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TITLE: Development and Use of Small-Molecule Activators of Neuroproteasomes to Treat ALS

PRINCIPAL INVESTIGATOR: Kapil Ramachandran

CONTRACTING ORGANIZATION: Columbia University, New York, NY

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Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)

Kapil Ramachandran

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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630 W 168TH ST FL 4
NEW YORK NY 10032-3725

8. PERFORMING ORGANIZATION REPORT NUMBER

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: To stay healthy and long-lived, all cells must maintain an intricate balance between protein synthesis, maintenance, and degradation. Dysfunctions in these mechanisms of protein homeostasis can lead to protein aggregation and cell death. Protein aggregation and death induced by proteostasis dysfunction is a common theme among almost all neurodegenerative diseases, including Amyotrophic Lateral Sclerosis (ALS). The dogma of selective protein degradation is that intracellular proteasomes degrade ubiquitin-tagged proteins tagged and destined for degradation. However, our laboratory has discovered a new node of protein homeostasis - a novel proteasome that is specific to neurons and does not require ubiquitylation of substrates. Our extensive previous findings reveal that these neuroproteasomes are embedded in neuronal plasma membranes and exposed to the extracellular space. Neuronal-specific plasma membrane-bound proteasomes (NMPs) represent an entirely new strategy for degrading proteins in the brain. Previous studies had alluded to a curious and important role for ubiquitin-independent proteasomes in mouse models of ALS as well as in patient samples. However, because the discovery of neuroproteasomes was so recent, it was not clear which species of proteasomes were dysregulated in ALS – the NMP neuroproteasomes or the canonical cytosolic proteasomes. Much to our surprise, when we studies two mouse models of ALS, as well as cellular models of ALS, we found that the neuroproteasomes were the only species of proteasome which were strongly dysregulated. Using NMP-specific inhibitors which we previously described and validated, we find that inhibition of NMP function causes protein aggregation consistent with ALS pathology. We also found that genetic risk factors for ALS act to inhibit neuroproteasome activity. Our collaborators independently were working on small molecules to boost ubiquitin-independent proteasome activity and required a physiological system in which to test these proteasome activators. Therefore, we considered that elevating proteostasis by activating NMPs would potentially delay the onset of or reduce the severity of ALS symptoms.

15. SUBJECT TERMS:
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	**14**	**19b. TELEPHONE NUMBER** *(include area code)*

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1. INTRODUCTION:

The goal of this project is to generate and test cell-impermeable 20S proteasome stimulators. The impermeable stimulators will be able to interact with the neuroproteasome and not the 20S proteasome within the cells. These new tools will help us to evaluate the role of the neuroproteasome.

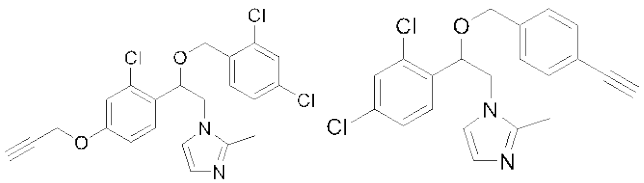
2. KEYWORDS:

Proteasome, Neuroproteasome, stimulator, cell-impermeable

3. ACCOMPLISHMENTS:

What were the major goals of the project?
The major goals in the first year was to 1) Synthesize analogs of miconazole to increase the potency of these compounds and 2) Convert these analogs to cell-impermeable analogs to specifically target the neuroproteasome.

What was accomplished under these goals?
We accomplished the entirety of the subtasks under Major Task 1, synthesizing novel analogs of the proteasome activators. The specific objectives were accomplished across the Trader and Ramachandran labs. Subtasks 1-3 were accomplished and we now have analogs with EC50s in the micromolar range as we promised. We then accomplished the goal of Major task 2, generating cell impermeable analogs as promised. We successfully achieved the milestones of Major task 1 and Major task 2, but saw some cell toxicity due to the linker choice that rendered the compounds cell impermeable. We are undertaking some minor optimization to this linker choice and should have final compounds to transition to in vivo studies shortly.



