Development of AMX0114, an Antisense Oligonucleotide Targeting Calpain-2

Poster #5210

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BACKGROUND

- Evidence supports axonal degeneration, first characterized by Augustus Waller in the 1850s, as one of the critical pathways underlying pathology in amyotrophic lateral sclerosis (ALS), other neurodegenerative diseases and axonopathies[1-4]
- Calpain-2 may be one of the critical effectors in the axonal degeneration pathway, as calpain family substrates include several cytoskeletal components[3]
  - Elevated immunoreactivity of calpains was detected in various myopathies and in muscle samples from people living with ALS and progressive muscular dystrophy[5]
  - Calpain-2 has been implicated in both acute neuronal injury and in various neurodegenerative conditions including ALS, Alzheimer's disease, and Wolfram syndrome[5,6]
  - In the pathogenesis of ALS, the role of calpain-2 is supported by both *in vitro* and *in vivo* findings including elevated calpain-2 messenger RNA (mRNA) in muscle samples[5] from people living with ALS, and the role of calpain-2 in cleaving neurofilament[3], a well-established biomarker in ALS
- In order to evaluate the potential benefit of calpain-2 modulation in neurodegenerative diseases, Amylyx Pharmaceuticals developed antisense oligonucleotides (ASOs) to target the gene encoding calpain-2 (*CAPN2*) *in vitro*

OBJECTIVES

- To evaluate the efficiency of knockdown of *CAPN2*-targeted ASOs
- To investigate the neuroprotective potential of the lead ASO candidate in neurotoxic compound–triggered neuropathy assays

CONCLUSION

- The *CAPN2*-targeted ASO, AMX0114, showed concentration-dependent knockdown of *CAPN2* mRNA (≤99% in human motor neurons)
- Pretreatment with AMX0114 partially prevented trigger-induced neuritic degeneration of βIII-tubulin staining and had no marked effect without toxicity trigger
- NfL excretion was further reduced upon pretreatment with AMX0114, and pretreatment 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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EXPERIMENTS

CAPN2 Expression in Human Glutamatergic Neurons

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

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

- The lead ASO candidate, AMX0114, reduced *CAPN2* expression by ~74% (**Figure 1A**) and showed no obvious cytotoxicity (**Figure 1B**)

Figure 1A. Top 15 ASO Candidates Reduced *CAPN2* mRNA Levels by ≥30%

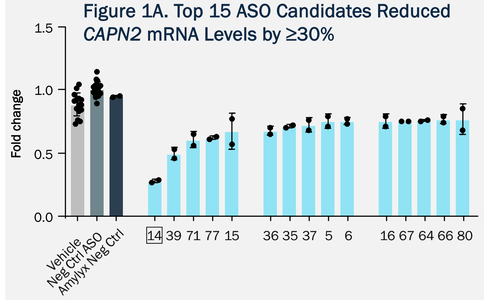

Figure 1B. Top 15 ASOs Did Not Display Obvious Cytotoxicity in Glutamatergic Neurons

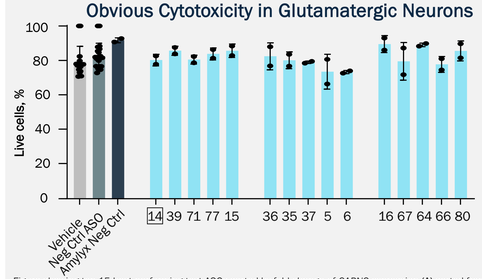

Figure showing top 15 best-performing test-ASOs sorted by fold-change of *CAPN2* expression (**A**) sorted from low to high fold change, and (**B**) percentage live cells. Blue bars on the right are ASO candidates identified by the number. On the left, gray is vehicle (5% TE Buffer), blue-gray is negative control ASO, and darker gray is Amylyx negative control. 5 µM of each ASO was used in the screen. Data represent mean ±SD of biological replicates; individual replicates are indicated by black dots. ASO, antisense oligonucleotide; mRNA, messenger RNA; TE, tris EDTA.

CAPN2 Expression in Human Motor Neurons

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

- Results in the human motor neuron cell line were consistent with glutamatergic cell line
- The lead candidate, AMX0114, showed concentration-dependent knockdown of *CAPN2* mRNA levels (≤99% at the 20-µM concentration)

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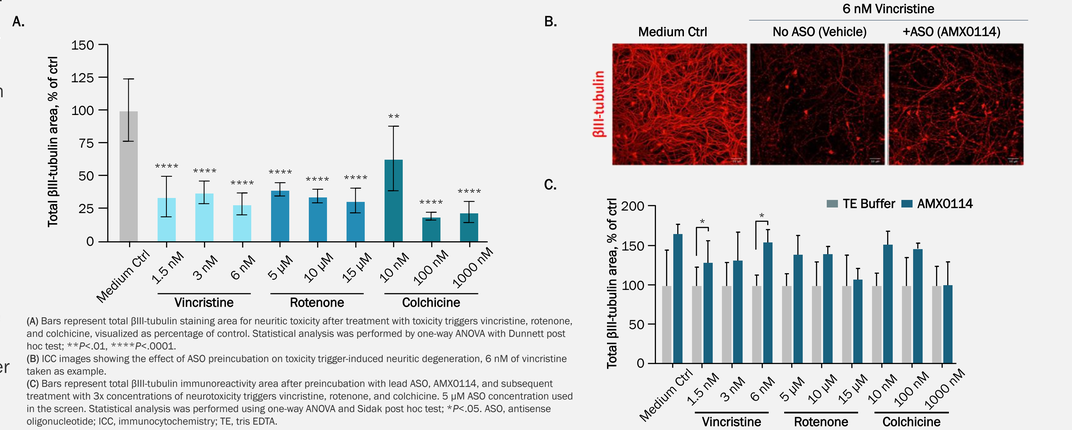
Neuropathy assays

