Development and Preclinical Assessment of Antisense Oligonucleotides Targeting Calpain-2, a Critical Effector of Axonal Degeneration

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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 1 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 messenger RNA (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 broadly researched biomarker in ALS
- Antisense oligonucleotides (ASOs) are small nucleic acid sequences (10-50 base pairs) that can bind directly to target mRNA prior to/during translation[7]
  - ASOs have been approved for the treatment of >10 conditions and have been tested in both animal models and clinical trials for the treatment of ALS
- Based on evidence supporting a potential benefit of calpain-2 modulation in ALS and other neurodegenerative diseases, Amylyx Pharmaceuticals developed ASOs aimed at targeting the gene encoding calpain-2 (*CAPN2*)

OBJECTIVES

- To evaluate ASOs targeting *CAPN2* and quantitatively assess their capacity to inhibit *CAPN2* mRNA expression and calpain-2 protein expression
- To establish a kinetic profile of the most potent ASO candidates, quantifying stability and duration of mRNA and protein knockdown

EXPERIMENTS

CAPN2 Expression in Human Glutamatergic Neurons

METHOD: Screened 80 ASOs for ability to reduce *CAPN2* expression in human glutamatergic neurons and for cytotoxicity[8,9]

- ASOs targeted to *CAPN2* had 5-10-5 gapmer chemistry with 2'-O-methoxy-ethyl wing modifications and fully phosphorothioated backbones
- ASOs 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 (RT-qPCR)
- Cytotoxicity was assessed by Hoechst (5 µg/mL) staining and imaging (2 days after ASO treatment)

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

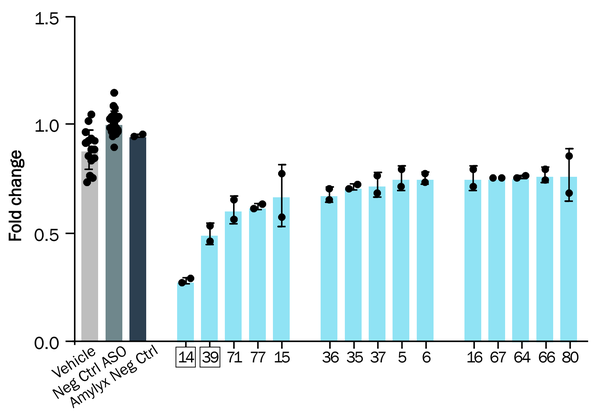
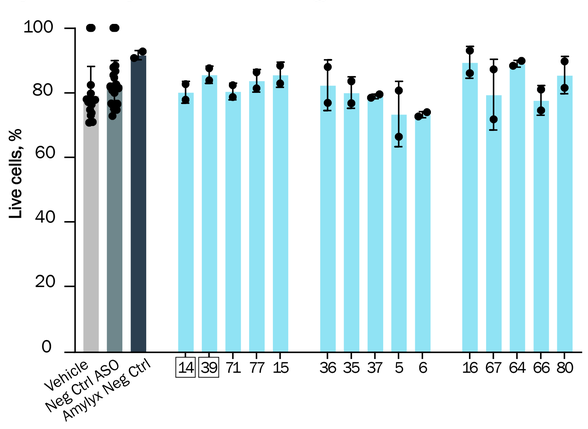


Figure 1B. Top 15 ASOs Did Not Display Measurable Cytotoxicity in Glutamatergic Neurons



- ASO14 and ASO39 were the top ASO candidates and were selected for further experiments based on preliminary *CAPN2* mRNA knockdown and absence of measurable cytotoxicity **(Figure 1)**

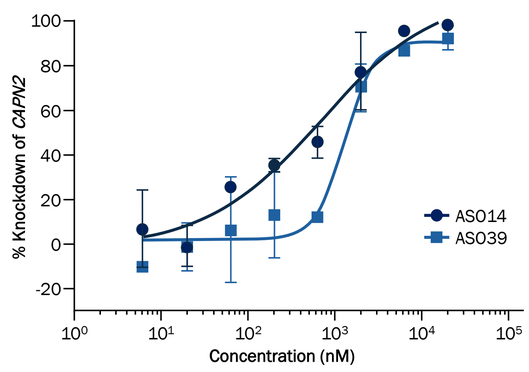
Figure showing top 15 best-performing test-ASOs sorted by fold-change of *CAPN2* expression (**A**) sorted from low to high fold change, and (**B**) percentage live cells. Blue bars on the right are ASO candidates identified by internal ID number. On the left, gray is vehicle (5% TE Buffer), blue-gray 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 ASO14 and ASO39 on *CAPN2* expression in human motor neurons