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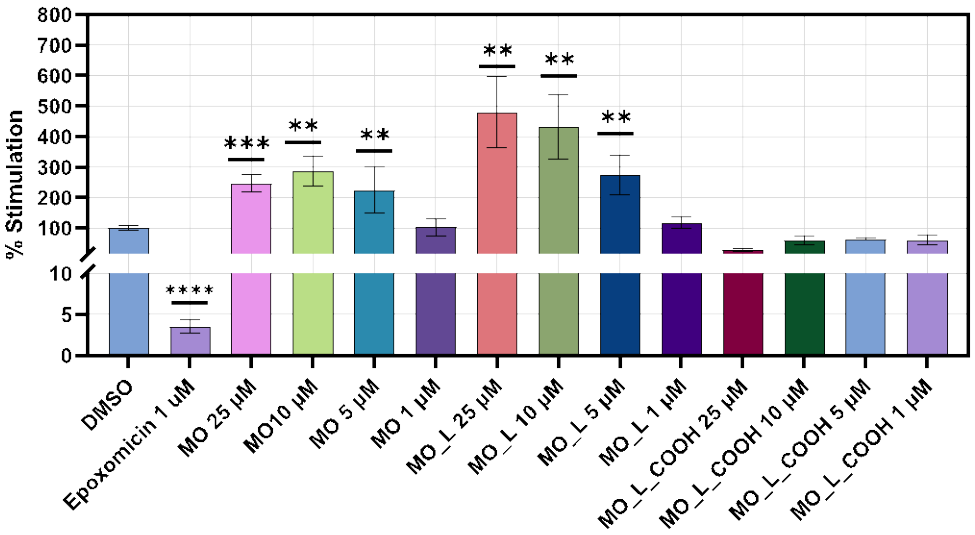
