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TITLE: Targeted Riluzole Delivery by Antioxidant Nanovectors for Treating Amyotrophic Lateral Sclerosis

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14. ABSTRACT
The goal of this proposal has been to determine whether a novel nanovector consisting of hydrophilic carbon clusters (HCCs) (pegylated) can serve as a therapeutic in a murine model of amyotrophic lateral sclerosis. HCCs were produced by Dr. James Tour of Rice University and provided to Dr. Grill to assess in his colony of G93A hSOD1 mutant mice. Aims were to determine whether PEG-HCCs functionalized with antibodies against the transferrin receptor could enhance lifespan, protect motoneurons and enhance motor function when delivered via sustained intravenous route at the first sign of disease. Second aim was to assess whether riluzole, in combination with functionalized PEG-HCCs could enhance the outcomes used in Aim 1. Progress in this grant was significantly reduced through two incidences with the Jackson Laboratory who had improperly sent us the wrong animals for the study. This resulted in a significant loss of time on two occasions as the colony needed to be refurbished. We report now, however that PEG-HCCs produce a significant enhancement in several indices of motor function, but did not rescue lumbar motoneurons nor enhance lifespan, when applied to the G93A mouse.

15. SUBJECT TERMS
ALS, inflammation, oxidative stress, motoneuron, nanovector, riluzole

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1. Introduction:

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that currently has no cure and results in a progressive neuropathology leading to death, usually within 3-5 years of diagnosis. Currently, riluzole is the only FDA-approved therapeutic for the treatment of ALS though it enhances lifespan by only a few months. There is a clear need for novel therapeutics that alone, or in combination with riluzole, can improve the lives of those living with ALS. Hydrophilic carbon clusters (HCCs) functionalized through the addition of polyethylene glycol subchains (PEG-HCCs) have recently been shown to: 1) exhibit potent antioxidant capabilities, and 2) possess the ability to serve as carriers for other potentially therapeutic molecules by James Tour, PhD of Rice University. The goal of this proposal was to determine whether functionalized PEG-HCCs could alone, or in combination with riluzole, enhance overall survival as well as preserve both motoneurons and behavioral function in mice that express a human mutated form of superoxide dismutase; a cause of ALS in a percentage of human familial cases of the disease. In this final report that covers the two years of the funding period, we show that PEG-HCCs, under sustained intravenous delivery, produced a significant enhancement in several indices of motor function. We did not, however, observe enhanced motor neuron sparing nor a prolongation of mouse life span.

2. **Keywords:** ALS, nanovectors, riluzole, G93a, locomotor, vascular, antioxidant

3. Accomplishments:

What were the major goals of the project: The goals of this proposal were: 1) to determine whether PEG-HCCs functionalized with antibodies against the transferrin receptor could enhance lifespan, protect motoneurons and enhance motor function when delivered via sustained intravenous route at the first sign of disease. Second aim was to assess whether riluzole, in combination with functionalized PEG-HCCs could enhance the outcomes used in Aim 1.

What was accomplished under these goals?
Due to problems encountered with Jackson Laboratories (described below) that adversely impacted the maintenance of our G93a mouse colony, our tasks for this grant were significantly reduced to assessing the therapeutic potential of PEG-HCCs in treating mice engineered to exhibit ALS-like symptoms. G93a mice were generated and maintained in our colony at UT-Health. Homozygous subjects were grafted with osmotic minipumps filled with either PEG-HCCs (condition A) created and provided by the Tour lab of Rice University or vehicle (Condition B). These osmotic minipumps were implanted between the shoulder blades of the mice with a cannula leading from the minipump to the jugular vein. Thus, the PEG-HCCs or vehicle were allowed to provide a sustained delivery from the time where motor deficits were observed (around week 6) until subjects required euthanasia (based on inability to self-feed). During the treatment period, subjects were monitored for: 1) body weight, 2) balance via Rotorod assessment, 3) a variety of motor indices as determined via Photobeam Activity System assessement, and 4) lifespan. In a separate cohort of mice, lumbar anterior horn motoneurons were counted and compared between PEG-HCC and vehicle-treated subjects following euthanasia at day 110 (post-birth). Our results are as follows:

