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TITLE: Rapid Flow Cytometry Screen for Identifying Novel ALS Drug Leads

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CONTRACTING ORGANIZATION: The University of Wollongong
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14. ABSTRACT

Amyotrophic lateral sclerosis (ALS) arises due to proteins misfolding inside motor neuron cells, leading to toxicity, cell death and loss of motor function. TDP-43 is an important protein known to misfold, leading to its clumping or "aggregating" in the cytoplasm to form large insoluble deposits (inclusions), which are associated with cell death and thereby causally associated with ALS pathology. This project uses a motor neuron cell model in which fluorescently-tagged TDP-43 forms cytoplasmic inclusions, in a high-throughput drug screen of thousands of chemicals to find potential drugs to treat ALS patients. A small number of "hits" have already been identified and will be screened in animal models of ALS, to identify a therapeutic to treat ALS patients.

15. SUBJECT TERMS					
motor neuron cell model; TDP-43 inclusions; flow cytometry; drug screen; chemical libraries; natural extracts; drug leads					
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The accumulation and aggregation of TDP-43 in the cytoplasm of cells is strongly associated with motor neurone loss in ALS. Owing to delays caused by covid, two no-cost extensions were approved during this grant. By September 2022, using an established flow cytometry assay for cytoplasmic TDP43 inclusions adapted to a high-throughput format, we had completed screening of 59,000 small molecules/fractions. From Sept 2022 – August 2023, animal testing of hit compounds was subsequently performed by collaborators in zebrafish and mouse models of ALS.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

TDP-43, ALS, Flow Cytometry, Proteostasis, Chemical Libraries

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals of this project were:

Aim 1. Screen chemical libraries for small molecules that reduce the burden of cytoplasmic TDP-43 inclusions and preserve motor neuron cell viability.

Aim 2. In ALS animal models, test leads identified in Aim 1 for their ability to reduce ALS pathology and improve motor function.

Both Aims are now 100% complete (completion dates: Aim 1 - 14 September 2022; Aim 2 - 14 August 2023).

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Aim 1

We completed screening of 3,517 FDA compounds and 48,732 NB fractions. In August 2022, we passed a list of 10 "hit" FDA (purified) compounds to our collaborators for testing in zebrafish and mouse models of ALS.

In our screening of NatureBank (NB) fractions, we identified three fractions that reduced the numbers of TDP-43 inclusions by ~ 70%. These top three hit fractions were freshly re-prepared from the original source organisms by a chemist at the Griffith Research Institute for Drug Discovery (GRIDD; Griffith University, Qld) and provided to us for additional testing. We identified an active HPLC fraction prepared from a marine sponge that in work beyond the scope of this grant will be the subject of further study - funding was obtained in 2023 from Motor Neuron Disease Research Australia to specifically support this work.

Aim 2

Our collaborators Dr Angela Laird (Macquarie University) and Dr Adam Walker (University of Queensland) were provided with a list of 10 hit compounds derived from the FDA-approved library screening. Dr Laird tested 9 FDA-approved compounds in a zebrafish model of ALS (one drug was excluded owing to toxicity). Owing to the much more labour-intensive nature of the mouse model, on the basis of known toxicity, pharmacokinetics and blood-brain-barrier permeability, Dr Walker selected 3 of these compounds for short-term testing in a mouse model of ALS, and then proceeded with a long-term study of one selected compound (IKK-16). **We were granted a (second) no-cost extension to this grant on 23 September 2022, to provide sufficient time for these animal testing experiments to be completed.**

Two of the nine drugs tested in the zebrafish model of ALS (INO-1001 & APTSTAT3-9R) significantly improved swimming distance and one of these (INO-1001) was shown to significantly reduce the levels of FUS detected in the fish. In the mouse model of ALS, the short-term treatment study of IKK-16 showed modest but significant neuroprotective effects, with inhibition of insoluble p-TDP-43 levels and restoration of *Eaat2* gene expression. However, the long-term treatment of IKK-16 did not significantly prevent pathological progression or improve the lifespan of treated mice. **For more detailed descriptions of results, see attached Appendices.**

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional *skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.*

Dr Nicholas Geraghty (post-doctoral scientist) - This project has enhanced Dr Geraghty's skills in flow cytometry, by working closely with Life Technologies in the optimization of the high-throughput flow cytometry method. Furthermore, this project has helped Dr Geraghty to develop further skills in mammalian cell culture and transfection, and in the analysis of large data sets. Finally, Dr Geraghty has recently trained in *C. elegans* techniques, which will aid his future career development.

Ms Nicole Miles (current PhD student) - Since starting her PhD, Miss Miles has been developing skills in cell culture, transfected cell models of disease, flow cytometry and data analysis, together with improving her writing skills by preparing written research reports as a compulsory part of her PhD. Miss Miles has continued to gain further experience in high resolutions light microscopy, and has actively helped validate the effects of compounds identified in Dr Geraghty's drug screening experiments.

Ms Julia Kam (research assistant, under supervision of Dr Angela Laird) - This project has provided Ms Kam with the opportunity to continue to develop her skills relating to zebrafish research and in particular to develop new skills in western blot analysis, microscopy and data analysis. As a result of acquiring these skills, she has now taken steps towards enrolling in a PhD degree.

Dr Wei Luan (post-doctoral scientist, under supervision of Dr Adam Walker) – This project has allowed Dr Luan to develop skills in small molecule pre-clinical testing in mouse models of ALS, and to develop project planning experience related to drug testing for ALS. These skills will greatly enhance Dr Luan's career development in ALS research.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Oral presentations by Dr Nicholas Geraghty at the Australia & New Zealand MND Research Symposium (Wollongong, Nov 17-18) and at the Molecular Horizons Symposium (UOW, Nov 16). Nicole Miles also presented a related poster at the same MND Research Symposium.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

This is the final report for this grant.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

After years of use, the flow cytometers used to enumerate fluorescent protein inclusions began to show unacceptable levels of variation between wells. We believe this to be the results of the long-term accumulation of nucleic acids and cell debris inside the fluidics of the machines. No amount of cleaning could completely remove this. Therefore, in the last 12 months we developed an alternative fluorescence microplate-based assay which has now been successfully implemented and is being used in ongoing screening work.

