AMX0035, a Novel Combination Therapeutic Candidate for Treatment of Primary Mitochondrial Diseases

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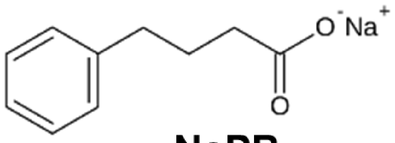
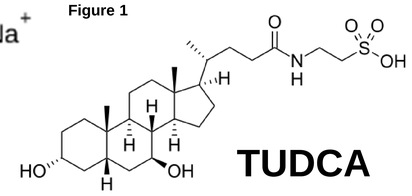
Introduction:
Amylyx has developed a novel therapeutic, AMX0035, a proprietary combination of two compounds, Sodium Phenylbutyrate (PB) and Tauroursodeoxycholic Acid (TUDCA), for the treatment of neurodegenerative diseases. The combination of agents were selected to block neuronal death, and downstream neuroinflammation, through simultaneous inhibition of endoplasmic reticulum (ER) stress and mitochondrial stress. Both PB and TUDCA have been evaluated individually in disease-specific models of AD and other neurodegenerative diseases, as well as models of ER Stress and mitochondrial dysfunction. PB is a class I and class II HDAC inhibitor that ameliorates ER stress through upregulation of DJ-1, a master chaperone regulator[1], and other chaperone proteins[2]. TUDCA has been shown to recover mitochondrial bioenergetic deficits through incorporation into the mitochondrial membrane, reducing Bax translocation to the mitochondrial membrane, reducing mitochondrial permeability, and increasing the apoptotic threshold of the cell.[3] The chemical structures of each molecule are presented in (**Figure 1**).

Figure 1



NaPB



TUDCA

Aim. Previous studies at Amylyx have provided evidence for a novel, synergistic efficacy in numerous models of neurodegenerative disease following simultaneous treatment with TUDCA and PB. Our aim was to investigate the effects of AMX0035 on transmitochondrial cybrid models and mouse embryonic fibroblasts (MEFs) derived from a mouse model of Leigh Syndrome (NDUFS4 KO).[4] These studies were designed to evaluate the potential efficacy of AMX0035 as a prospective therapeutic agent to treat primary mitochondrial diseases.

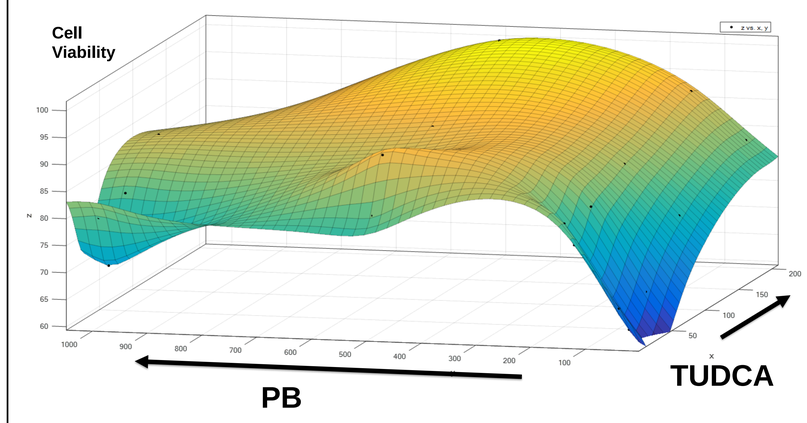


Figure 2. Primary rate cortical neuronal viability is synergistic increased at specific concentrations of TUDCA and PB. Primary cortical neurons were treated with 26.5µM H2O2 for 1 hour and treated with the combination of compounds in a dose-matrix study for 24 hours prior to and after H2O2 exposure. H2O2 dose was titrated to kill approximately 60% of neurons. 20 concentration ratios of TUDCA and PB were evaluated to investigate the dosage range at which optimal efficacy is achieved. TUDCA was shown to demonstrate optimal efficacy in combination at concentrations between 50µM - 200µM and PB at concentrations between 150µM - 500µM. At optimal concentrations, ratios of TUDCA and PB resulted in neuronal cellular viabilities >95%.