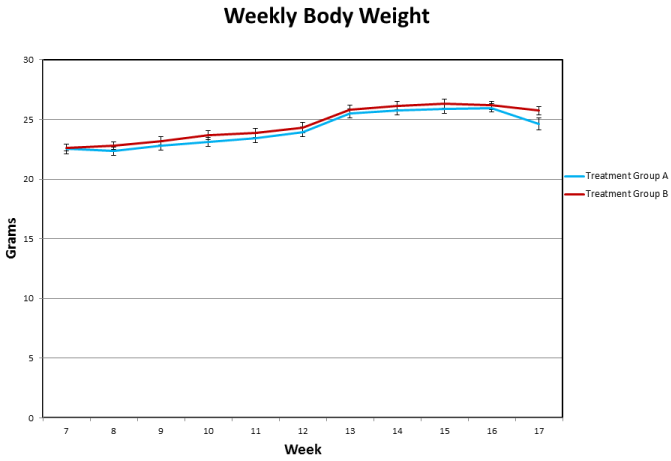


Fig. 1: G93A mutant mice treated with PEG-HCC's do not exhibit any difference in body weight compared to vehicle-treated mice. Blue line=PEG-HCC-treatment group vs. red line (vehicle).

Body weight: We observe no difference in overall body weight throughout the treatment period between PEG-HCC- vs. vehicle treated subjects (Fig. 1).

Rotorod (balance): G93a mice treated with PEG-HCCs showed a strong trend towards increased latency to fall on the rotorod test from week 7-15 with a statistically-significant enhancement detectable at week 14 (Fig. 2).

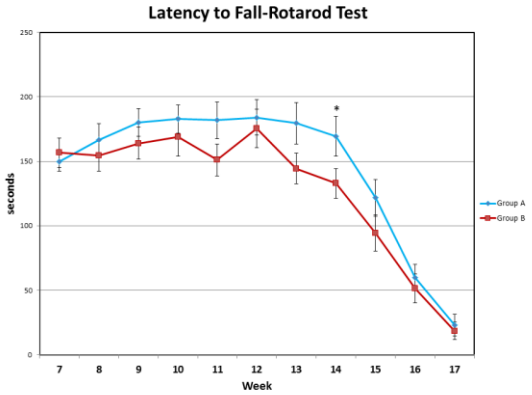


Fig. 2: Rotorod-test. PEG-HCC-treated g93A mice exhibited a statistically-significant improvement on the rotorod test only during week 14 of life (with treatment beginning around day 75-80).

PAS test: Average Distance Walked As with the rotorod test, PEG-HCC-treated mice showed a trend towards greater distances traveled compared to vehicle-treated subjects from weeks 7-16 with the enhancement becoming significant at week 15 (Fig. 3).

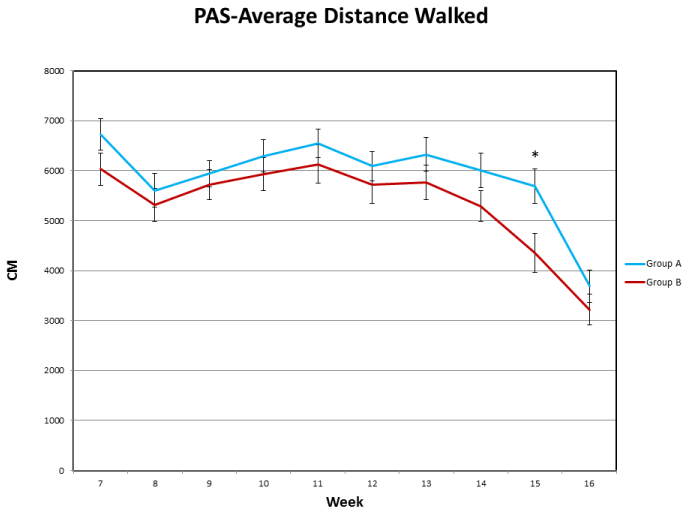


Fig. 3: Average distance walked: As with rotorod assessment above, PEG-HCC-treated g93a mice exhibited a significant enhancement in overall distance walked compared to vehicle-treated controls when assessed on week 15.

PAS test: Average speed

While placed in the PAS device, mice are allowed to ambulate freely for the entire period. The device measures the overall average speed of these movements. We observe a significant difference in speed favoring PEG-HCC-treated mice at week 15 compared with vehicle-treated (Fig. 4).