Work within this study helped the zebrafish team to develop skills with high throughput microscopy (to measure the subcellular localization of human ataxin-3) that had flow on effects enhancing the development of high throughout microscopy assays for expression levels of other transgenically expressed proteins.

In this project, we have enhanced and optimized our ALS mouse model pre-clinical testing pipeline, which will allow for faster testing of other therapeutics in future ALS research projects.

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The new fluorescence microplate-based assay we developed to measure insoluble fluorescent protein inclusions may find applications in other drug-screening platforms.

We are using the other high-throughput assays in other drug-screening studies to rapidly screen drug libraries in vivo.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

The covid-19 pandemic resulted in closures of the University (UOW) for a period of months during 2020 and 2021 and restrictions on people movements and access to buildings and equipment. The circumstances significantly slowed progress and had flow-on delays for the animal model work. The covid restrictions also directly affected the continuity of operations at Macquarie University (the workplace of Dr Angela Laird, our collaborator working with zebrafish) and at the University of Queensland (the workplace of Dr Adam Walker, our mouse model collaborator), compounding the delays.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Commencement of the animal model testing, to be conducted by collaborators at Macquarie University (Sydney; Dr Angela Laird, zebrafish) and the University of Queensland (Brisbane; Dr Adam Walker, mouse model) was deferred, and then delayed by the finalisation of inter-institutional agreements, and began in August 2022. **A (second) no-cost extension, approved 23 September 2022, allowed completion of the animal testing by September 2023.**

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

None.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

None.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

The project is highlighted on the Molecular Horizons, University of Wollongong webpage under the "Wilson Lab" tab (https://www.uow.edu.au/research-and-innovation/our-research/research-institutes-and-facilities/molecular-horizons/themes-disciplines/protein-aggregation-related-diseases/). Additionally, a YouTube video on the SCMB channel also explains the project and the associated flow cytometric technique in lay terms (https://youtu.be/U2YwoOYnoRY).

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

A new fluorescence microplate-based assay to measure insoluble intracellular protein inclusions was developed. This may be reported in a publication within the next 12 months.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

None.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

ALS mouse model

Name: Dr Leon Luan
Project role: Employed as Postdoctoral Fellow
ORCID ID: 0000-0003-2478-9790
Nearest person month worked: 9.6
Contribution to project: Performed testing of drugs in mouse model of ALS.
Funding support: Salary funded by this grant (AL180040).

Name: Adam Walker
Project role: PI
ORCID ID: 0000-0001-7954-5801
Nearest person month worked: 1.2
Contribution to project: Supervised drug testing in mouse model of ALS.
Funding support: Salary funded by FightMND Mid-Career Research Fellowship, Ross Maclean Fellowship, and National Health and Medical Research Career Development Fellowship.

Zebrafish ALS model

Name: Julia Kam
Project role: Employed Research Assistant
ORCID ID: NA
Nearest person month worked: 14.
Contribution to project: Performed testing of drugs in zebrafish model of ALS including working on performing drug testing, western blotting, microscopy and data analysis.

Funding support: Salary funded by this grant (AL180040).

Name: Kristy Yuan
Project role: Research Assistant
ORCID ID: NA
Nearest person month worked: 2
Contribution to project: Assisted with zebrafish care and drug testing pipeline.
Funding support: Salary paid by funds external to this grant.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding

agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Partners:

1. Organisation: Macquarie University (Sydney, Australia). Collaborator: Dr Angela Laird. Performed drug testing in zebrafish model of ALS.
2. Organisation: University of Queensland (Brisbane, Australia). Collaborator: Dr Adam Walker. Performed drug testing in a mouse model of ALS.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

Nothing to report.

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Detailed reports of outcomes from drug testing in zebrafish and mouse models of ALS are attached.

Appendix A: AL180040 Final Report, zebrafish ALS model results (Angela Laird, Macquarie University)

I. Treatment with 1µM INO-1001 & 5µM APTSTAT3-9R significantly improves swimming behaviour in a transgenic human FUS-R521C zebrafish model

Transgenic zebrafish larvae expressing human FUS-R521C mutant protein (hFUS-R521C) were treated with various concentrations of a candidate drug or vehicle for 24 hours (Figures 1A-B; see Table 1 for the specific concentrations tested for each candidate). At 2 days post-fertilisation (2dpf), the photo-motor response of each zebrafish larva was tracked using a 96-well plate assay within the ZebraTower device, and the total distance swum by each larva was calculated (Figure 1B). Vehicle-treated larvae carrying hFUS-R521C swam significantly shorter distances compared to non-transgenic siblings (p=0.0223), whereas treatment with 1µM INO-1001 or 5µM APSTAT3-9R resulted in a significant increase in distances swum (p=0.0003 & p=0.0374 respectively; Figures 1C-D).

Table 1: Vehicle and concentrations of each candidate tested

Candidate name	**Vehicle**	**Doses Tested**
IKK-16	DMSO	0.5, 1 & 2µM
ZM336372	DMSO	2.5, 5 & 10µM
GW5074	DMSO	0.5, 1, 2 & 5µM Toxicity issues with doses >1µM at 2-3dpf & 0.5uM at 6dpf. Testing discontinued.
INO-1001	DMSO	0.5, 1, 5 & 10µM
3-aminobenzamide	DMSO	1 & 5µM
AZD1080	DMSO	0.5 & 1µM
APTSTAT3-9R	Water	0.5, 1 & 5µM
Chicago Sky Blue 6B	Water	5 & 10µM
Procarbazine HCl	Water	5 & 10µM