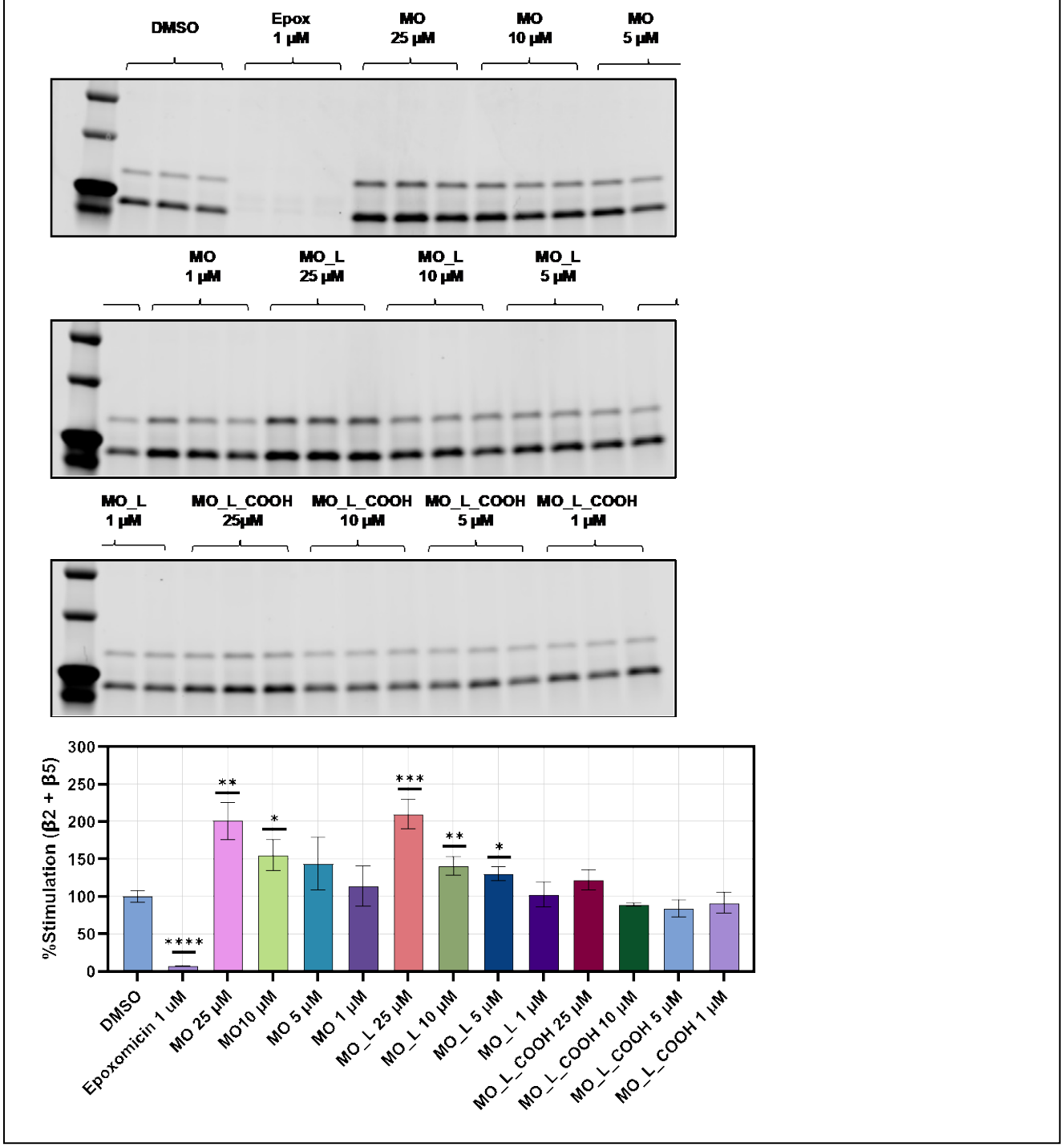
Molecular Weight: 449,7560