METHOD: Evaluated effect of AMX0114 on neuritic degeneration

- βIII-tubulin immunoreactivity area was assessed by immunostaining (chicken anti-βIII tubulin [Millipore Sigma])
- A new hierarchical cluster analysis algorithm was established for more robust quantification of staining

- Total area and density × area but not average staining intensity of βIII-tubulin immunoreactivity were significantly diminished after trigger
- With the exception of 10nM colchicine, all treatments resulted in similar degrees of neurotoxicity (60%–80% effect size) (**Figure 2A**)
- Pretreatment with 5 µM AMX0114 partially prevented trigger-induced neuritic degeneration of βIII-tubulin staining (**Figure 2B and C**)
- Significant prevention of trigger-induced loss of βIII-tubulin staining by AMX0114 was observed in combination with 1.5 nM vincristine and 5 µM rotenone
- ASO treatment without toxicity trigger had no marked effect on βIII-tubulin immunoreactivity

Figure 2A-C. Pretreatment With AMX0114 Partially Prevented Trigger-Induced Neuritic Degeneration of βIII-tubulin Staining



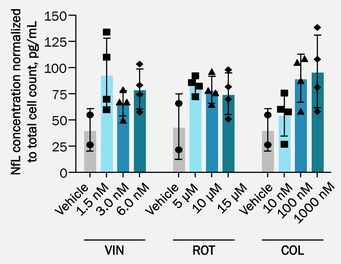
(**A**) Bars represent total βIII-tubulin staining area for neuritic toxicity after treatment with toxicity triggers vincristine, rotenone, and colchicine, visualized as percentage of control. Statistical analysis was performed by one-way ANOVA with Dunnett post hoc test; ***P*<.01, *****P*<.0001.
(**B**) ICC images showing the effect of ASO preincubation on toxicity trigger-induced neuritic degeneration, 6 nM of vincristine taken as example.
(**C**) Bars represent total βIII-tubulin immunoreactivity area after preincubation with lead ASO, AMX0114, and subsequent treatment with 3x concentrations of neurotoxicity triggers vincristine, rotenone, and colchicine. 5 µM ASO concentration used in the screen. Statistical analysis was performed using one-way ANOVA and Sidak post hoc test; **P*<.05. ASO, antisense oligonucleotide; ICC, immunocytochemistry; TE, tris EDTA.

METHOD: Evaluated effect of AMX0114 on mitigating neurofilament light chain (NfL) excretion 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)

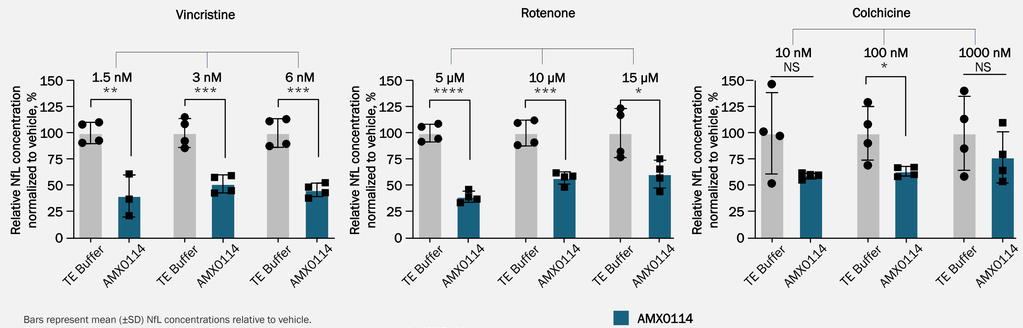
- Treatment with toxicity triggers vincristine, rotenone, and colchicine resulted in an increase in extracellular NfL levels (assay window = 1.5–2 fold) (**Figure 3**)
- Pretreatment with 20 µM AMX0114 significantly reduced neurotoxic trigger–induced NfL excretion (**Figure 4**)

Figure 3. 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.

Figure 4. 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*>.05. **P*<.05. ***P*<.01, ****P*<.001, *****P*<.0001.
NfL, neurofilament light chain; NS, not significant; TE, tris EDTA.

Disclosures
JC and JK are co-CEOs of and have stock option ownership in Amylyx Pharmaceuticals, Inc. ML and EM are full-time employees of and have stock option ownership in Amylyx Pharmaceuticals, Inc. TMC, SD, FvV, ME, MH, RR, 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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