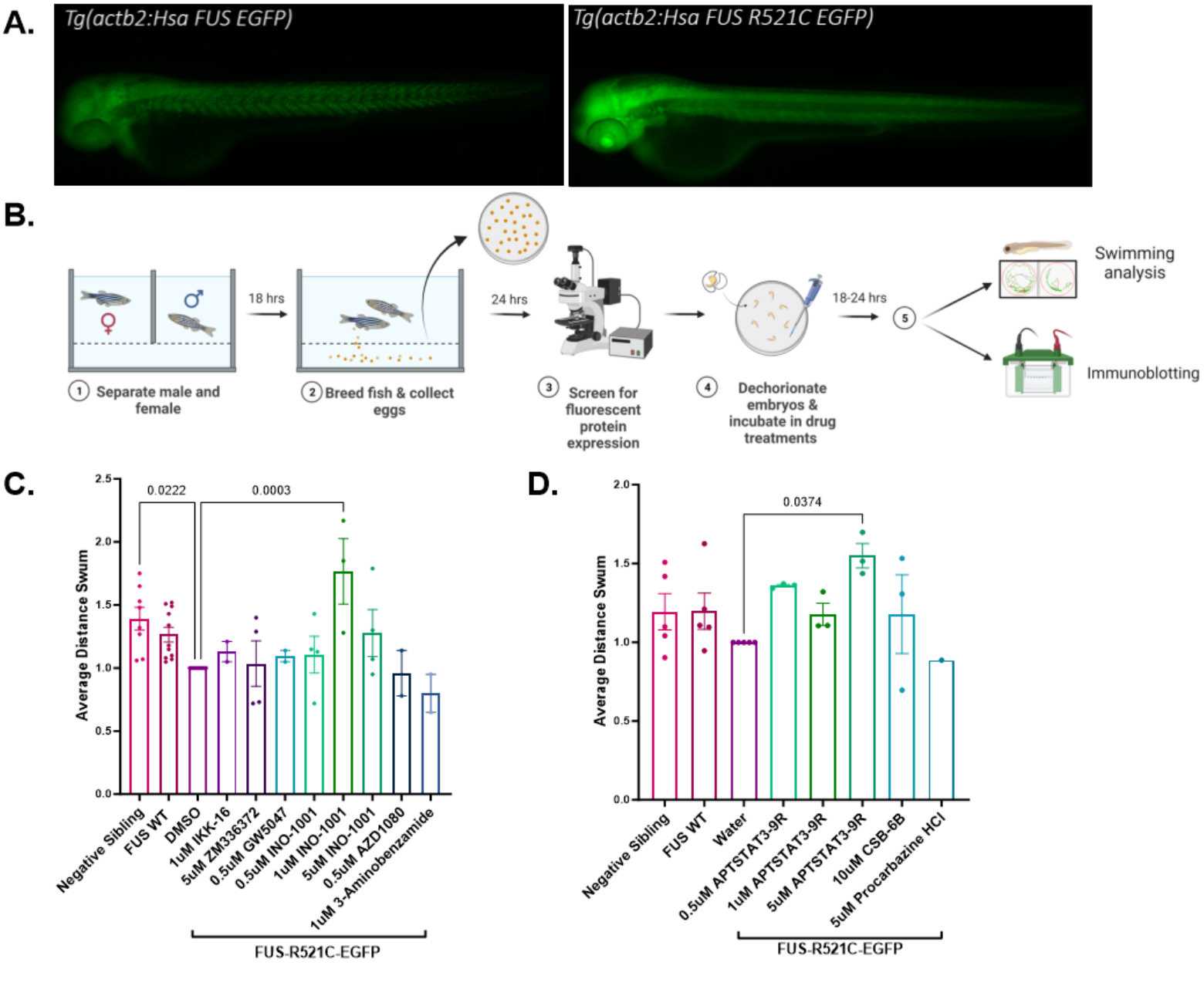

Figure 1: Quantification of swimming distance in FUS-R521C-EGFP embryos following treatment with candidate compounds. A. Representative images showing 2dpf *Tg(actb2:Hsa FUS-EGFP)* and *Tg(actb2:Hsa FUS-R521C-EGFP)* larvae expressing human FUS wild type and human FUS-R521C mutant protein tagged to EGFP respectively. Magnification used: 10x. **B.** Schematic of experimental workflow. **C-D.** Average distance swum by each treatment condition (n=24 per condition) normalised to the average distance swum by vehicle-treated FUS-R521C-EGFP larvae. Each data point represents an experimental replicate and error bars indicate ±SEM. Statistical analysis was performed on all concentrations tested, however, aside from INO-1001 and APTSTAT3-9R, only the concentration that improved swim behaviour most is shown. p-values were calculated by performing a one-way ANOVA. **C.** Candidate compounds dissolved in DMSO. **D.** Candidate compounds dissolved in water.

II. INO-1001 treatment significantly reduced full length human FUS protein levels

To assess whether the improved swim behaviour of FUS-R521C-EGFP larvae treated with INO-1001 was accompanied by changes in abundance of mutant FUS, protein extraction and western blotting was performed following behavioural tracking. A band corresponding to the full-length (FL) human FUS-R521C protein fused with EGFP was observed around 90kDa (predicted 102kDa), as well as a fragment of cleaved FUS around 65kDa (Figure 2A). Treatment with 0.5µM INO-1001 significantly reduced levels of FL-FUS-R521C-EGFP ($p=0.0267$; Figure 2B). We quantified whether the amount of amount of FUS-R521C-EGFP cleavage products had decreased with INO-1001 treatment, but found that it had not ($p=0.3482$; Figure 2C). Larvae treated with 1µM INO-1001 also demonstrated a reduction in FL-FUS-R521C-EGFP, but this was not statistically significant ($p=0.0743$; Figure 2B)

