Investigating the effect of a novel mitophagy activator on mitochondrial function and mutant load in cybrid cell lines

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Background and Aims

Approximately 1 in 4300 individuals suffer from mitochondrial DNA (mtDNA) mutations that impair functioning of the respiratory chain and present debilitating consequences, with no currently known treatments [1]. Patients carrying mtDNA mutations commonly display heteroplasmy, where they possess both mutated and wild-type mtDNA, with relative proportions defined by the mutant load. Symptoms are known to manifest once heteroplasmy levels surpass a certain biochemical threshold [2], and reducing mutant load is therefore an attractive possible treatment strategy. Notably, recent evidence has indicated an upregulation of PI3K-Akt-mTORC1 signalling in cells harbouring the m.3243A>G mutation, which was suggested to downregulate mitophagy, enabling maintenance of dysfunctional mitochondria [3,4]. Inhibition of mTORC1 signalling was shown to restore bioenergetic function and decrease mutant load [3]. This same effect of mTORC1 inhibition was not observed in m.8993T>G cybrids, suggesting that altered signalling pathways are likely mutation-dependent [3]. Therefore, compounds stimulating mitophagy may hold therapeutic potential for certain mitochondrial disorders, by mediating clearance of mutant mtDNA to a level where patients become asymptomatic.

Accordingly, we have been provided with a novel mitophagy activator, CAP-1902 from Capacity Bio. This project aims to treat cybrid cell lines displaying heteroplasmy with 3 different concentrations of this drug, verify that mitophagy has been activated, and subsequently determine whether mutant load has decreased and bioenergetic function has been rescued.

Methods

Cell Lines: A549 cybrids carrying the m.3243A>G (MT-TL1) mutation, and 143B cybrids (N50 and N80) harbouring the m.8993T>G (MT-ATP6) mutation were cultured in high glucose DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% (v/v) penicillin–streptomycin (Gibco), in an incubator at 37°C and 5% CO_2.
Cell Culture: Cells were passaged every 5-7 days at 70-80% confluence, using 0.25% trypsin-EDTA (Gibco). Media was changed 3-4 times a week. A 100 µM stock solution of CAP-1902 in DMSO was diluted in media to achieve concentrations of 20 nM, 100 nM and 1 µM. Untreated cells were cultured in media containing a respective volume of DMSO.
ARMS-qPCR: DNA was extracted from generated cell pellets with DNeasy Blood and Tissue Kit (Qiagen) and samples standardized to 0.4 ng/µl for ARMS-qPCR. Master mixes were prepared with 1 µL each of forward and reverse ARMS primers (5 µM working solution) and 5 µL of SYBR Green. 7 µL of master mix and 3 µL of DNA sample was added per well in a 96-well PCR plate, with triplicates for each sample. The CFX96 Touch Real-Time PCR Detection System (Bio-Rad) was used for PCR amplification.
Respirometry: ECAR and OCR was measured using the XF Cell MitoStress Kit and Seahorse Bioscience XFe96 bioanalyzer. Cells were seeded in a Seahorse XF Cell Culture Microplate for 2 days. Prior to measurements, media was removed and replaced with 175 µL Seahorse XF Base Medium (with added 10mM glucose, 1 mM pyruvate and 2 mM glutamine), then incubated for 1hr at 37°C (without CO_2). A BCA assay was performed to quantify protein levels for normalisation.
Fluorescence Microscopy: Cells were seeded in 35 mm FluoroDishes and incubated for 48hrs at 37°C and 5% CO_2. Media was replaced with 1 mL phenol red-free high glucose DMEM prior to imaging with an LSM 880 (Carl Zeiss) confocal microscope.
Image Analysis: F_{543}/F_{458} ratiometric images were generated on ImageJ following subtraction of mean background signal.

Results

Result 1: Upregulated mitophagy in 20 nM drug-treated m.3243A>G cybrids

A statistically significant increase in mean F_{543}/F_{458} ratio from 3.14 ± 1.68 in untreated to 4.19 ± 2.36 in 20 nM-treated m.3243A>G cybrids was observed, indicating higher number of mitochondria engulfed in lysosomes for degradation (Fig. 1). Though imaging was performed on 100 nM and 1 µM-treated cybrids, no signal was visualized, likely due to unsuccessful transduction with mt-Keima.

Result 2: Increased respiratory capacity in 20 nM drug-treated m.3243A>G cybrids after 2 weeks

100 nM and 1 µM treatments did not yield observable changes in OCR in relation to untreated (Fig. 2). Conversely, 20 nM-treated m.3243A>G cybrids displayed a significant increase in maximal respiratory capacity and respiratory reserve compared to untreated (Fig. 2f-g), indicating possible improvements in bioenergetic capability.

