Development and Preclinical Assessment of AMX0114: an Antisense Oligonucleotide Targeting Calpain-2, a Critical Effector of Axonal Degeneration

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Poster #11

BACKGROUND

- Axonal degeneration has been increasingly recognized as a key early contributor to the clinical presentation and pathogenesis of amyotrophic lateral sclerosis (ALS)[1-3]
- Activation of the calcium-dependent protease calpain-2 is proposed as one of the critical effectors of axonal degeneration[3]
- Calpain-2 has been implicated in the pathogenesis of ALS based on:
  - Findings of elevated calpain-2 mRNA in muscle samples[4] and calpain-specific TAR DNA binding protein 43 cleavage product concentrations in postmortem spinal cord samples[5] from people living with ALS
  - Therapeutic benefit of calpain-2 activity modulation in animal models of ALS[6]
  - The role of calpain-2 in cleaving neurofilament,[3] a well-established biomarker in ALS
- Based on evidence supporting a potential benefit of calpain-2 modulation in ALS and other neurodegenerative diseases, Amylyx Pharmaceuticals developed antisense oligonucleotides (ASOs) aimed at targeting the gene encoding calpain-2 (*CAPN2*)

OBJECTIVES

- To quantitatively assess the capacity of *CAPN2*-targeted ASO candidates to inhibit *CAPN2* expression
- To evaluate the neuroprotective effects of the lead ASO candidate in in vitro motor neuropathy assays

EXPERIMENTS

CAPN2 Expression in Human Glutamatergic Neurons

METHOD: Screened 80 ASOs for ability to reduce *CAPN2* expression in human glutamatergic neurons

- ASOs targeted to *CAPN2* were applied via gymnosis to human induced pluripotent stem cell (iPSC)–derived glutamatergic neurons (ioGlutamatergic Neurons; bit.bio); neurons were then incubated for 48 hours
- *CAPN2* mRNA levels were assessed by real-time quantitative polymerase chain reaction (RT-qPCR)
- Cytotoxicity was assessed by Hoechst (5 µg/mL) staining and imaging (2 days after ASO treatment)

- A total of 6 ASO candidates reduced *CAPN2* messenger RNA (mRNA) levels by ≥30% without evident cytotoxicity
- The lead ASO candidate, AMX0114, reduced CAPN2 expression by ~74%

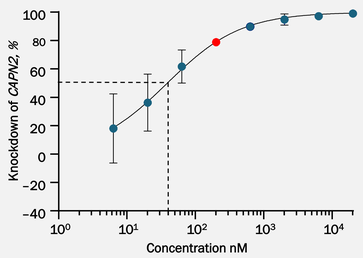
CAPN2 Expression in Human Motor Neurons

METHOD: Assessed effect of AMX0114 on *CAPN2* expression in human motor neurons

- AMX0114 was applied in varying concentrations (0.006, 0.02, 0.06, 0.2, 0.63, 2.0, 6.32, and 20 µM) by gymnosis to an iPSC-derived human spinal motor neuron cell line (iCell Motor Neurons; FUJIFILM Cellular Dynamics, Inc.); neurons were then incubated for 72 hours
- *CAPN2* mRNA levels were assessed by RT-qPCR

- AMX0114 reduced *CAPN2* mRNA levels in a dose-dependent manner up to 99% at the 20-µM concentration
- The potency of AMX0114 (half maximal effective concentration [EC_{50}]) was ~40 nM (**Figure 1**)

Figure 1. AMX0114 Potency Screen



Red data point indicates value obtained from only a single replicate. Dotted line indicates EC_{50}.

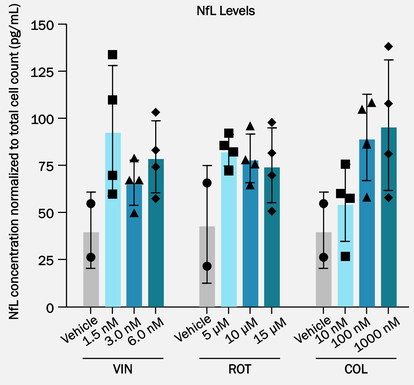
Neuropathy Assays

METHOD: Evaluated effect of AMX0114 on mitigating neurofilament light chain (NfL) excretion and neurite degeneration in neurotoxic compound–triggered neuropathy assays