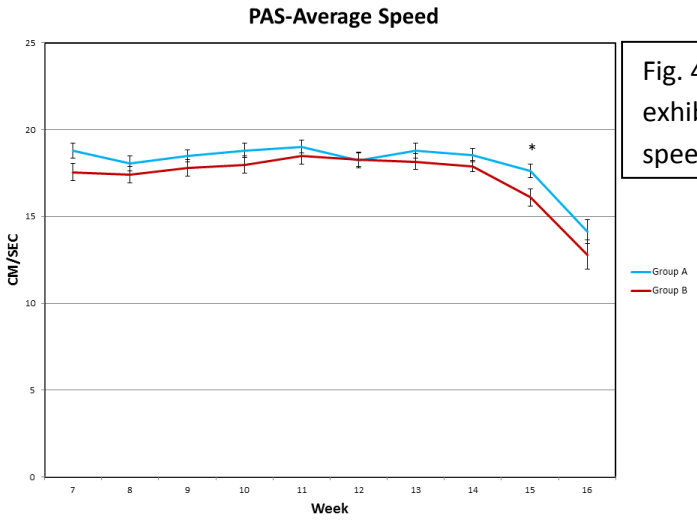
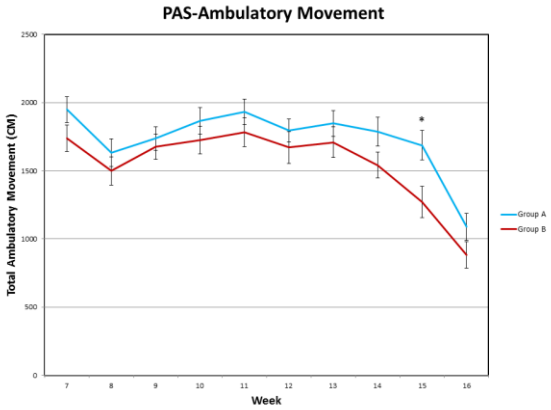


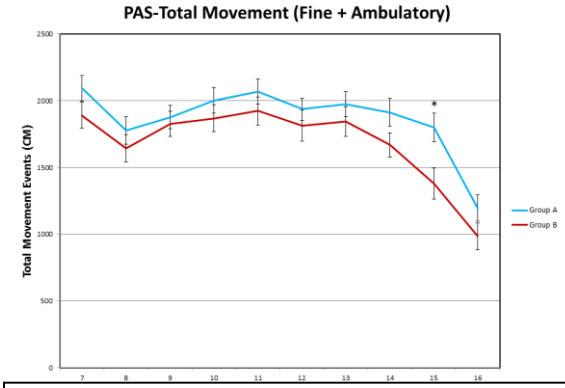
Fig. 4: Average speed: Again, PEG-HCC-treated subjects exhibited a significant improvement in average locomotor speed only at week 15 compared to vehicle-treated mice.

PAS-Ambulatory Movement

Ambulatory movement represents the total number of gross movements that the computer recognizes as longitudinal movement. Again, as with other previously described metrices, PEG-HCC-treated subjects exhibit a trend towards enhanced activity throughout the post-graft period while showing significantly enhanced ambulatory movement at week 15 post-grafting (Fig. 5).

Fig. 5: Ambulatory Movement: PEG-HCC-treated mice show a statistically-greater number of movements as part of ambulatory behavior compared to vehicle-treated subjects at week 15.





PAS-Total Movement (fine + ambulatory)

The PAS can also combine both fine and total ambulatory movements into one matrix. As you can see, PEG-HCC treated subjects again show a trend towards elevated movement with statistical significance achieved compared to vehicle-treated at week 15 (Fig. 6). Also similar to the other

Fig. 6: Total movement (both fine and ambulatory): Perhaps a better assessment of overall #'s of various types of movements, we again see a statistical-improvement in movement in PEG-HCC-treated mice vs. vehicle at week 15.

outcomes, both PEG-HCC and vehicle-treated subjects both undergo time-dependent loss of function regardless of therapeutic.

PEG-HCC vs. Vehicle-treatment: Preservation of spinal lumbar motoneurons

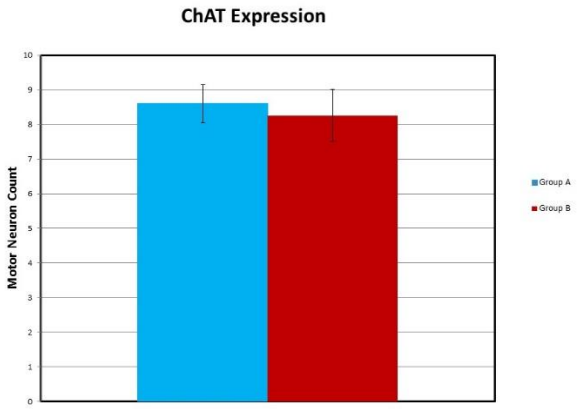


Fig. 7: Motoneuron survival: We do not detect any difference in numbers of ChAT+ motoneurons in the lumbar spinal cords of PEG-HCC vs. vehicle-treated subjects.