Exact Mass: 384,0796
Molecular Weight: 385,2880

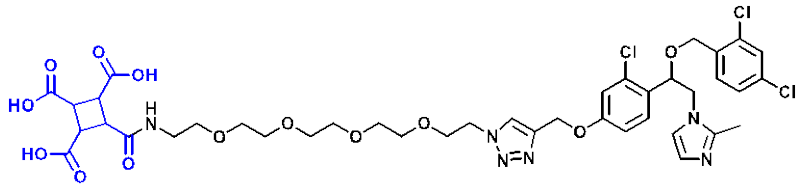


Rh-based kinetic assay with 20S proteasome for 60 min. at 4 concentrations (25, 10, 5 and 1 µM)

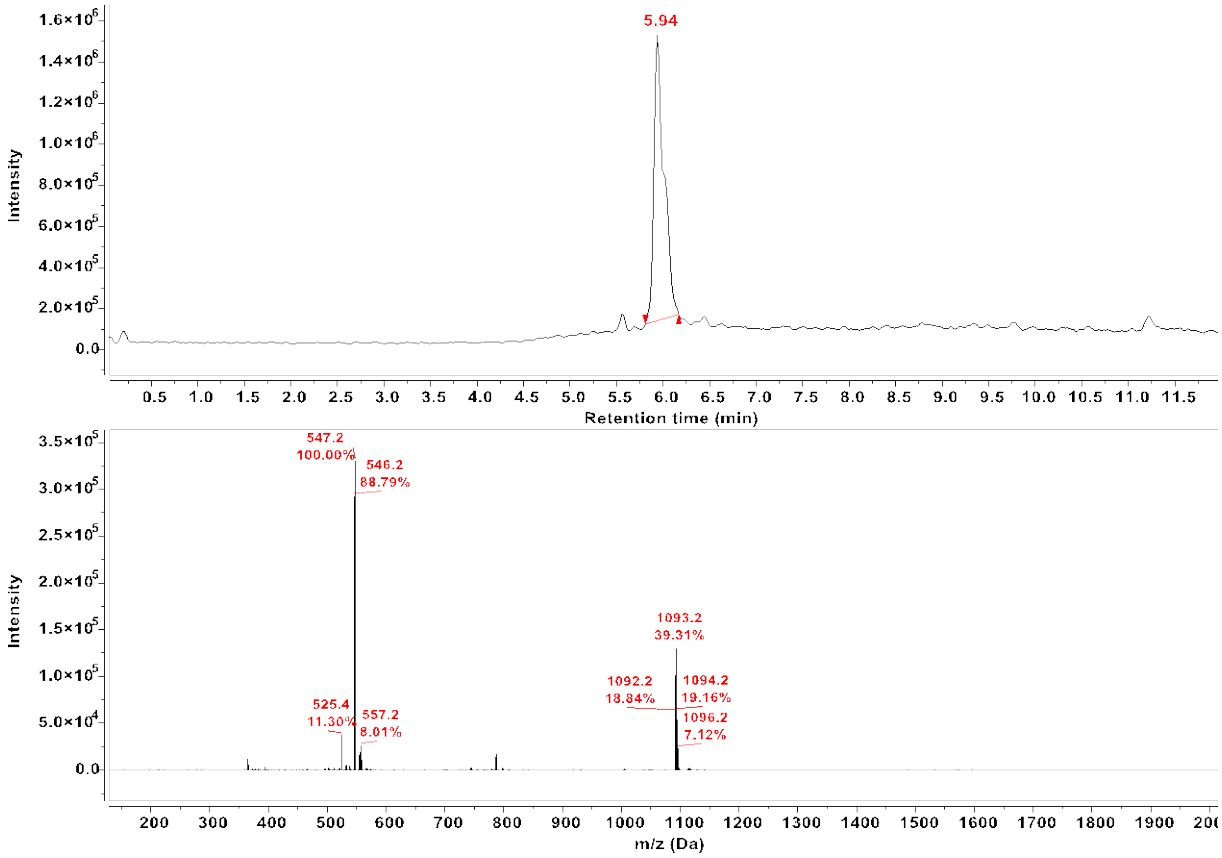
Here, we demonstrate the two new molecules generated and then SAR by modifying varying rings and creating new analogs. We chose to stick with Mo-L which increased potency both in vitro (above) and in cellulo (below).