- iPSC-derived human motor neurons were incubated with varying concentrations of the neurotoxic compounds vincristine, rotenone, and colchicine for 24 hours after preincubation with AMX0114 for 48 hours
- Extracellular NfL levels were measured by Meso Scale Discovery chemiluminescence assay
- For the neurotoxic triggers, NfL levels were normalized to total cell count and compared with those in the presence of vehicle (H_2O for vincristine and colchicine assays and dimethyl sulfoxide for rotenone assays)
- The effect of pretreatment with AMX0114 on NfL excretion in each neurotoxic compound–triggered assay was assessed and compared between AMX0114 and vehicle using 1-way analysis of variance
- βIII-tubulin immunoreactivity area was assessed by immunostaining (chicken anti-βIII tubulin [Millipore Sigma])

- Treatment with the neurotoxic triggers increased extracellular NfL levels by 1.5- to 2-fold, though differences were not statistically significant compared with vehicle control owing to high replicate variability (**Figure 2**)

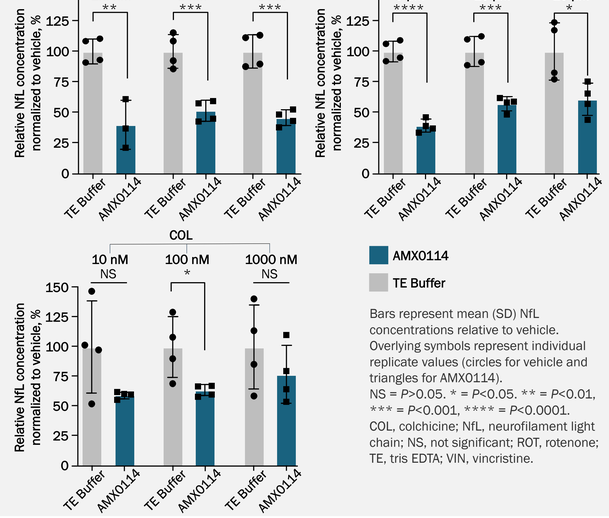
Figure 2. Extracellular NfL Levels in the Presence of Neurotoxic Triggers



Bars represent mean (SD) extracellular NfL concentrations normalized to total cell count. Overlying symbols represent individual replicate values (circles for vehicle and squares, triangles, and diamonds for ascending individual concentrations of neurotoxic compounds). COL, colchicine; NfL, neurofilament light chain; ROT, rotenone; VIN, vincristine.

- Pretreatment with 20 µM AMX0114 significantly reduced neurotoxic trigger–induced NfL excretion (**Figure 3**)
- Pretreatment with AMX0114 also partially prevented trigger-induced neuritic degeneration as quantified by βIII-tubulin staining (data not shown)

Figure 3. Effect of AMX0114 Pretreatment on Neurotoxic Trigger–Induced NfL Excretion



Bars represent mean (SD) NfL concentrations relative to vehicle. Overlying symbols represent individual replicate values (circles for vehicle and triangles for AMX0114).
NS = $P>0.05$. * = $P<0.05$. ** = $P<0.01$, *** = $P<0.001$, **** = $P<0.0001$.
COL, colchicine; NfL, neurofilament light chain; NS, not significant; ROT, rotenone; TE, tris EDTA; VIN, vincristine.

CONCLUSION

- The *CAPN2*-targeted ASO AMX0114 showed concentration-dependent knockdown of *CAPN2* mRNA (up to 99% in human motor neurons)
- Pretreatment with AMX0114 reduced NfL excretion and partially prevented neuritic degeneration in neurotoxic trigger–induced models of motor neuropathy
- Studies in additional models relevant to neurodegenerative diseases are planned to further assess the functional efficacy of AMX0114
- AMX0114 is an investigational agent not approved for use by the FDA or any other regulatory agency but is currently in IND-enabling studies

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Disclosures
JC and JK are co-CEOs of and own stock in Amylyx Pharmaceuticals, Inc. EM and JT are full-time employees of Amylyx. TMC, SD, FvV, ME, MH, RSR, MBT, RdW, and SdM are employees of Charles River Laboratories, which was contracted by Amylyx to perform the experiments described herein.

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