPE function.

The sigma-2 receptor (S2R, TMEM97) has been linked to dry age-related macular degeneration (AMD) in genome-wide association studies[4]. We demonstrated that small molecule modulators of S2R, including investigational therapeutic CT1812 (zervimesine), rescue RPE POS trafficking deficits *in vitro*[5]. Studies in heterologous cell lines have shown that S2R interacts with proteins involved in lipid trafficking, such as LDLR and Niemann-Pick C Protein 1 (NPC1)[6,7].



Schema 1. The sigma-2 receptor complex is comprised of TMEM97 and PGRMC1 and interacts with the LDL receptor (LDLR) (*purple*). We have shown that S2R modulators (*cyan*) prevent amyloid-β oligomer-induced POS trafficking deficits in RPE[5]. The effects of S2R modulators on LDLR-mediated signaling remain to be elucidated.

GOAL: Test the hypothesis that S2R modulators alter LDLR-mediated lipid trafficking in RPE cells to further illuminate S2R modulator mechanisms

S2R modulators elevate LDL uptake in a TMEM97-dependent manner[8]

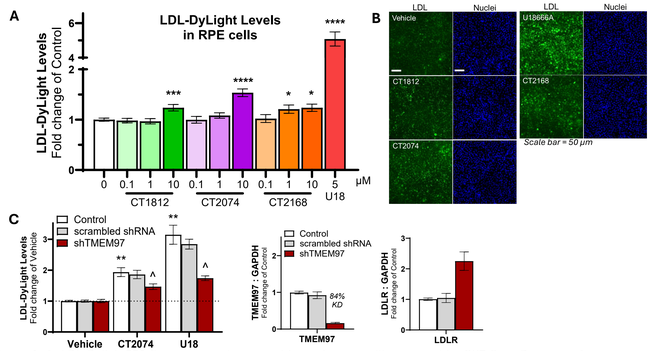


Figure 1. A. LDL-Dylight550 fluorescence (spot total intensity per cell) assessed by CX7 Spot Detector after treatment with CT1812, CT2074, and CT2168. U18 5 µM treatment was used as positive control. N=5 independent experiments, normalized to vehicle (DMSO); mean +/- SEM, one-way ANOVA. *p<0.05; **p<0.01; ***p<0.001; **** p<0.0001. B. Representative images of Hoechst-positive nuclei and LDL-DyLight550 fluorescence in cultures treated with vehicle, S2R modulator (10 µM), or U18 (5 µM). C. LDL levels were assessed 7 days after transduction of scrambled shRNA or TMEM97-targeting shRNA (shTMEM97). TMEM97 and LDLR transcript levels were assessed by qRT-PCR normalized to GAPDH. N=3 experiments; mean +/-SEM, two-way ANOVA, **p<0.01 modulator vs vehicle, ^p<0.05 shTMEM97 vs control.

RESULTS

S2R modulator effect on LDL levels requires LDLR expression

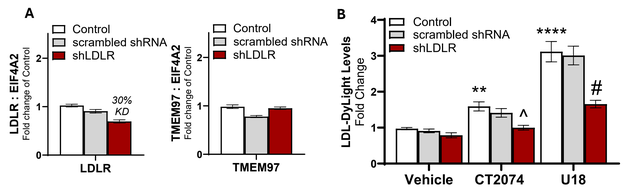


Figure 2. A. LDLR and TMEM97 mRNA transcript levels were assessed by qRT-PCR, normalized to EIF4A2 as control. B. LDL-Dylight550 fluorescence (spot total intensity per cell) assessed by CX7 Spot Detector after treatment with vehicle (DMSO), CT2074 (10 µM) or U18 (5µM). N=4 experiments, normalized to vehicle; mean +/- SEM, two-way ANOVA. **p<0.01 Control CT2074 vs vehicle, ****p<0.0001 Control U18 vs vehicle ^p<0.05, CT2074 shLDLR vs control, #p<0.01 U18 shLDLR vs control.

S2R modulator effects are dependent on levels of plasma membrane-localized LDLR

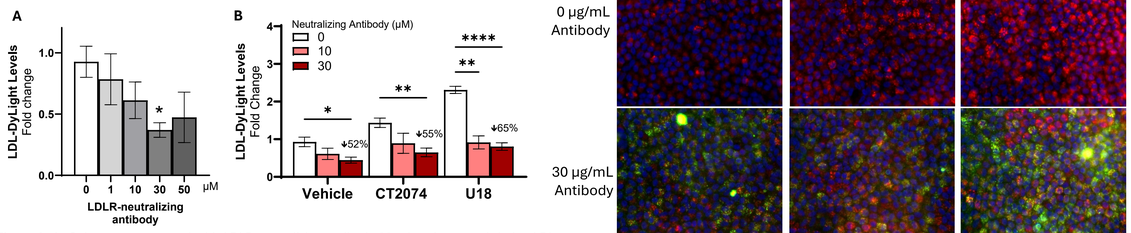


Figure 3. A. Cultures were treated with LDLR-neutralizing antibody 30 min prior to and during LDL-DyLight550 assay. Fluorescence intensity was assessed and normalized to vehicle (0 µM antibody). N=3 experiments, mean +/- SEM, one-way ANOVA, *p<0.05. B. LDL-Dylight550 fluorescence intensity after treatment with vehicle (DMSO), CT2074 (10 µM) or U18 (5µM) and LDLR-neutralizing antibody. N=3 experiments, mean +/- SEM, two-way ANOVA. *p<0.05, **p<0.01, ****p<0.0001. C. Representative images of immunofluorescence staining for LDLR-neutralizing antibody, with LDL-DyLight550 and Hoechst-positive nuclei (20x magnification).