- ASOs were 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

Figure 2. ASO Potency Screen



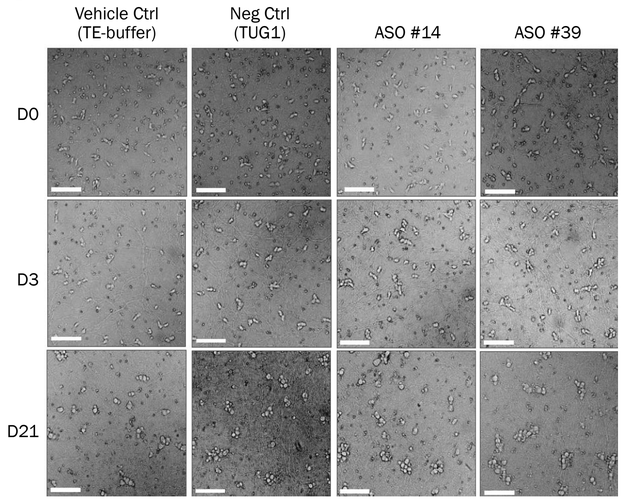
- Both lead ASOs reduced *CAPN2* mRNA levels in a dose-dependent manner, reaching ≥90% knockdown without observed cytotoxicity at the highest concentrations tested **(Figure 2)**
- ASO14 and ASO39 exhibited mean potencies of ≈100 nM (EC_{50}, n=2) and ≈600 nM (EC_{50}, n=2), respectively **(Figure 2)**

Figure showing representative concentration-response curves for candidate ASOs screened against CAPN2. Data points represent mean ±SD of replicate treatments. ASO, antisense oligonucleotide; EC_{50}, half-maximal effective concentration.

Kinetic Profiling Assays

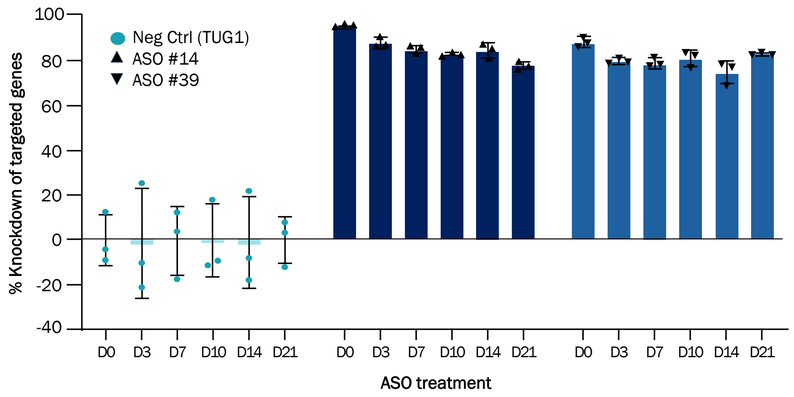
- Kinetic profiling was performed by incubating iPSC-derived human spinal motor neurons with a given ASO candidate for 48 hours
- Levels of *CAPN2* mRNA (RT-qPCR) and calpain-2 protein (size-based automated capillary Western blot assays [Jess Simple Western™, Biotechne]) were compared with a negative control ASO at 0, 3, 7, 10, 14, and 21 days following ASO removal
- No apparent differences in cell morphology were observed upon treatment with either ASO for ≤21 days **(Figure 3)**

Figure 3. ASO Treatment Did Not Affect Cell Morphology



Scale bar represents 100µm. ASO, antisense oligonucleotide; TE, tris EDTA.