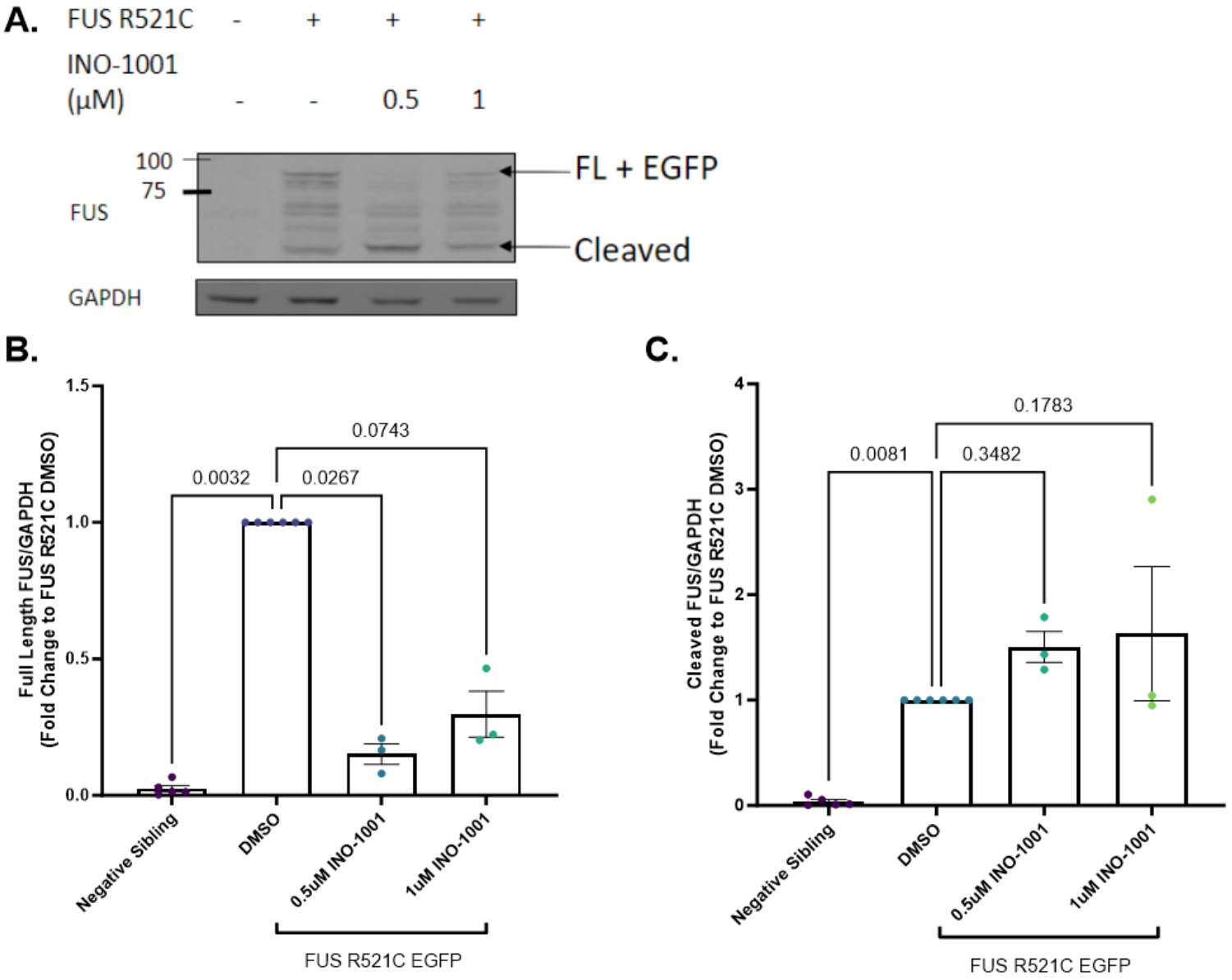

Figure 2: Western blotting to characterise changes in FUS-R521C-EGFP following INO-1001 treatment. **A.** Representative image of a nitrocellulose membrane containing protein from FUS-R521C-EGFP embryos treated with INO-1001 or vehicle (n=24/condition), probed with human-TLS/FUS antibody. **B.** Full-length FUS-R521C-EGFP, and **C.** Cleaved FUS-R521C levels expressed as a fold change to the mutant-vehicle treatment group. Each data point represents an experimental replicate and error bars indicate ±SEM. p-values were calculated by performing a one-way ANOVA.

Appendix B: AL180040 Final Report, Adam Walker ALS mouse model results

This work was led by Dr Wei (Leon) Luan.

Thorough research on the background for the 10 top hits that inhibited TDP-43 inclusion in the in vitro assay was conducted, focusing on suitability for progression to mammalian in vivo testing. These compounds include IKK-16, AZD1080, Chicago Sky blue, Procarbazine HCl, 3-Aminobenzamide, AG-1478, Iniparib (BS-201), ZM336372, LJH586, and APTSTAT3-9R. Based on the pharmacokinetic (PK) properties, the unknown effects and potential toxicity of these compounds, three compounds were selected for the short-term studies in the rNLS8 mice, aiming to test the effects of these compounds on TDP-43 pathology and disease-associated motor dysfunction in rNLS8 mice. Three compounds were IKK-16, GW5074 (substitute for ZM336372 due to better PK properties), INO-1001 (substitute for 3-Aminobenzamide due to less toxicity).

Walker lab conducted administration of IKK-16 (1mg/kg, *i.p.,* daily), GW5074 (10mg/kg, *i.p.,* twice per day) and INO-1001 (10mg/kg, *i.p.,* twice per day) for rNLS8 mice and corresponding vehicles for their littermate controls for two weeks since the removal of Doxycycline diet (Dox), the disease onset of this rNLS8 mouse model (n = 4). Walker lab assessed 1) the body mass change and the hindlimb clasping (a key feature of motor dysfunction of rNLS8 mice), 2) biochemically the soluble and insoluble levels of TDP-43, and the insoluble phosphorylated TPD-43 (p-TDP-43, Ser409/410) in the brain of treated rNLS8 mice using immunoblotting, 3) altered cell stress, neuroinflammatory, apoptotic and reactive glial genes using real-time PCR.

The results show that in this 2 week study:

1) IKK-16 treatment showed a tendency to prevent hindlimb clasping by two weeks off Dox, however, worsened the body mass loss compared to the vehicle treatment group;
2) None of the three compounds affected the soluble level of TDP-43, however, all tended to reduce the level of insoluble TPD-43 in the brain of treated rNLS8 mice (although this difference was not statistically significant);
3) IKK-16 treatment significantly reduced the level of insoluble p-TDP-43 in the brain of treated rNLS8 mice compared to the vehicle treatment group. GW5074 and INO-1001 showed similar trends without statistical significance.
4) IKK-16 treatment prevented the downregulation of excitatory amino acid transporter 2 (EAAT2) in the brain of treated rNLS8 mice compared to the vehicle treatment group. However, none of the compounds affected the expression of cell stress response genes (Atf4, Chop, Xbp1, Trp53).

Based on the above data of the short-term study, IKK-16 showed promising effects in preventing TDP-43 pathology and restoring the expression of *Eaat2* gene, and was thus selected for the long-term study.