DMSO
Epox 1 µM
MO 25 µM
MO 10 µM
MO 5 µM
MO 1 µM
MO_L 25 µM
MO_L 10 µM
MO_L 5 µM
MO_L 1 µM
MO_L_COOH 25µM
MO_L_COOH 10 µM
MO_L_COOH 5 µM
MO_L_COOH 1 µM
%Stimulation (β2 + β5)
300
250
200
150
100
50
0
****
**
*
***
**
*
DMSO
Epoxomicin 1 uM
MO 25 µM
MO10 µM
MO 5 µM
MO 1 µM
MO_L 25 µM
MO_L 10 µM
MO_L 5 µM
MO_L 1 µM
MO_L_COOH 25 µM
MO_L_COOH 10 µM
MO_L_COOH 5 µM
MO_L_COOH 1 µM

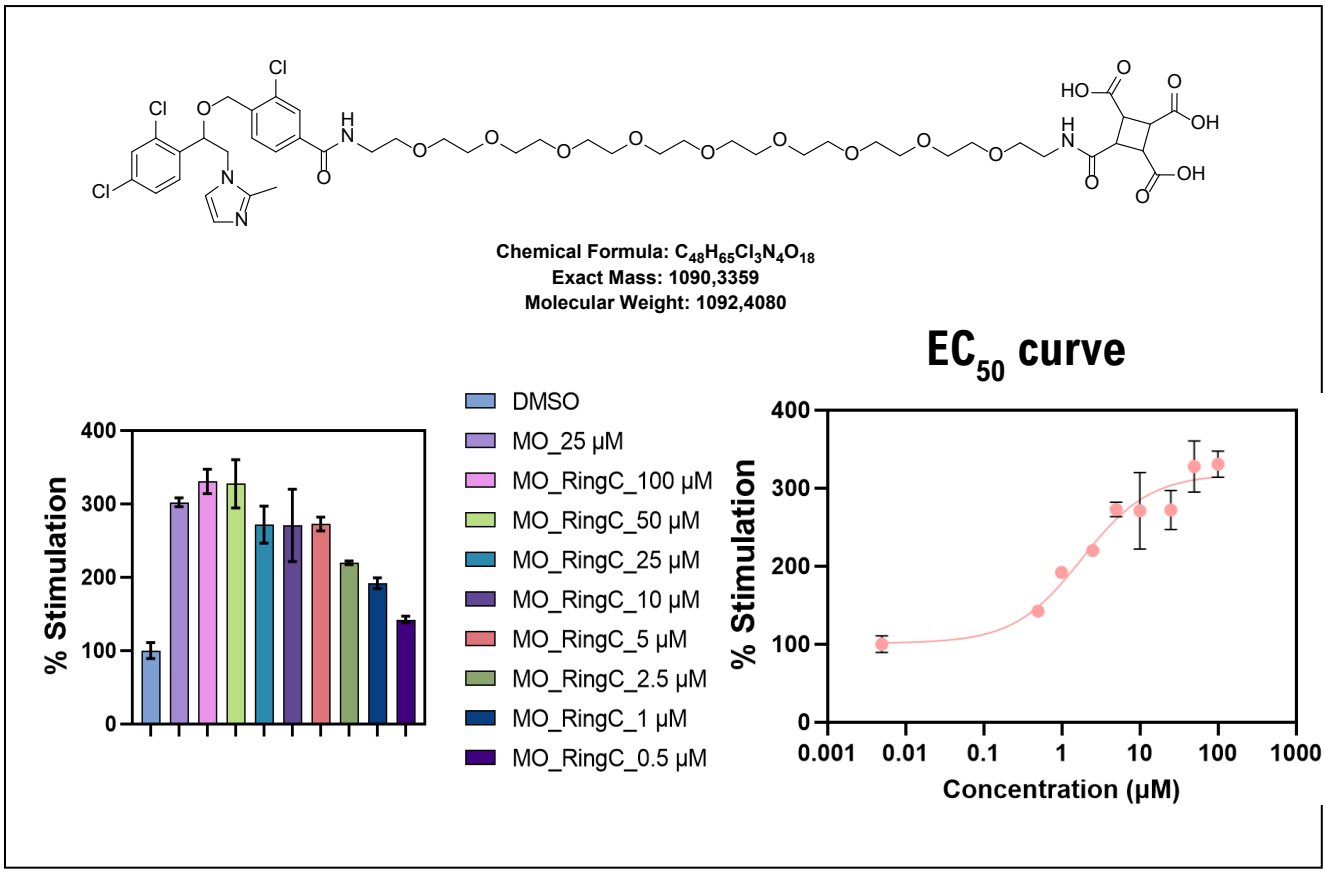
Stimulator 1



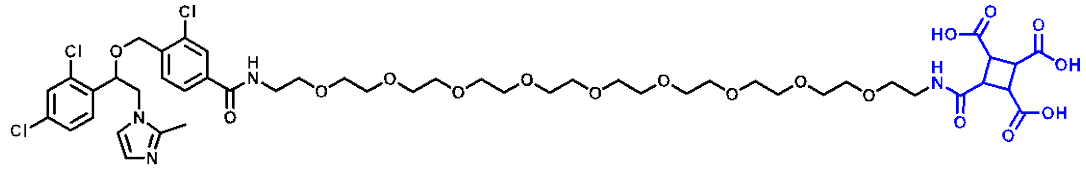
Exact Mass: 924,2267
Molecular Weight: 926,1950