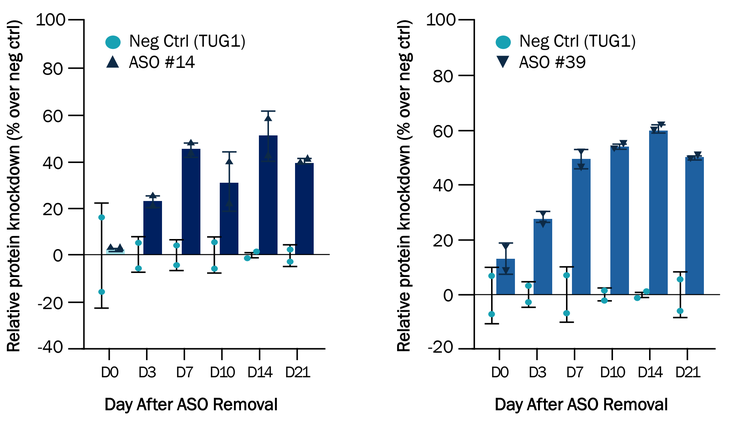
Figure 4. Efficacious and Robust mRNA Knockdown Was Achieved at All Timepoints



RT-qPCR analysis of *CAPN2* mRNA expression after incubation with ASO14 or ASO39 for 48 hours. Knockdown of *CAPN2* was compared to a negative control TUG1 (taurine up-regulated gene 1). Data represent mean ± SD of replicate treatments, and each replicate is indicated by a dot or triangle. ASO, antisense oligonucleotide; mRNA, messenger RNA.

- ASO14 treatment resulted in a maximum *CAPN2* mRNA knockdown of 94% on day 0 (48 hours after gymnosis) with a slight decrease over time to 77% on day 21 **(Figure 4)**
- Treatment with ASO39 resulted in a maximum mRNA knockdown of 87% on day 0 with negligible decrease over time **(Figure 4)**

Figure 5. Stable Protein Knockdown Was Maintained With Both ASOs



Relative knockdown of calpain-2 protein levels after incubation with ASO14 for 48 hours. Reduction of calpain-2 protein levels was compared to a negative control (TUG1). Data represent mean ± SD of replicate treatments, and each replicate is indicated by a dot or triangle.

Relative knockdown of calpain-2 protein levels after incubation with ASO39 for 48 hours. Reduction of calpain-2 protein levels was compared to a negative control (TUG1). Data represent mean ± SD of replicate treatments, and each replicate is indicated by a dot or triangle.

- Stable knockdown of calpain-2 protein was achieved with both ASO candidates, ASO14 and ASO39, compared to the negative control ASO
- The highest relative knockdown of calpain-2 protein achieved with ASO14 was 51% on day 14, and knockdown remained stable from day 7 through day 21 **(Figure 5, left)**
- Treatment with ASO39 resulted in ≈60% knockdown of calpain-2 protein on day 14 with stable knockdown maintained from day 7 onward **(Figure 5, right)**

CONCLUSIONS

- ASOs targeting *CAPN2* were successfully developed and demonstrated concentration-dependent knockdown of *CAPN2* mRNA
- In kinetic profiling experiments, efficacious and robust target mRNA and protein knockdown were observed following treatment of human motor neurons with *CAPN2*-targeting ASOs ASO14 and ASO39
- The extent of knockdown conferred by both ASOs exhibited little decrease over time, suggesting favorable stability
- No adverse effects on cell morphology were observed following treatment with either ASO
- These findings will inform future *in vivo* dosing protocols in models of ALS
- ASO14 and ASO39 are investigational agents not approved for use by the FDA or any other regulatory agency but are currently in IND–enabling studies

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Disclosures

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

References

1. Moloney EB, et al. *Front Neurosci*. 2014;8:252. **2.** Suzuki N, et al. *Front Neurosci*. 2020;14:194. **3.** Ma M. *Neurobiol Dis*. 2013;60:61-79. **4.** Ueyama H, et al. *J Neurol Sci*. 1998;155(2):163-169. **5.** Yamashita T, et al. *Nat Commun*. 2012;3:1307. **6.** Rao MV, et al. *J Neurochem*. 2016;137(2):253-265. **7.** Borros BD, et al. *Neurotherapeutics*. 2022;19(4):1145-1158. **8.** Cohen J, et al. Poster presented at: the 21st Annual NEALS Meeting; November 1-3, 2022; Clearwater, FL and virtual (hybrid). **9.** Leinders M, et al. Poster presented at: Neuroscience 2022; November 12-16, 2022; San Diego, CA.

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