Walker Lab therefore conducted an end-stage studying by administrating IKK-16 to rNLS8 mice (1mg/kg, i.p., daily) to assess the neuroprotective effects of IKK-16 on the neurological progression, the non-motor phenotypes and the lifespan of treated rNLS8 mice. Walker Lab assessed 1) the body mass change, 2) the lifespan of rNLS8 mice in treatment groups, 3) the

hindlimb clasping phenotypes, 4) the motor dysfunction measured by rotarod performance and the rearing# in the open field test, 5) the hyperactivity and anxiety-like non-motor phenotypes in treated rNLS8 mice. The groups include vehicle-treated control mice (n = 22, 7 males and 15 females), vehicle treated rNLS8 mice (n=19, 7 males and 12 females) and IKK16 treated rNLS8 mice (n=19, 7 males and 12 females). Two male rNLS8 mice were excluded at the beginning of the study due to unexpected sickness unrelated to expected motor phenotype or drug treatment and their data was not included.

The results showed that:
1) IKK-16 treatment did not significantly prevent the body mass decline of treated male or female rNLS8 mice;
2) IKK-16 treatment did not improve the lifespan of treated male or female rNLS8 mice;
3) IKK-16 treatment did not prevent the hindlimb deficit in treated male or female rNLS8 mice;
4) IKK-16 treatment did not improve the rotarod performance or the rearing # of treated male or female rNLS8 mice;
5) IKK-16 treatment did not reverse the hyperactivity or the anxiety-like behaviours in treated male or female rNLS8 mice.

In conclusion, the short-term treatment study of IKK-16 show modest but significant neuroprotective effects in rNLS8 mice, with inhibition of insoluble p-TDP-43 levels and restoration of *Eaat2* gene expression. However, the long-term treatment of IKK-16 did not significantly prevent pathological progression or improve the lifespan of treated rNLS8 mice.

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Report for USDOD study

Dr Wei Luan, Dr Adam Walker

Neurodegeneration Pathobiology Laboratory, Queensland Brain Institute

October 2023

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Outline

- Top hit compound list
- Short-term study in rNLS8 mice (Adam Walker group)
- Long-term study in rNLS8 mice (Adam Walker group)

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Compounds for rNLS8 mouse experiments

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Compounds for rNLS8 mouse experiments

Ranking for in vivo work	Target	Dose (mg/kg)	Frequency	Route	Ethics approved (Y/N)	Short-term study	Long-tern study
1) IKK-16	IKß kinase	1	Daily	i.p.	Y	2 weeks off Dox in rNLS8	Survival study completed
2) INO-1001	PARP	10	Twice/day	i.p.	**Y**	2 weeks off Dox in rNLS8	NA
3) GW5074	c-Raf/LRRK2	10	Twice/day	i.p.	Y	2 weeks off Dox in rNLS8	NA
4) AZD1080	GSK3ß	20	Twice/day	Oral gavage	Y	NA	NA
5) CHICAGO SKY BLUE	Unknown, VGLUT inhibitor	70	Daily	i.p.	Y	NA	NA
6) Procarbazine HCl	Alkylating anticancer agent	150-450	Weekly	IP	Y	NA	NA
7) 3-Aminobenzamide	PARP	20-320	Acute	i.p.; internal jugular vein (i.j.v.)	Y	NA	NA
9) AG-1478	Tyrosine kinase Kv1.5 channel	20	Daily	Chow	Y	NA	NA
10) Iniparib (BSI-201)	Not via PARP1 inhibition	8, 25, 50, 100	Twice/day	IP, IV	Y	NA	NA
11) ZM336372	c-Raf/LRRK2	NA	NA	NA	Y	NA	NA
12) LJH685	RSK	NA	NA	NA	N	NA	NA
8) APTSTAT3-9R	STAT3 binding peptide	0.4µg/µl	Acute	Intrathecal injection	N	NA	NA

Note: Highlighted in green are drugs approved for use in the Walker lab by animal ethics.

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USDOD short-term study (2 weeks off Dox)

Adam Walker's lab

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USDOD short-term study: pathology study

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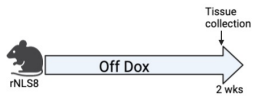
Aims:
To examine the inhibitory effects of drugs on TDP-43 aggregation
To examine the toxicity of drugs in *in vivo* studies



Methods: rNLS8 mice received treatments for two weeks (off Dox) as below.

Target	Compound	Dose/Route/ in vivo model	Frequency	Route	Vehicle
IKß kinase inhibitor	IKK-16	1mg/kg	Daily	i.p.	10% DMSO >> 40% PEG400 >> 5% Tween-80 >> 45% saline
c-Raf/LRRK2	GW5074	10mg/kg;	Twice/day	i.p.	10% DMSO >> 40% PEG400 >> 5% Tween-80 >> 45% saline
Poly (ADP-ribose) polymerase (PARP)	INO-1001	10mg/kg	Twice/day	i.p.	10% DMSO >> 40% PEG400 >> 5% Tween-80 >> 45% saline

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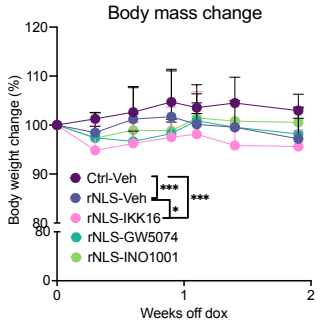
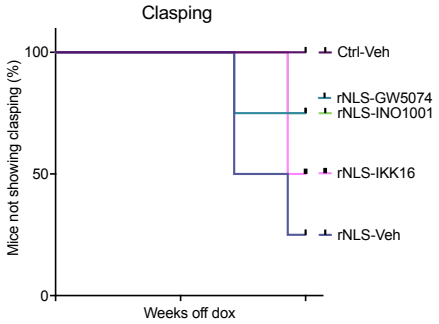
Neurological results





- IKK-16 negative affects body mass of treated-rNLS8 mice compared to vehicle-treated group and control group
- There is tendency that drug treatments slow motor deficits (clasping), thought underpowered in statistic analysis.