S2R modulators do not significantly impact total LDLR protein levels in RPE cells

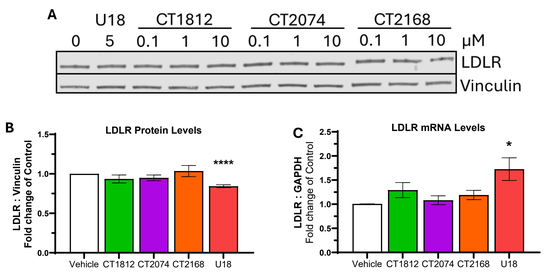


Figure 4. A. Representative western blots of LDLR (140 kDa) and membrane-associated protein vinculin (120 kDa) as loading control. B. Densitometry of western blots was performed, with LDLR normalized to vinculin, normalized within experiment as fold change of DMSO control (0 µM). C. Transcript levels of LDLR were assessed by qRT-PCR after treatments. GAPDH was assessed as housekeeping gene control. Graphs depict the mean fold change +/- SEM of each S2R modulator (10 µM) or U18 (5 µM) from N=5 experiments. Significance assessed by one-way ANOVA of S2R modulator vs vehicle or two-tailed t-test U18 vs vehicle. *p<0.05; ****p<0.0001.

S2R modulators may exhibit differential effects on proteins regulating LDL uptake

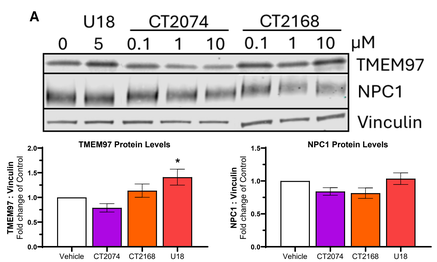
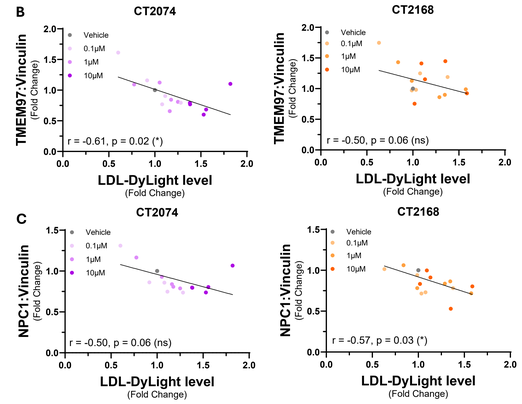


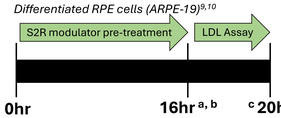
Figure 5. A. Representative western blots of TMEM97 (21 kDa), NPC1 (160 kDa), and vinculin (120 kDa) as loading control. Densitometry of western blots was performed, with TMEM97 or NPC1 normalized to vinculin, normalized within experiment as fold change of vehicle (0 µM). Graphs depict the mean fold change +/- SEM of each S2R modulator (10 µM) or U18 (5 µM) from N=5 experiments; one-way ANOVA of S2R modulator vs vehicle or two-tailed t-test U18 vs vehicle, *p<0.05. Normalized TMEM97 (B) or NPC1 (C) protein levels were plotted as a correlation with LDL-DyLight550 fold change within experiment. N=5 experiments, significance assessed by Pearson correlation analysis. Additional analyses are underway for characterizing CT1812 effects.



METHODS

Aim 1: Characterize S2R modulator effects on LDL levels *in vitro* in relation to levels of S2R components and LDL trafficking proteins

Differentiated RPE cells (ARPE-19)[9,10]

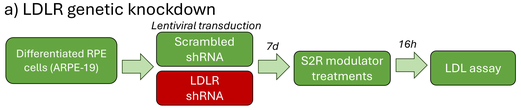


Endpoints
a. Western Blot
b. Quantification of LDL-DyLight550 fluorescence
c. Correlation analysis of protein levels with LDL levels

- S2R modulators CT1812, CT2074, and CT2168 (0.1, 1, or 10 µM) demonstrate S2R binding affinity (K_i) at low nanomolar levels[5].
- U18666A (5 µM), an NPC1 inhibitor, was used as an assay control.

Aim 2: Determine whether LDLR is essential for S2R modulator mechanism of action in regulating LDL uptake

a) LDLR genetic knockdown



b) LDLR functional blockade using neutralizing α-LDLR antibody

CONCLUSIONS

- S2R modulators increase LDL uptake in an LDLR-dependent manner
- LDL uptake via S2R modulators CT2074 and CT2168 correlates with levels of TMEM97 or the S2R interacting protein NPC1
- These studies highlight a potentially important biological mechanism for S2R modulators in lipid trafficking, which is dysregulated as part of dry AMD pathology
- Ongoing work aims to further identify mechanisms underlying S2R modulator effects on cellular lipid trafficking functions and cell health in neurodegenerative diseases

*See **Poster #A0175** for additional studies conducted at CogRx*

Sigma-2 Receptor Modulation Promotes Retinal Pigment Epithelial Cell Survival Following Chronic 7-Ketocholesterol Exposure
Thursday May 8, 11:45AM – 1:30PM

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