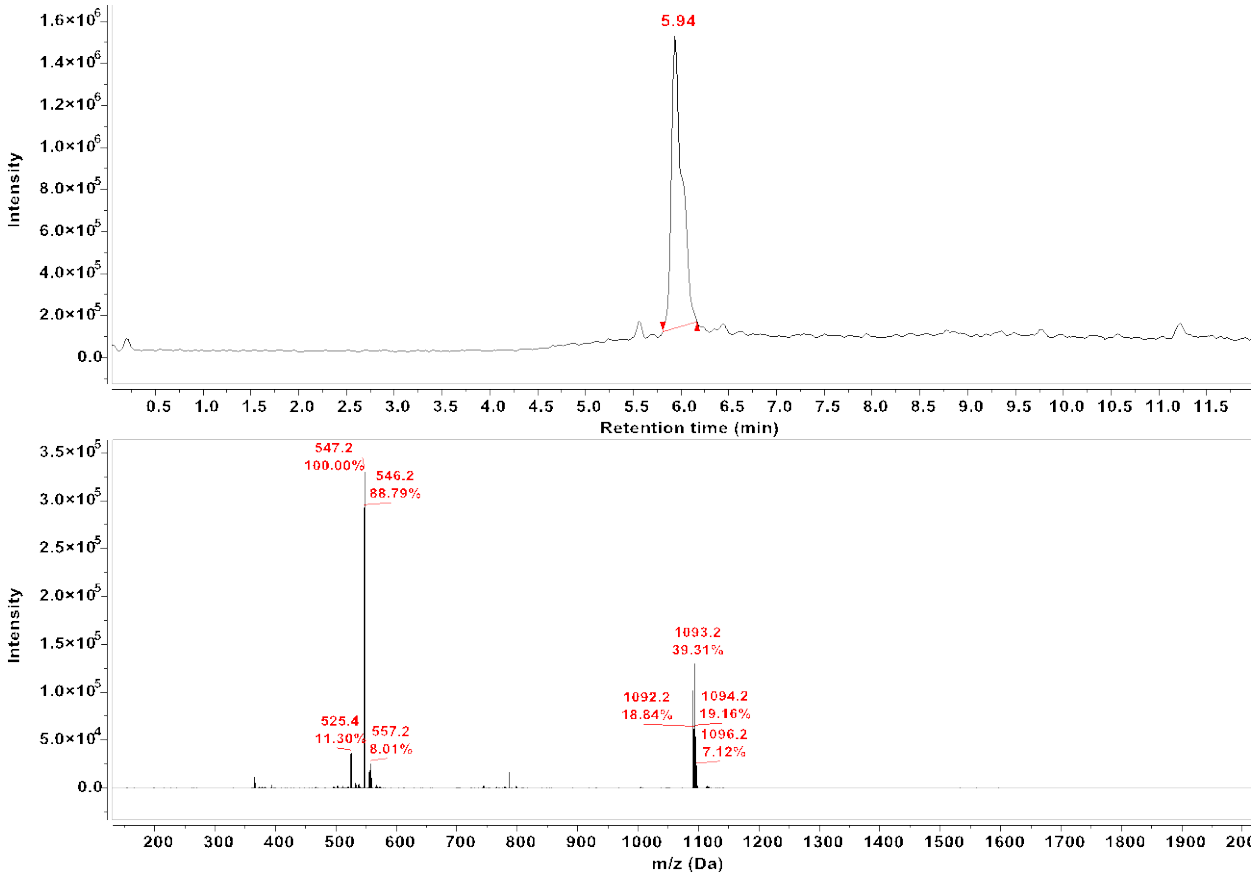
We then generated two different stimualtor molecules, the first shown here and the biochemical and cellular EC50s shown below. This was incredibly challenging and required extensive optimization of linkers, peg lengths, and solubility. This was the first set of molecules that gave us robust stimulation in neurons but did not induce stimulation in HEK cells, suggesting they were cell impermeable. This was also the case for stimualtor 2 on the following page.


Cl
Cl
Cl
O
N
N
H
N
O
O
O
O
O
O
O
O
O
O
O
HO
O
OH
OH
O
O
H
N
Chemical Formula: C48H65Cl3N4O18
Exact Mass: 1090,3359
Molecular Weight: 1092,4080
% Stimulation
400
300
200
100
0
DMSO
MO_25 µM
MO_RingC_100 µM
MO_RingC_50 µM
MO_RingC_25 µM
MO_RingC_10 µM
MO_RingC_5 µM
MO_RingC_2.5 µM
MO_RingC_1 µM
MO_RingC_0.5 µM
EC50 curve
% Stimulation
400
300
200
100
0
0.001
0.01
0.1
1
10
100
1000
Concentration (µM)

Stimulator 2



Exact Mass: 1090,34
Molecular Weight: 1092,41



However, to our surprise, cell-impermeable proteasome activators were extremely detrimental to neuronal health compared to cell-permeable proteasome activators. This may reflect interesting biology that overstimulating the catalytic activity of the membrane proteasome may lead to detrimental phenotypes, but this could also be due to the specific linker we used.