Mean + SD, n = 4
* $p < 0.05$; *** $p < 0.001$

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Biochemical results

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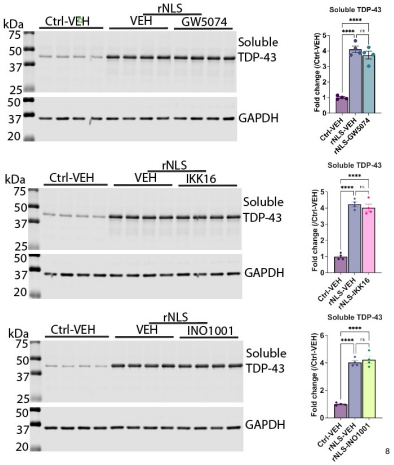
- None of tested drugs significantly affected the level of soluble pan-TDP-43 (human and mouse) protein in the cortex of treated rNLS8 mice, compared to vehicle-treated rNLS8 mice.

Cortex, soluble pan-TDP-43 (human and mouse TDP-43)



Mean + SEM, n = 4
*** $p < 0.0001$

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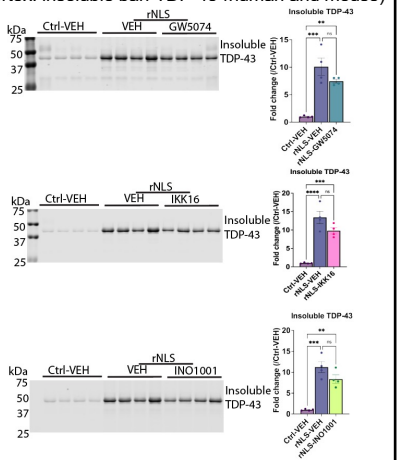
Biochemical results

- There is tendency that tested drugs seem to reduce the level of insoluble pan-TDP-43 (human and mouse) protein in the cortex of treated rNLS8 mice, compared to vehicle-treated rNLS8 mice.

Cortex, insoluble pan-TDP-43 (human and mouse)



Mean + SEM, n = 4
* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$

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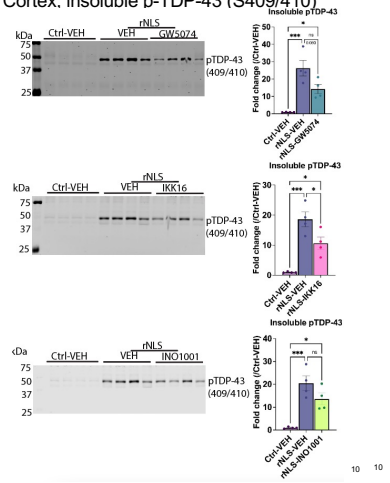
Biochemical results

- IKK-16 treatment significantly reduce the level of phosphorylated TDP-43 (p-TDP-43) protein in the cortex of treated rNLS8 mice, compared to vehicle-treated rNLS8 mice.
- There is tendency GW5074 treatment also decrease the level of phosphorylated TDP-43 (p-TDP-43) protein in the cortex of treated rNLS8 mice, compared to vehicle-treated rNLS8 mice (p = 0.060).
- INO1001 treatment does not significantly affect the level of phosphorylated TDP-43 (p-TDP-43) protein in the cortex of treated rNLS8 mice, compared to vehicle-treated rNLS8 mice.

Cortex, insoluble p-TDP-43 (S409/410)



Mean + SEM, n = 4
* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$

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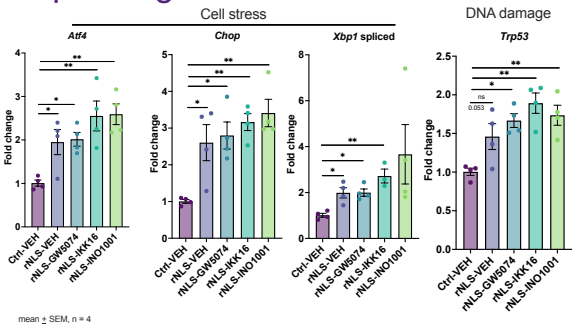
qPCR results

Cell stress response genes



- Drug treatments do not significantly prevent upregulation of stress response genes (*Atf4, Chop, Xbp1, Trp53*) in the cortex treated rNLS8 mice, compared to vehicle-treated rNLS8 mice.

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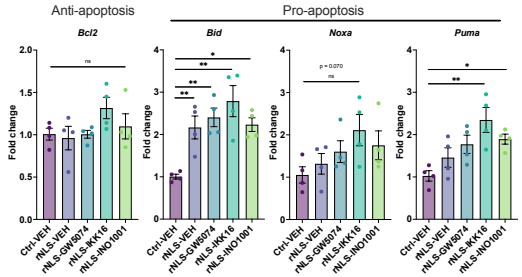
qPCR results

Apoptosis



- Drug treatments do not significantly influence the expression of anti-apoptotic Bcl2 gene, or pro-apoptotic Noxa gene in the cortex treated rNLS8 mice, compared to control or vehicle-treated rNLS8 mice.
- Drug treatments do not prevent upregulation of pro-apoptotic Bid gene in the cortex treated rNLS8 mice, compared to vehicle-treated rNLS8 mice.
- IKK-16 or INO-1001 treatment seems to increase the expression of pro-apoptotic *Puma* gene in the treated rNLS8 mice, compared to vehicle-control mice.

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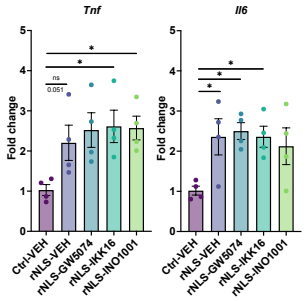
qPCR results
Neuroinflammation



- Drug treatments do not significantly influence the expression of anti-apoptotic neuroinflammatory genes (*Tnf* and *Il6*) in the cortex treated rNLS8 mice, compared to vehicle-treated rNLS8 mice.

mean ± SEM, n = 4

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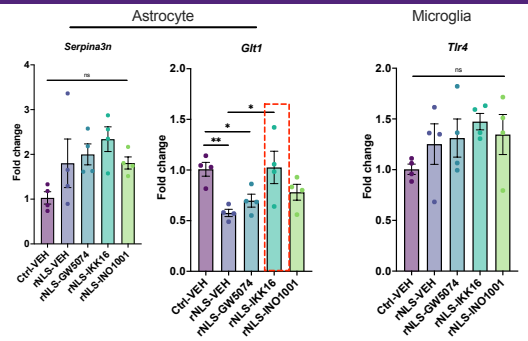
qPCR results
Glial genes



- IKK treatment significantly restore the expression of glutamate transporter-1 (*Glt1*) gene in the cortex treated rNLS8 mice to the level of control mice, compared to vehicle-treated rNLS8 mice.