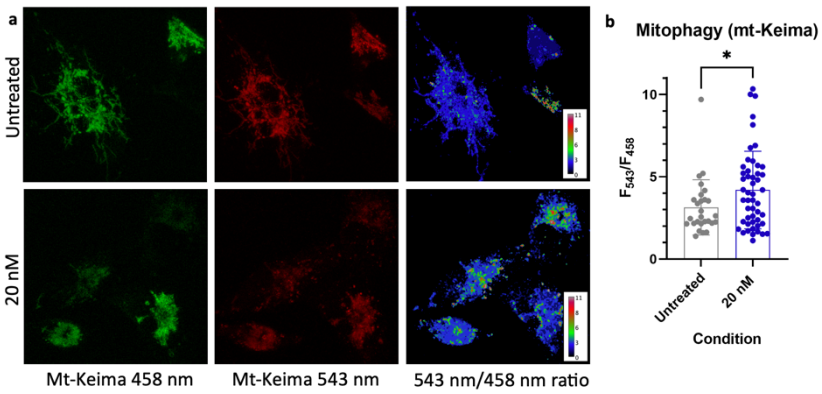

Fig 1. Activation of mitophagy in m.3243A>G cybrids treated for 2 weeks. (a) Representative images of mt-Keima emission at 458 nm and 543 nm following subtraction of mean background signal. Ratiometric images were generated on ImageJ with addition of a median filter for visualization purposes. **(b)** F_{543}/F_{458} ratios for ROIs were plotted and represented as mean ± SD. Data underwent two-tail *t*-test analysis, $n \geq 27$ ($^*p < 0.05$).

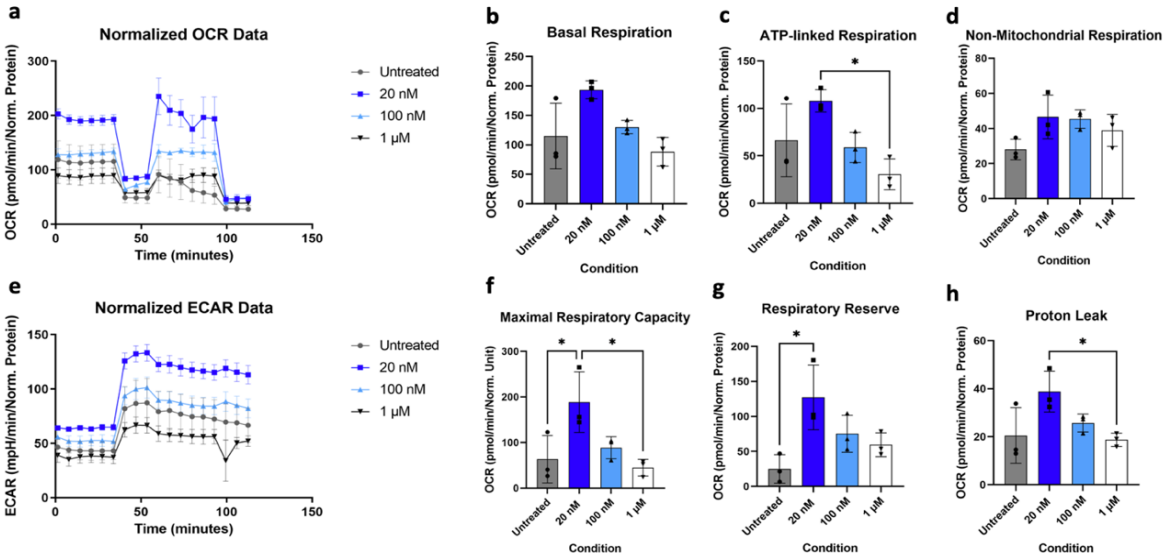

Fig 2. Mitochondrial respiration parameters of m.3243A>G cybrids. Seahorse XFe96 Analyser was used to measure **(a)** oxygen respiratory capacity (OCR) and **(e)** extracellular acidification rate (ECAR) of m.3243A>G cybrids treated for 2-weeks with mitophagy activator (20 nM, 100 nM or 1 µM) or untreated (n = 3 culture wells). **(b)** Basal respiration, **(c)** ATP-linked respiration, **(d)** non-mitochondrial respiration, **(f)** maximal respiratory capacity, **(g)** respiratory reserve and **(h)** proton leak values calculated are plotted, represented as mean ± SD. All data underwent one-way ANOVA analysis with Tukey's multiple comparison test, n = 3 culture wells, (*$p < 0.05$).