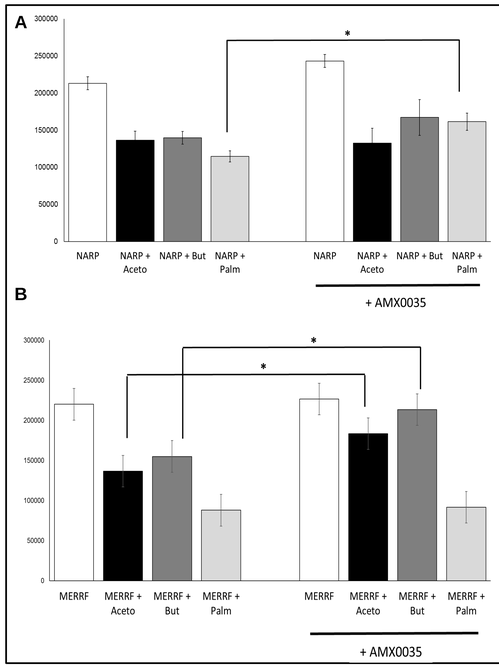
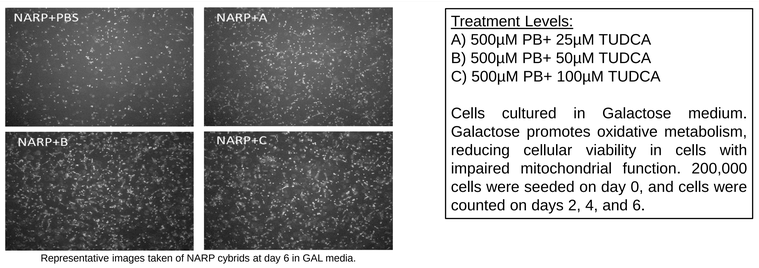


Figure 3. Effect of AMX0035 treatment in transmitochondrial cybrid cells grown in non-fermentable substrate. Cellular growth and viability was assessed following growth in acetoacetate ("aceto"), butyric acid ("but"), and palmitate ("palm"). Cells were cultured in DMEM media containing 0.5mM GLU +dFBS + Pyr + glut + P/S with the addition of either: 10mM acetoacetate, 5mM butyrate, or 200µM palmitate. 200,000 cells were seeded on day 0 and cells were counted by Coulter counter at day 2. Cybrids were treated with AMX0035, dosed at 500µM PB and 50µM TUDCA, the most effective dose observed in cell viability assays (Fig. 5). **a)** Cybrid cells homoplasmic for the m.8993T>G mutation in ATP6 subunit of the ATP synthase complex were evaluated. Statistically significant ($p < 0.05$) improvements in cell viability were seen in cells cultured in 200µM palmitate treated with AMX0035. **b)** MERRF cybrids viability at 48 hours demonstrated statistically significant improvements following culturing in media containing 10mM acetoacetate and 5mM butyrate. Experiments were completed in triplicate.



Representative images taken of NARP cybrids at day 6 in GAL media.

Treatment Levels:
A) 500µM PB+ 25µM TUDCA
B) 500µM PB+ 50µM TUDCA
C) 500µM PB+ 100µM TUDCA

Cells cultured in Galactose medium. Galactose promotes oxidative metabolism, reducing cellular viability in cells with impaired mitochondrial function. 200,000 cells were seeded on day 0, and cells were counted on days 2, 4, and 6.

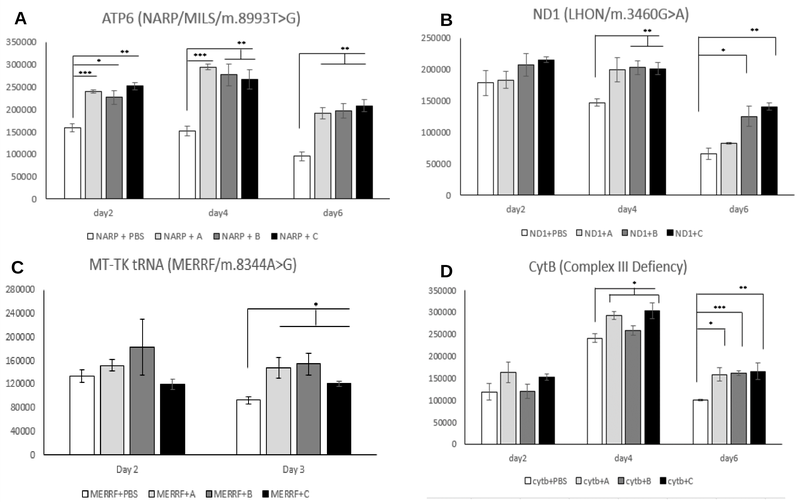
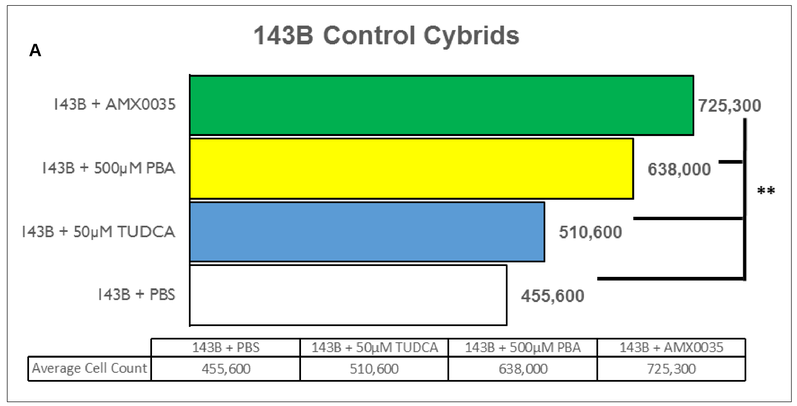


Figure 4. AMX0035 improves cell viability in transmitochondrial cybrid models. Three ratios of AMX0035 were evaluated in cybrid models grown in DMEM + 10mM GAL + dFBS + Pyr + glut + P/S. Cell viability was assessed in **a)** cybrid cells homoplasmic for the m.8993T>G mutation in ATP6 subunit of the ATP synthase complex, **b)** a cybrid model of Leber's Hereditary Optic Neuropathy (LHON) containing the m.3460G>A mutation in ND1, a mitochondrial-encoded subunit of Complex I, **c)** a cybrid model containing a cytochrome B truncation in the *MT-CYB* gene. *MT-CYB* encodes a subunit of Cytochrome C oxidoreductase. **d)** Cell viability was also assessed in homoplasmic mutant MERRF cybrids with a mutation in the mitochondrially-encoded lysine transfer RNA. MERRF cybrids contain 100% homoplasmic mutated mitochondria and were unable to survive in the absence of glucose. MERRF cybrids were grown in GAL media supplemented with 0.5mM glucose. Experiments were completed in triplicate.

Figure 5. AMX0035 improves cell viability in a synergistic manner. 143B control cybrids and NARP (m.8997T>G) cybrids were grown in GAL media and treated with PBS (control), 50µM TUDCA, 500µM PB, and AMX0035 (50µM TUDCA + 500µM PB) to assess whether the combination of PB and TUDCA improves cell viability relative to the individual compounds. Cells were cultured for 3 days and counted using coulter counter. **a)** AMX0035 resulted in a statistically significant improvement in cell viability (** = p<0.001) over treatment with PB, TUDCA, or control. **b)** Treatment with AMX0035 improves cell viability (114% improvement relative to PBS) vs. PB (43%) or TUDCA (21%) individually (*** = p<0.0001). Experiments were completed in triplicate.



Average Cell Count	143B + PBS	143B + 50µM TUDCA	143B + 500µM PBA	143B + AMX0035
	455,600	510,600	638,000	725,300

Average Cell Count	JCP + PBS	JCP + 50µM TUDCA	JCP + 500µM PBA	JCP + AMX0035
	178,600	215,300	254,700	382,300

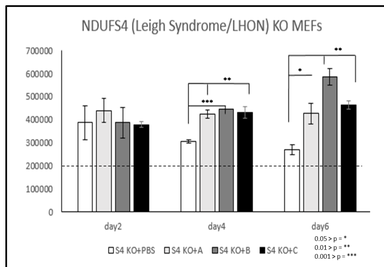


Figure 6. AMX0035 treatment improves cell viability in mouse embryonic fibroblasts (MEFs) derived from a mouse model of mitochondrial Complex I deficiency (NDUFS4 KO mouse). Three ratios of AMX0035 were evaluated in embryonic fibroblasts grown in DMEM + 10mM GAL + dFBS + Pyr + glut + P/S. Three doses of AMX0035 were evaluated (those tested in Fig. 5) with a combination of 500µM PB and 50µM TUDCA emerging as the most effective dose.

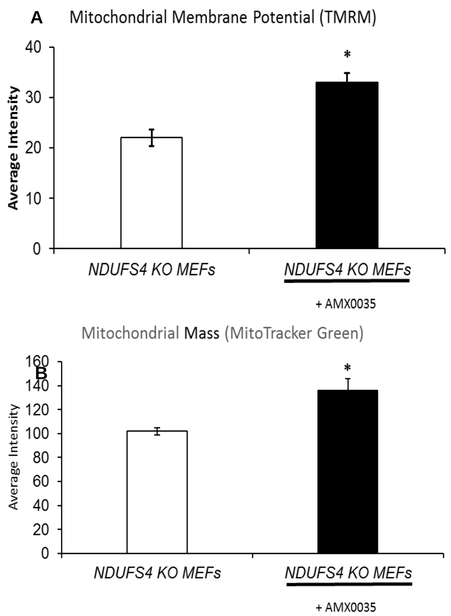


Figure 7. AMX0035 treatment in NDUFS4 KOs results in increased mitochondrial membrane potential and increased mitochondrial mass. Mouse embryonic fibroblasts were seeded at the density of 20,000 cell/well in quadruplicate in 48-well tissue culture plates. The next day, cells were loaded with 50 nM tetramethylrhodamine methyl ester (TMRM, 544ex; 590em, Life Technologies) and 25 nM MitoTrackerGreen (MTG, 490ex; 516em, Life Technologies) for 30 minutes at 37°C in phenol-free DMEM containing 5 mM glucose, 4 mM glutamine and 1 mM pyruvate. AMX0035 treatment, at the dose evaluated in Figures 4 and 5, increased **a)** mitochondrial membrane potential (TMRM), and **b)** mitochondrial mass (MTG).

Conclusion;

- AMX0035 is a combination of Sodium phenylbutyrate and Tauroursodeoxycholic Acid.
- AMX0035 improves cell viability in neurons following oxidative insult.
- AMX0035 improves cell viability in cybrid cells grown in metabolic-stress media
- AM0035 improves cell viability, in a statistically significant manner better than either PB or TUDCA, in primary mitochondrial disease models.
- AMX0035 increases measures of mitochondrial function and mass.

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2. Wright, Jerry M., et al. "Gene expression profile analysis of 4-phenylbutyrate treatment of IB3-1 bronchial epithelial cell line demonstrates a major influence on heat-shock proteins." Physiological Genomics 16.2 (2004): 204-211.
3. Rodrigues, CM. et al. Tauroursodeoxycholic acid prevents Bax-induced membrane perturbation and cytochrome C release in isolated mitochondria. Biochemistry. 2003.
4. Kruse, Shane E., et al. "Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy." Cell metabolism 7.4 (2008): 312-320.