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Summary

- IKK-16 treatment decreases the body mass of treated rNLS8 mice over 2 weeks.
- IKK-16 treatment reduce the level of insoluble p-TDP-43 protein in treated rNLS8 mice at 2 week off Dox.
- IKK-16 rescues the expression of Glt1 gene in treated rNLS8 mice at 2 week off Dox.
- The data suggests IKK-16 is the promising drug to test for long-term studies.

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USDOD long-term study

Adam Walker's lab

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USDOD long-term study: survival study

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Aims:
To examine the effects of IKK-16 on neurological outcomes and lifespan in rNLS8

Methods: rNLS8 mice received treatments

Target	Compound	Dose/Route/ in vivo model	Frequency	Route	Vehicle
IKß kinase inhibitor	IKK-16	1mg/kg	Daily	i.p.	10% DMSO >> 40% PEG400 >> 5% Tween-80 >> 45% saline

End-point criteria on Ethics (details available in the attached pdf)

1. ALL ANIMALS MUST BE CHECKED DAILY, AND WEIGHED AND FULLY ASSESSED THREE TIMES PER WEEK (usually Mon/Wed/Fri) AFTER REMOVAL OF DOX FROM FEED.
2. BLADDER DISTENTION MUST BE CHECKED IN MALES THREE TIMES PER WEEK (usually Mon/Wed/Fri) AFTER MND SYMPTOM ONSET AND DAILY THEREAFTER IF DETECTED. BLADDER TO BE GENTLY PALPATED TO ENCOURAGE URINATION IF RETENTION DETECTED. IF URINATION CANNOT BE ENCOURAGED, SEEK VETERINARY ASSISTANCE.
3. IF WEIGHT LOSS ≥10% FROM PREVIOUS RECORDING OR ≥20% FROM STARTING WEIGHT OR NS = 3, INCREASE WEIGHING AND FULL ASSESSMENT FREQUENCY TO EVERY DAY.
4. EUTHANASIA = WEIGHT LOSS ≥10% FROM PREVIOUS RECORIDNG FOR 24H OR IF WEIGHT <70% OF STARTING BODY WEIGHT IN THREE CONSECUTIVE DAILY MEASUREMENTS OR SCORE = 4 ACCORDING TO NEUROLOGICAL SCORE CRITERIA.
5. DETAILS OF ANY ABNORMAL (OUTSIDE THE TYPICAL MUTANT PHENOTYPE) FINDINGS MUST BE RECORDED, AND ANIMALS MUST BE SCORED ONCE THEY REACH A '1' IN ANY CATEGORY.
6. ACCORDING TO THE FOLLOWING OTHER ABNORMAL FINDINGS CONDITION DESCRIPTORS: TOTAL OAF SCORE > 5 REQUIRES MONITORING MORNING AND LATE AFTERNOON; ANY ONE OAF SCORE = 3 REQUIRES EUTHANASIA.

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Update for the long-term study

We in the middle of the long-term study to assess whether IKK-16 treatment can prevent disease progression or prolong the lifespan of treated rNLS8 mice in an end-stage study. The end-stage study include two waves of recruitment.

- Ctrl-VEH(n = 22, 7M, 15F), rNLS-VEH(n=19, 7M, 12F), rNLS-IKK16(n=19, 7M, 12F)
- Exclusion: two male rNLS mice were excluded due to sickness at the beginning of the study, and their data was excluded for the data analyses.

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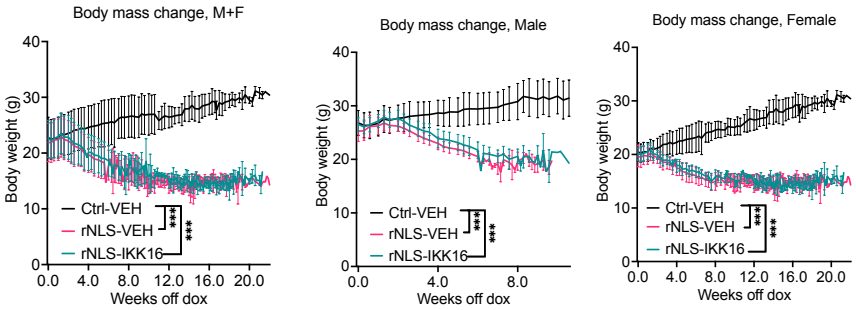
Long-term study: body mass results



- IKK-16 treatment did not significantly affect body mass change of treated male or female rNLS8 mice, comparing to the vehicle control group.

*mean±SD, *** p < 0.001 by repeated ANOVA*
Ctrl-VEH(n = 22, 7M, 15F), rNLS-VEH(n=19, 7M, 12F), rNLS-IKK16(n=19, 7M, 12F)

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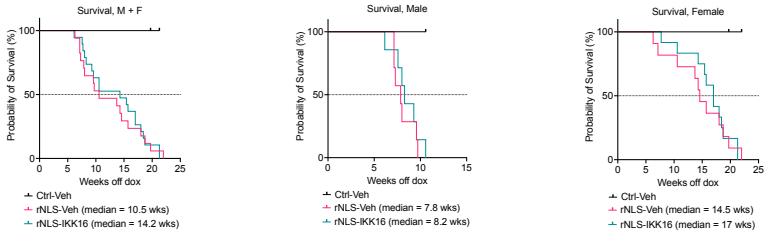
Long-term study: survival results



- IKK-16 treatment did not prolong the lifespan of treated male or female rNLS8 mice compared to the vehicle group ($p > 0.05$ by Log rank test). However, IKK-16 treated mice showed higher median survivals (*indicated by the dash line*) than the vehicle group, especially in female groups, suggesting that IKK-16 might have protective effects against TDP-43 toxicity.
- Male mice reached the humane end-point earlier than females due to common bladder tension in disease course

Kaplan-Meier curve, *by Log rank test*
Ctrl-VEH(n = 22, 7M, 15F), rNLS-VEH(n=19, 7M, 12F), rNLS-IKK16(n=19, 7M, 12F)