Result 3: Unchanged mutant load following treatment with mitophagy activator

There were no observable changes in mutant load subsequent to a 19-day treatment for m.3243A>G cybrids, and 15-day treatment for N50 and N80 cybrids (Fig. 3).

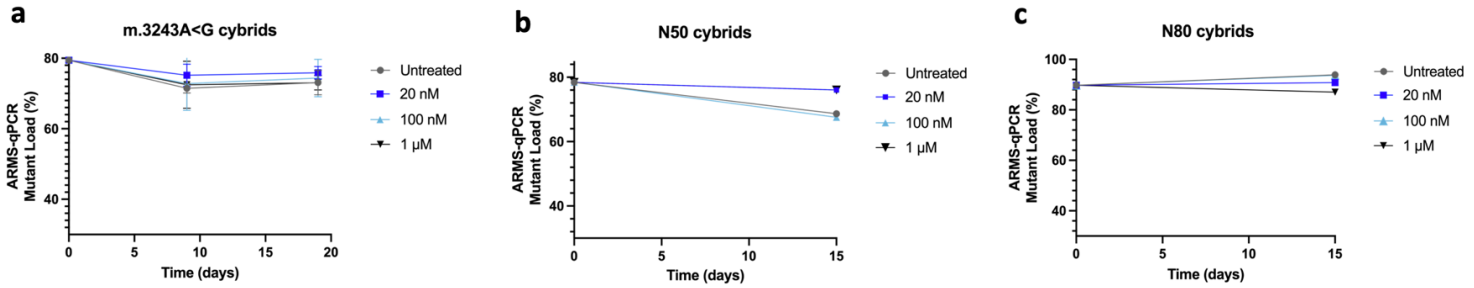

Fig 3. ARMS-qPCR results indicating no change in mutant load following sustained treatment. (a) m.3243A>G cybrids were treated for 19 days with mitophagy activator (20 nM, 100 nM or 1 µM) or untreated (n = 2 biological repeats). **(b)** N50 (n = 1) and **(c)** N80 (n = 1) cybrids underwent 15-day treatment.

Discussion and Future Directions

A sustained 2–3-week treatment with a mitophagy activator (CAP-1902) was not shown to reduce mutant load in cybrids harbouring m.3243A>G or m.8993T>G mutations. Results in the latter case are in line with previous findings that inhibiting PI3K-Akt-mTORC1 signalling in m.8993T>G cybrids does not decrease mutant load [3]. Therefore, pharmacological interventions for mitochondrial disorders will likely require targeting of mutation-specific signalling pathways. Alternatively, this could indicate that mitophagy activation alone is insufficient to clear mutant mtDNA. Interestingly, improvements in respiratory capacity were observed in 20nm-treated m.3243A>G cybrids, possibly suggesting that more prolonged exposure to CAP-1902 may be needed to see concomitant changes in mutant load. Future experiments should further investigate the potential for CAP-1902 to rescue bioenergetic function.

Fig 4. Anitta Chacko (PhD student), Michael Duchen (project supervisor), Alim Devecioglu and Gabriel Valdebenito (PhD student). From left to right.

Departures from Original Project

In the first 4 weeks we treated patient-derived fibroblasts which had to be discarded, as we discovered original cell culture flasks were cross-contaminated. We moved to using cybrids due to their faster proliferation, for data collection in a shorter time span.

Studentship Value

Student: I have gained experience in performing numerous molecular biology techniques, mammalian cell culture and image analysis, which has allowed me to develop confidence in a laboratory setting that will be vital for my future studies and 3rd year project. I also cultivated transferable skills such as organisation and time management – by conducting experiments in a timely manner – and problem-solving from several cases of troubleshooting.

Lab: The laboratory has generated preliminary data assessing the viability of a mitophagy activator as a therapeutic strategy for treating mitochondrial disorders. The improved respiratory parameters are encouraging and offer direction for future studies.

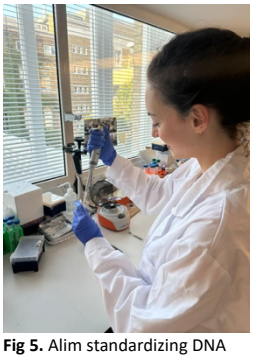

Fig 5. Alim standardizing DNA samples for ARMS-qPCR.

References

1. Gorman et al., (2015), *Ann Neurol*. **77**(5):753-759.
2. Wei and Chinnery, (2020), *J Intern Med*. **287**(6):634-644.
3. Chung et al., (2021), *Nat Commun*. **12**(1):6409.
4. Chung et al., (2022), *Trends Cell Biol*. **32**(5):391-405.