Due to the established antioxidant properties of PEG-HCCs, we hypothesized that sustained IV treatment would promote sparing of motoneurons at risk for degeneration in ALS as well as within the G93A mouse model. A separate cohort of mice were generated for the purpose of assessing motoneuron survival at day 110 in PEG-HCC vs vehicle-treated mice. Those motoneuron counts are based on immunohistochemical localization of a neuronal marker (choline acetyltransferase) and quantified image analysis. Motoneurons were counted in the anterior horn region of the lumbar spinal cord (both sides, then averaged). We do not detect a statistical difference in surviving motoneurons between PEG-HCC and vehicle-treated subjects (Fig. 7).

Lifespan: IV-delivered PEG-HCC, initiated when motor deficits detected, did not produce an improvement in overall subject lifespan compared to vehicle-treated controls. Based on previous studies that demonstrated the potent anti-oxidant properties of PEG-HCC's, we hypothesized that a sustained treatment regimen of PEG-HCC's would result in an enhancement of overall lifespan in the g93a ALS mouse compared to vehicle-treated controls. PEG-HCCs or vehicle-treatment was initiated around day 75-80. Using Meier-Kaplan metrics, we did not observe any PEG-HCC-dependent benefits in sustaining lifespan in these mutant mice (Fig. 8).

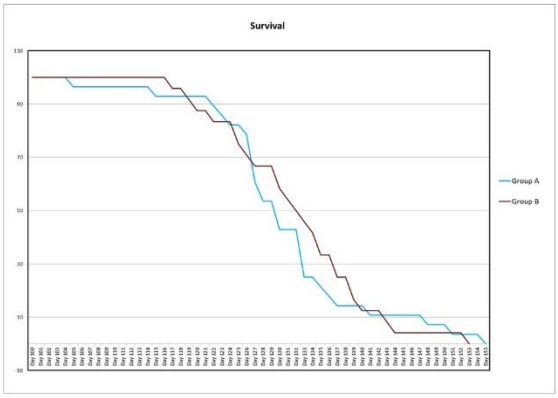


Fig. 8: Lifespan. We do not observe any preservation of overall lifespan resulting from PEG-HCC treatment when compared with vehicle in g93A ALS mice.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?
As of yet, these data have not been introduced to the general research community.

What do you plan to do during the next reporting period to accomplish these goals?
This is a final report.

4. Impact

What was the impact on the development of the principle disciplines of the project?
Our observed results suggest that while PEG-HCCs may have beneficial properties based on transient improvements in several aspects of motor function. These improvements, however, were not sustained and did not further translate into enhanced lifespan nor preserved motor neurons. It is conceivable that these transient improvements might be capitalized to further improve outcome either via combination with other therapeutics (one such drug, licofelone, has shown great promise as a therapeutic in our lab) or when administered via a different treatment route (i.e., subcutaneous vs. long-term, sustained IV treatment). Due to

adverse issues associated with year 1 and the colony, were only able to assess PEG-HCC vs vehicle treatment. However, this comparison suggests a beneficial role of the bare PEG-HCC nanovector without further functionalization. It is our hope that we may continue developing functionalized PEG-HCCs with Dr.Tour's group beyond this particular funding mechanism in order to better develop PEG-HCCs as a novel and effective treatment for ALS.

What was the impact on other disciplines?
The potent anti-inflammatory properties of PEG-HCCs represent a novel potential therapeutic for spinal cord- and traumatic brain injury studies, another area of research interest in my laboratory. The PI, Dr. Grill, helped establish an ongoing collaboration between the Tour lab of Rice University and the Herrera Lab of UT-Health to begin assessing the potential therapeutic efficacy of PEG-HCC treatment in rodent models of spinal cord injury.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

5. Changes/Problems

Changes in approach and reasons for change.
As described in a previous report, we encountered two problems with animal purchases through Jackson Laboratories. On two consecutive occasions, they provided us with the wrong animals which lead to our generating animals that lacked the human mutated SOD 1 gene. While Jackson Laboratories agreed to replace the animals originally purchased, we were unable to get them to recompense our loss of time and effort. As these studies require a significant amount of time to set up (purchase, breeding, behavioral assessments, etc), this resulted in a significant setback for our originally designed studies. Despite this, we have completed the behavioral assessment of PEG-HCCs vs. vehicle-filled osmotic minipumps; further allowing us to perform our planned assessment of the potential for PEG-HCC to preserve motor neuron survival as well as lifespan.