By dropping the concentration 2 fold, we have narrowed in on a specific concentration of our activators that functions to activate the neuroproteasome without affecting cell health. Additionally, another linker design will be undertaken that prevents cell permeability and may be able to limit any unwanted toxicity. Once we have final versions of these molecules, we anticipate a seamless set of tests in vivo.

Describe opportunities for training and professional development
One postdoctoral fellow and one technician worked one-on-one with Dr. Ramachandran and one graduate student worked one-on-one with Dr. Trader. Dr. Trader's graduate student was able to recently present stimulation data at the Purdue-Norte Dame Medicinal Chemistry Symposium (Oct. 2022). Dr. Ramachandran's technician presented work at the CSHL neurodegeneration meeting as did Dr. Ramachandran.

How were the results disseminated to communities of interest?
Dr. Ramachandran's technician presented work at the CSHL neurodegeneration meeting as did Dr. Ramachandran. Dr. Ramachandran's lab also posted a preprint on related work.

What do you plan to do during the next reporting period to accomplish the goals?
As we promised, we seek to finish testing in both primary neurons as well as testing these compounds in vivo by cannulating animals. We will also be publishing the manuscript with these findings. The Trader lab will continue to supply stimulators and modify the linkers or stimulators that could limit unwanted neuron death.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Protein degradation through the proteasome is the dominant mechanism for how proteins are turned over in all cells. We found a completely new form of the proteasome in neurons which are critical to help neurons protect themselves against proteotoxic stress. Currently, no good methods exist to stimulate the proteasome, or specifically, the neuroproteasome. Therefore, definitionally, there is no way to determine whether stimulating their activity is sufficient to clear the neuroproteasome. These advances define progress in this field, with broad ranging implications to understand how to enhance protein clearance in neurodegenerative diseases like ALS.

What was the impact on other disciplines?
Beyond neurodegeneration, these advances will define how to determine the function of proteasome activation in any cell type or tissue. We hope from these studies we will be able to design neuroproteasome specific inhibitors, stimulators, and probes that we can disseminate to the larger chemical biology/neuroscience field.

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:

There have not been significant ranges in approach, but building cell-impermeable activators that are not toxic is a challenge. We have gotten the cell impermeability down, but the toxicity is still a challenge. We are hopeful that by using more potent activators, we will be able to use less compound and drop toxicity.

Changes in approach and reasons for change
None

Actual or anticipated problems or delays and actions or plans to resolve them
The covid-19 crisis has caused major delays in shipping and reagent availability, which has caused some delays. These are completely out of anyone's control, but has set us back. We hope now with stimulators in hand we can move more rapidly with our neuroproteasome studies.

Changes that had a significant impact on expenditures
There were significant delays in hiring, but now we are staffed up.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects
None

Significant changes in use or care of vertebrate animals
None

Significant changes in use of biohazards and/or select agents
None

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

1. He, HH*, Ahsan A, Bera R, McLain N, Faulkner R, **Ramachandran KV**, Margolis SS, Cline HT*. Neuronal membrane proteasomes regulate neuronal circuit activity in vivo and are required for learning-induced behavioral plasticity. 2022, PNAS, *in press*
2. Paradise V, Sabu M, Bafia J, Sharif NA, Nguyen C, Dhanraj Mukim R, Wang X, Fu J, Ndubisi J, Maldonado G, Strickland M, Figueroa H, Almeida D, Hyman B, Holtzman DM, Nuriel T, **Ramachandran KV***. ApoE isoforms differentially regulate neuronal membrane proteasomes to shift the threshold for pathological aggregation of endogenous Tau, in review, Science 2022 (on Biorxiv)

Books or other non-periodical, one-time publications.
N/A

Other publications, conference papers and presentations.
CSHL Neurodegeneration meeting, Ramachandran and colleagues

- **Website(s) or other Internet site(s)**

- **Technologies or techniques**

- **Inventions, patent applications, and/or licenses**
  - Patent application from Ramachandran and Columbia on transgenic mice to purify the neuroproteasome

- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Kapil Ramachandran
Project Role: Principal Investigator
Researcher Identifier: https://orcid.org/0000-0002-4944-3553
Nearest person month worked: 1.12 CM
Contribution to Project:

Name: Darci Trader
Project Role: Co-Investigator
Researcher Identifier: 0000-0002-0607-1243
Nearest person month worked: 1
Contribution to Project: Supervised student being supported on grant to synthesize molecules and coordinated meetings with Ramachandran lab.

