Identifying novel pharmacological drugs to eliminate pathogenic heteroplasmic mtDNA by using a novel quantitative assay of mitophagy

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Introduction

Mitochondrial diseases such as mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) are frequently heteroplasmic, where mutant m.3243A>G mitochondrial DNA (mtDNA) co-exists with normal mtDNA. These diseases are usually progressive, the load of mutant mtDNA determining disease severity. Mitophagy is a cellular mechanism for the recycling of redundant or dysfunctional mitochondrial fragments. This process may be able to improve mtDNA quality in heteroplasmic disease and influence disease progression. Homoplasmic mitochondrial diseases also exist, where the mutant mtDNA is 100%. We reasoned that modulating mitophagy could be beneficial for patients with mitochondrial disease. We therefore screened 447 drugs from the NCC1 drug library for effects on mitophagy in fibroblasts using a high throughput imaging systems (IN Cell analyser (GE)).

Methodology

We used fibroblasts derived from a patient harbouring the common m.3243A>G mtDNA mutation and a healthy control. Treatment with idebenone was also performed on fibroblasts derived from a patient with the m.13051G>A mtDNA mutation. Patient derived fibroblasts were cultured either in standard glucose media (25mM glucose) or galactose media (no glucose). Cells were immunostained for the autophagy marker LC3 (rabbit, 1/500, PM036, MBL) and the mitochondrial protein TOM20 (mouse, 1/200, sc-136211, SantaCruz) and analysed with IN Cell 1000. For the detailed protocol of the heteroplasmy assay please refer to Diot et al., 2015.

Results

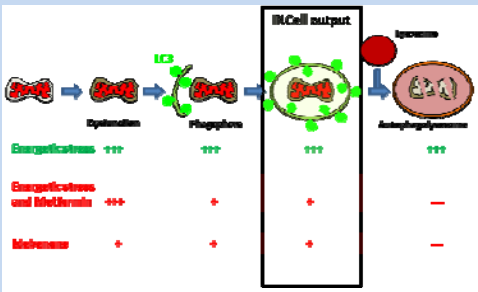

Figure 1. Cartoon illustrating mitophagy and the readout of the INCell analysis.
A dysfunctional mitochondrial fragment is targeted to a developing autophagosome (phagophore) which engulfs it, forming an autophagosome. The LC3-II protein is a hallmark of the autophagosomal membrane. The autophagosome then fuses with a lysosome and its content is degraded. The signal we detect with the INCell 1000 analyser is indicated (INCell output).
The coloured bars below indicate the effect of modulators of mitophagy on each component of the pathway. The boxed region highlights the output measured by INCell. In this study energetic stress (forcing cells to use oxidative phosphorylation by growing them on glucose-free galactose based media) increases mitophagy. Both the inhibitors discussed in this study appear to slow down the whole pathway, metformin by phagophore availability, idebenone by reducing the energetic stress.

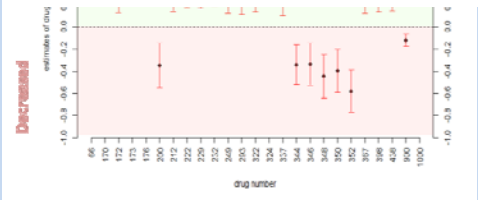

Figure 3. Modulators of mitophagy identified through the drug screen. Based on the number of co-localization events between mitochondria and LC3-punctae 18 drug were identified as mitophagy inducers and 7 drug were found to decrease mitophagy (bars represent 95% confidence intervals).

