AWARD NUMBER: **W81XWH-22-1-0224**

TITLE: **Quinolinones as a Novel Therapeutic Strategy in ALS**

PRINCIPAL INVESTIGATOR: **Prof. Julie Atkin, Ph.D**.

CONTRACTING ORGANIZATION: **MACQUARIE UNIVERSITY, Sydney, Australia**

REPORT DATE: **October 2023**

TYPE OF REPORT: **ANNUAL**

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2023	ANNUAL	01Sep2022-31Aug2023

4. TITLE AND SUBTITLE

Quinolinones as a Novel Therapeutic Strategy in ALS

5a. CONTRACT NUMBER
W81XWH-22-1-0224

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

JULIE ATKIN, BELINDA ABBOTT, SONAM PARAKH

E-Mail: julie.atkin@mq.edu.au

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

MACQUARIE UNIVERSITY
NORTH RYDE
SYDNEY
AUSTRALIA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project investigates quinolinone analogues as a novel therapeutic strategy for ALS. We previously demonstrated that these compounds display broad efficacy in cellular and zebrafish models. This study aims (i) to identify the primary biological target, (ii) optimize formulation and delivery, and (iii) investigate efficacy and best modes of delivery of the lead molecule, ML4-10. In the first year of the project, we completed the Safety Screen analysis of ML4-10. We identified the $GABA_A$ receptor as a possible primary target and identified 3-4 possible off-target hit proteins. Using proteomics, we identified 16 interacting partners of ML4-10, nine of which were localised in the nucleus, and these are currently being prioritized. We also synthesized and tested new ML4-10 analogues in disease models, including salt/prodrug forms to optimize formulation/delivery. However, ML4-10 remains the most active compound to date although additional analogues are being examined.

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	15	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal and severely debilitating neurodegenerative disorder that overlaps significantly with Frontotemporal dementia (FTD). There are few effective treatments for ALS, highlighting the urgent need to develop novel therapeutic strategies. The formation of misfolded protein inclusions is the key pathological hallmark of both sporadic and familial forms of ALS/FTD, and TDP-43 and FUS are RNA/DNA binding proteins that are strongly linked to pathophysiology. In fact, pathological forms of TDP-43, involving the accumulation of abnormally phosphorylated, aggregated, ubiquitinated and truncated TDP-43 in the cytoplasm, are strongly associated with toxicity in almost all ALS (~97%), and ~45% FTD cases. Hence, alterations in TDP-43 distribution and inclusion formation are important contributors to disease. In familial forms of ALS, SOD1 mutations account for a major proportion of cases (20%), and in the ALS/FTD spectrum, mutations in *TARDBP* (encoding TDP-43), *FUS* and *CCNF* (encoding cyclin F), each account for ~3-5% of cases. Molecules that inhibit the formation of pathological events linked to these important proteins could therefore be used for the development of new drugs to treat ALS/FTD. We have identified a novel group of compounds, quinolinones, that are protective against multiple pathological phenotypes highly relevant to ALS/FTD in neuronal cells, including the formation of inclusions formed by mutant forms of TDP-43, FUS and SOD1, and the mis-localization of TDP-43 and FUS to the cytoplasm. In addition, quinolinones are protective against motor impairment in zebrafish expressing ALS/FTD-linked forms of mutant *CCNF*. This implies that these compounds have efficacy against major pathological events central to ALS. This project investigates the effectiveness of quinolinone analogues as a novel and highly innovative therapeutic strategy. This study aims (i) identify the primary biological target, (ii) optimise formulation and delivery, and (iii) investigate efficacy and best modes of delivery of the lead molecule.

2. Keywords

Amyotrophic lateral sclerosis (ALS), TDP-43 (TAR-DNA binding protein-43), neurodegeneration, quinolinones, proteostasis, ML4-10

3. Accomplishments

Specific Aim 1: Target Identification and Safety Profile of ML4-10

Major Task 1: Safety Profile of ML4-10

In this reporting period we examined the safety profile of our existing lead compound, ML4-10, using Safety Screen 44 (Eurofins Panlabs, Inc). Using enzyme and radioligand binding assays, the activity of ML4-10 was determined to identify significant non-specific interactions with major biological target proteins. The 44 selected targets are recommended by four major pharmaceutical companies, and includes ion channels, nuclear receptors, kinases, GPCRs, transporters and other non-kinase enzymes. Reference standards were run as an integral part of each assay to ensure the validity of the results obtained.