Name: Andres Salazar
Project Role: Graduate Student
Researcher Identifier: N/A
Nearest person month worked: 6 months
Contribution to Project: Synthesized and tested 4 molecules for proteasome stimulation in biochemical assay and cell-based assay.

Name: Rijuta Dhanraj Mukim
Project Role: Technician B
Researcher Identifier: N/A
Nearest person month worked: 4.87 CM
Contribution to Project:

Name: Nyle Sharif
Project Role: Technician B
Researcher Identifier: N/A
Nearest person month worked: 10.18 CM
Contribution to Project:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

PI: Kapil Ramachandran, Ph.D.
Completed research supports since the last reporting
Title: Contributions and mechanisms of neuroproteasomes to brain aging
Effort:0 CM
Supporting Agency: Glenn Foundation for Medical Research
Performance Period: 10/1/19 – 9/30/21
Funding Amount: $48,500
Project Goals: To determine how neuroproteasomes change across the aging brain
Specific Aims: 1) Identify the profile of neuroproteasome function over the course of mouse aging in the brain and 2) Prematurely inhibit neuroproteasomes to determine whether aging is accelerated
Overlap: None

New research supports since the last reporting
Title: Contributions of neuroproteasome-mediated degradation to Alzheimer's Disease
Effort: 0.72 CM
Supporting Agency: Eli Lilly & Co. Proejct Number: ELCO CU21-3489
Grants Officer: Cindy Healey <chealey@lilly.com>
Performance Period: 07/01/2022-06/30/2024
Funding Amount: $318,000
Project Goals: Determine whether ApoE isoforms differentially bind to and activate neuroproteasomes using proteomic apporaches developed at Lilly.
Overlap: None

Title: Mechanisms of co-translational degradation through neuronal membrane proteasomes
Effort: 0.12 CM
Supporting Agency: Fidelity Biosciences Research Initiative (FBRI) Project Number: FBRI CU22-0128
Grant Officer: Matthew Boersma mboersma@fbri.com
Performance Period: 01/01/2022-12/31/2022
Funding Amount: $35,041
Project Goals: Study the mechanisms by which neuroproteasomes recognize and degrade substrates independent of ubiquitylation
Overlap: None

Title: Establishing neuroproteasomes as a new mechanism of neuronal proeostasis and a core dysregulated mechanism of the aging brain
Effort: 1.20 CM
Supporting Agency: Norn Group, INC (the Longevity Impetus Grants)
Grants Officer: Impetus Grants Team: team@impetusgrants.com
Performance Period: 11/01/2021-04/30/2023
Funding Amount: $220,000
Project Goals: to delineate the contribution of NMPs to aging and to determine if inhibition induces a reduction in lifespan
Overlap: None

Co-Investigator: Darci Trader, PhD
Completed research supports since the last reporting
Tite: Development Of Activity-Based Chemical Reporters To Differentiate Proteasome Isoforms In Cells
Supporting Agency: Phs-Nih Nat Inst Of General Medical Sci
PI: Trader, Darci J
Time Commitment: Academic Month: 1.80 Summer Month: 0.60
Name and Address for Grant Officer: Jiong Yang, jiong.yang@nih.gov
Performance Period: 09/01/2019 - 08/31/2020
Level of Funding: $426,250
Description of Major Goal: The Major Goals Development of probes that can be used in cells to differentiate the different activities of the various proteasome isoforms.
List of Specific Aims: 20S activity probe and 26S activity probe
Indicate overlap if any: None

New research supports since the last reporting
None

What other organizations were involved as partners?
Organization Name: Purdue University
Location of Organization: 155 South Grant Street, West Lafayette, Indiana 47907-2114
Partner's contribution to the project
- Collaboration (e.g., partner's staff work with project staff on the project). Dr Darci Trader in Purdue University served as a co-Investigator in this project via subaward.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: