Title: Pre-clinical testing of novel small molecules inhibit neurodegeneration – implications for the pathogenesis and treatment of MS

Cole D. Libner, Hannah E. Salapa, Joseph-Patrick W. E. Clarke, Patricia A. Thibault, Michael C. Levin

Background: Neurodegeneration, the death and damage to neurons and axons, drives disease progression and permanent disability in MS, yet there are currently no therapies that specifically target neurodegeneration. We identified dysfunction of the RNA binding protein heterogeneous nuclear ribonucleoprotein A1 (A1) as a key contributor to neuronal death and damage in MS and its models *(Nature Communications, 2024)*. Using *in silico* computer modeling and biochemical assays we identified multiple small molecules capable of interacting with A1 and inhibiting neurodegeneration.

Objectives: To assess efficacy, toxicity, and the pharmacokinetic (PK) properties of A1 targeted small molecules using clinically relevant *in vitro* and *in vivo* models to determine their therapeutic potential and suitability for clinical trials.

Methods: Primary neurons were subjected to a physiological stressor and treated with six different small molecules at doses of 25-100ug/mL and examined by immunocytochemistry for A1 dysfunction (its mislocalization from the nucleus to the cytoplasm) and neurite loss, a marker of neurodegeneration. Small molecule binding affinity for A1 was assessed by biochemical assays. Because of its *in vitro* efficacy, small molecule 3 was selected for *in vivo* analyses and was tested for toxicity in naïve mice, efficacy in experimental autoimmune encephalomyelitis (EAE), and PK properties via tandem liquid chromatography/mass spectrometry (LC-MS/MS) in rats.

Results: Of the six A1 specific small molecules assessed, four of them, including small molecule 3, significantly reduced A1 dysfunction (demonstrated by nuclear retention of A1 (p<.001)), and inhibited neurodegeneration (preserved neurite length (p<.01)) in primary neurons. Small molecule 3, which was most efficacious *in vitro*, demonstrated the highest affinity binding to A1 when analyzed by thermal shift assay. Preliminary *in vivo* experiments demonstrate molecule 3 was non-toxic in naïve mice, efficacious in mice with EAE ($p<0.01$) and showed a favorable PK profile in rats.

Conclusions: Small molecules targeting A1 restored A1 function, inhibited neurodegeneration, and showed a favorable PK profile in relevant pre-clinical MS models. These data confirm the role of A1 dysfunction in pathogenesis of MS and indicate that small molecules that target A1 are a novel and viable option for clinical trials in MS designed to inhibit neurodegeneration, reduce disability, and improve the lives of persons living with MS.