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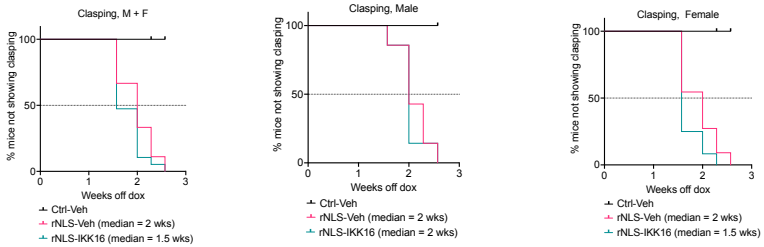
Long-term study: neurological results



- IKK-16 treatment did not significantly prevent clasping phenotype in treated male or female rNLS8 mice compared to the vehicle group

Kaplan-Meier curve, *by Log rank test*
Ctrl-VEH(n = 22, 7M, 15F), rNLS-VEH(n=19, 7M, 12F), rNLS-IKK16(n=19, 7M, 12F)

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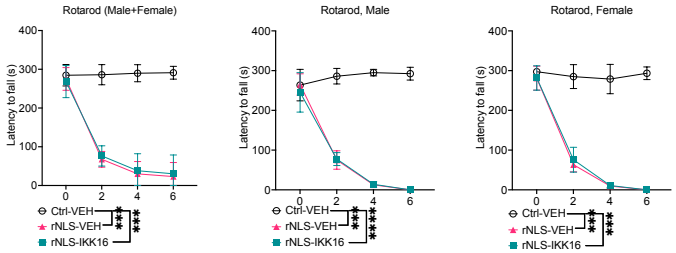
Long-term study results



- IKK-16 treatment did not affect the rotarod performance of treated male or female rNLS8 mice compared to vehicle-treated mice.

*mean±SD, ***, p < 0.001, **** p < 0.0001, by one-way ANOVA*
Ctrl-VEH(n = 21, 7M, 14F), rNLS-VEH(n=19, 7M, 12F), rNLS-IKK16(n=19, 7M, 12F)

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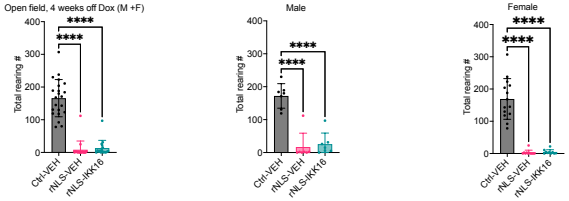
Long-term study results



- IKK-16 treatment did not affect the exploratory vertical movement (measured as rearing # in the open field test) of treated male or female rNLS8 mice at 4 weeks off Dox, compared to the vehicle group

*mean±SD, **** p < 0.0001 by one-way ANOVA*
Ctrl-VEH(n = 22, 7M, 15F), rNLS-VEH(n=19, 7M, 12F), rNLS-IKK16(n=19, 7M, 12F)

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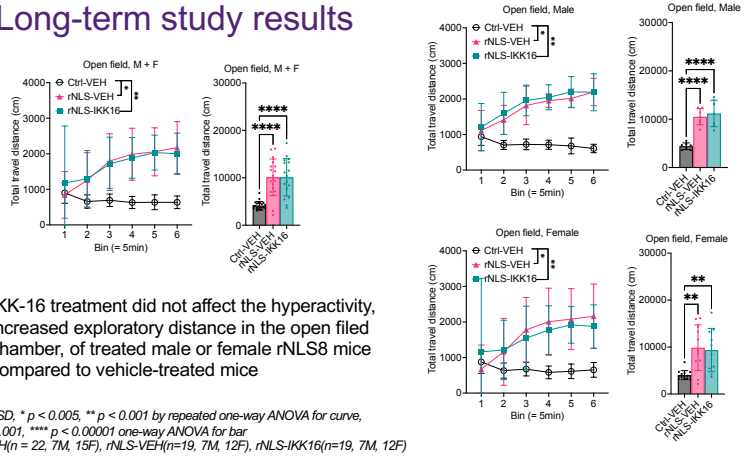
Long-term study results



- IKK-16 treatment did not affect the hyperactivity, increased exploratory distance in the open filed chamber, of treated male or female rNLS8 mice compared to vehicle-treated mice

*mean±SD, * p < 0.005, ** p < 0.001, **** p < 0.00001 one-way ANOVA for bar*
Ctrl-VEH(n = 22, 7M, 15F), rNLS-VEH(n=19, 7M, 12F), rNLS-IKK16(n=19, 7M, 12F)

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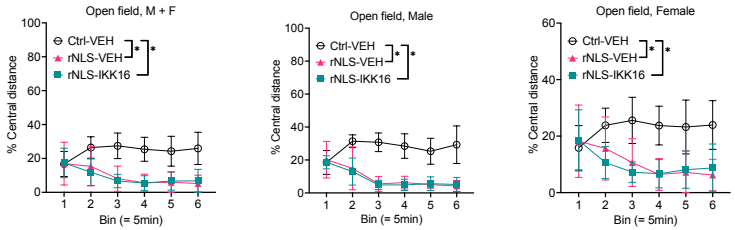
Long-term study results



- IKK-16 treatment did not affect anxiety-like phenotype (decreased central exploration in the open filed chamber) of treated male or female rNLS8 mice compared to vehicle-treated mice

*mean+SD, * p < 0.005 by repeated one-way ANOVA*
Ctrl-VEH(n = 22, 7M, 15F), rNLS-VEH(n=19, 7M, 12F), rNLS-IKK16(n=19, 7M, 12F)

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Summary of the long-term study for IKK-16 treatment

- IKK-16 treatment did not affect the survival of treated male or female rNLS8 mice, compared to the vehicle treatment group
- IKK-16 treatment did not affect the body mass of treated male or female rNLS8 mice, compared to the vehicle treatment group
- IKK-16 treatment did not affect the neuropathological progress (measured as clasping) or motor ability (measured by rotarod and open field tests) in treated male or female rNLS8 mice, compared to the vehicle treatment group
- IKK-16 treatment did not affect the hyperactivity or anxiety-like phenotype in treated male or female rNLS8 mice at 4 weeks off Dox, compared to the vehicle treatment group

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