Actual or anticipated problems or delays and actions or plans to resolve them
See immediately above.

Changes that had a significant impact on expenditures
We were left with finances of which we have requested a small amount to use towards personnel effort applied for the described ongoing cell counts; now completed.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
None to report.

Significant changes in use or care of human subjects
None to report.

Significant changes in use or care of vertebrate animals
none to report

Significant changes in use of biohazards and/or select agents
none to report

6. Products

publications, conference papers, and presentations
None to report. .

websites or other internet sites
None to report

Technologies or techniques
None to report.

Inventions, patent applications and or licenses
Data did not support further pursuit of PEG-HCCs as a treatment for ALS.

Other Products:
None to report

7. Participants and Other Collaborating Organizations:

What individuals have worked on the project?
James Tour, PhD
Co-PI
0.25 Summer months
Dr. Tour developed and provided the PEG-HCCs as needed throughout the projet
Funding for this effort provided through this DOD-sponsored grant.

Has there been a change in the active other support of trhe PD/PI or senior/key personnel since the last reporting period?
None to report.

What other organizations were involved as partners?
None to report.

8. Special Reporting Requirements

Collaborative Awards
Dr. Tour's group has been responsible for providing Dr. Grill's group with the PEG-HCCs throughout year 1 and 2 of the project.

Quad Charts
See attached

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PI: Raymond J. Grill **Org:** UT-Health **Award Amount:** $259,045

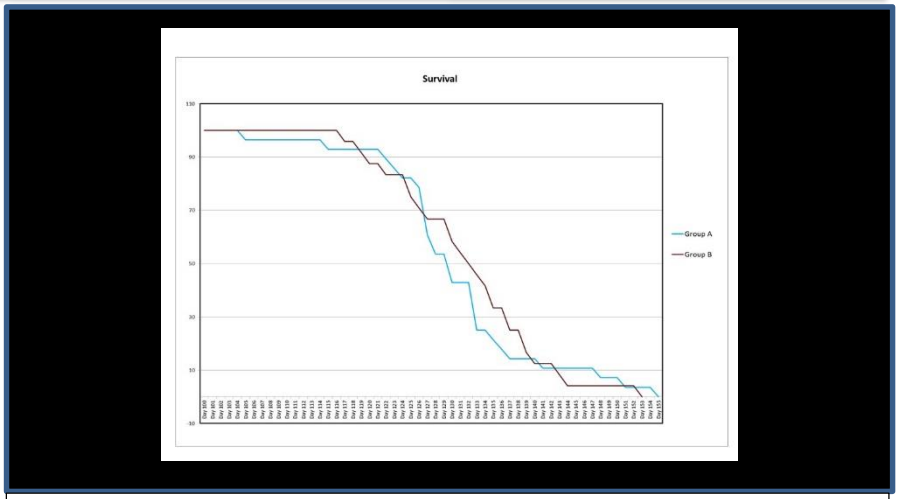
Study/Product Aim(s)

- **Specific Aim 1:** Determine whether PEG-HCCs functionalized with antibodies against the transferrin receptor could enhance lifespan, protect motoneurons and enhance motor function when delivered via sustained intravenous route.

- **Specific Aim 2:** Determine whether riluzole, in combination with functionalized PEG-HCCs could enhance the outcomes employed in Aim 1.

Approach

Determine whether IV-delivered :PEG-HCCs can improve outcome when administered in a mouse model of amyotrophic lateral sclerosis.



Accomplishment: PEG-HCCs delivered IV promote did not promote an enhancement in overall lifespan as originally hypothesized.

Timeline and Cost

Activities CY	12/14	13/15
Aim 1: Performed assessment of PEG-HCCs on behavioral outcome measures using G93A mouse model of ALS		
Performed quantification of behavioral analyses; neuronal counts and survival analysis completed		
Estimated Budget ($K)	**$259,045**	

Updated:

Goals/Milestones (Example)

CY14/15 Goal – Initiate Aim 1 of project

☑ Rebuild the G93A colony

☐ generate sufficient numbers of mice to: 1) evalulate PEG-HCC vs ☐vehicle treatment with behavioral outcomes and 2) perform neuronal survival analyses (collection at day 110).

☐ Perform quantification of all animal behavioral data

☐ Perform quantification of all neuronal cell counts: 1) histopathology, 2) immunolabeling, 3) image acquisition, 4) image analysis/quantification

☐survival analysis and motoneuron counts are now complete. This should be complete by 01/31/2015.