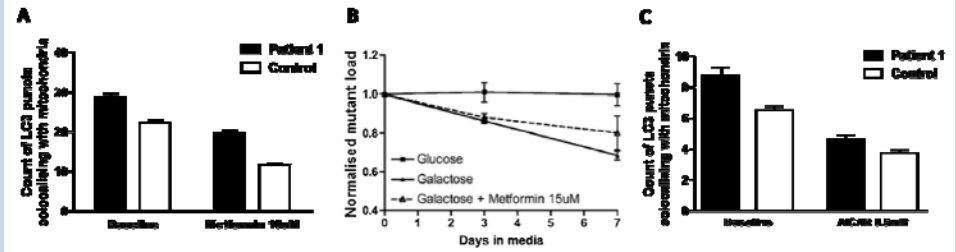

Figure 2. Metformin is a mitophagy inhibitor. Metformin, the most commonly prescribed antidiabetic drug is one of the mitophagy inhibitors identified. Metformin decreases the number of co-localisation events between LC3 puncta and mitochondria under 3 days energetic stress (galactose media) in both control and m. 3243A>G cultures (A). Culture of m. 3243A>G cells in galactose media promotes the removal of pathogenic mtDNA via mitophagy therefore decreases the mutant load. Treatment with metformin inhibits the removal of pathogenic mtDNA (B). AICAR, a drug that is believed to act through AMP-activated protein kinase like metformin, has a similar effect on mitophagy. Treatment of cells for 24h decreases the number of co-localization events in galactose media.

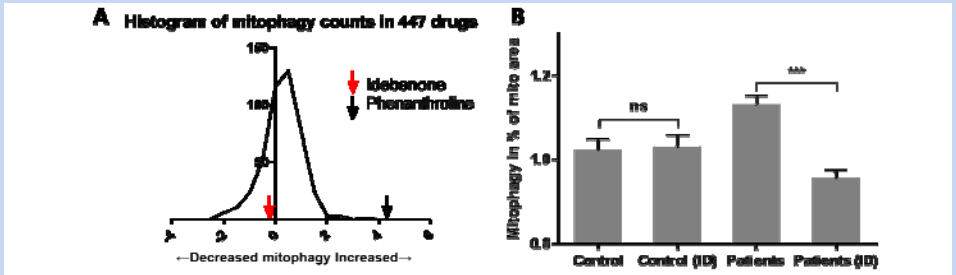

Figure 4. Idebenone is also a mitophagy inhibitor Exposure of m.3243A>G cells to Idebenone, an organic compound of the quinone family, mildly inhibited mitophagy. Phenanthroline, a very strong non-selective mitophagy inducer is also indicated on the histogram for comparison (A). Fibroblasts harbouring the m.13051G>A mtDNA mutation showed a decrease in mitophagy after treatment with idebenone (1µM) when compared to control under energetic stress (B).

Conclusions

We found that metformin, the most commonly prescribed antidiabetic drug that is still sometimes used in Maternally Inherited Diabetes and Deafness (MIDD), inhibits mitophagy at clinically relevant concentrations under energetic stress (figures 2A and 2B). This was recapitulated by exposing the same cells to AICAR (figure 2C), suggesting that metformin inhibits mitophagy by activating AMPK. Metformin may be damaging to patients with heteroplasmic mtDNA disease because it inhibits mitophagy.
Idebenone, a drug used for treating patients with Leber's Hereditary Optic Neuropathy (LHON) was a weak inhibitor of mitophagy in cultures from MELAS patients carrying the m.3243A>G mutation, but not in controls. It significantly inhibited mitophagy in LHON/Leigh's disease due to the rare m.13051G>A mutation under energetic stress.
While metformin probably disadvantages patients by inhibiting the clearance of pathogenic m. 3243A>G mutant mtDNA under energetic stress, idebenone appears to be beneficial in m.13051G>A homoplasmic mtDNA disease, both in culture and clinically. This is probably because idebenone benefits the impaired respiratory chain that underlies LHON pathology and potentially reduces the mitochondrial damage that results from m.13051G>A. Understanding how drugs affect mitophagy in specific mitochondrial diseases is important for clinicians treating mitochondrial patients.

References
Diot A, Hinks-Roberts A, Lodge T, et al. A novel quantitative assay of mitophagy: Combining high content fluorescence microscopy and mitochondrial DNA load to quantify mitophagy and identify novel pharmacological tools against pathogenic heteroplasmic mtDNA. Pharmacol Res 2015;100:24-35. doi: 10.1016/j.phrs.2015.07.014.