Significant responses (≥ 50% inhibition or stimulation for biochemical assays) were identified in assays for the following targets;

Phosphodiesterase PDE3A human 10 µM - % inhibition = 52%
Phosphodiesterase PDE4D2 hum 10 µM - % inhibition = 81%
$GABA_A$, Flunitrazepam, Central rat 10 µM - % inhibition = 86%
Transporter, Dopamine (DAT) hum 10 µM - % inhibition = 77%
Transporter, Norepinephrine (NET) hum 10 µM - % inhibition = 58%

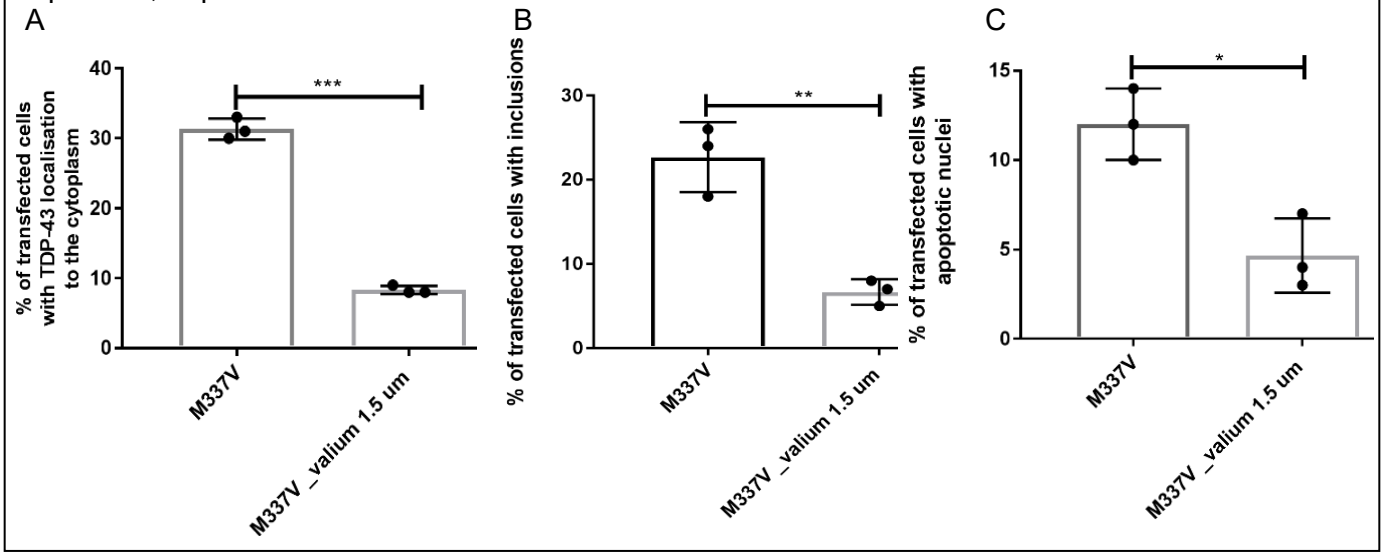
Target assessment

A major aim of this project is to identify the primary biological target of ML4-10. Hence, we considered the possibility that the one or more of these proteins may represent a valid drug target. Here we focussed on

the $GABA_A$ Flunitrazepam receptor given that hyperexcitability and excitotoxicity are implicated strongly in the pathophysiology of ALS. Furthermore, several analogues of ML4-10 display structural similarity to Diazepam (Valium) and ML4-10 displayed the most biological activity against the $GABA_A$ Flunitrazepam receptor compared to the other four targets identified (85% inhibition). Hence, we then examined whether Diazepam (Valium) was protective in cellular models of ALS.

Cells were transfected with TDP-43^{M337V} and at 6hr post-transfection Neuro-2a cells were treated with

Figure 1. Diazepam (Valium) is protective against ALS-associated phenotypes in cellular models Neuro-2a cells were transfected with TDP-43^{M337V} and treated with 1.5 μm Diazepam (Valium), 6 hr post-transfection. Diazepam (Valium) significantly decreased A) mislocalisation (***$p < 0.001$), B) inclusion formation (**$p < 0.01$) and C) apoptosis (*$p < 0.01$) compared to M337V treated with DMSO only. 100 cells were counted, n=3, values represent mean ± SD, n = 3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.



either 1.5 μm Diazepam (Valium) or DMSO as control. At 72 hr post-transfection cells were fixed and GFP-positive inclusions, TDP-43^{M337V} mislocalisation and apoptotic nuclei were examined using fluorescent microscopy. Quantification of TDP-43 positive cells demonstrated that Diazepam (Valium) was significantly protective against TDP-43^{M337V}-induced cellular defects in neuronal cells (**Figure 1**). **Hence, interestingly, these findings raise the possibility that the $GABA_A$ Flunitrazepam receptor could be the primary drug target of ML4-10. They also imply that Valium could be repurposed as a novel therapeutic strategy for ALS.** Hence, we also treated zebrafish models of ALS with Diazepam (Valium). However, we found that it resulted in a sedating effect in a photomotor response assay. Hence it was not possible to detect efficacy against ALS-relevant phenotypes using this model (data not shown), because our data suggested that the sedation effect is stronger than any potential therapeutic effects in zebrafish. Thus, further studies *in vivo* in higher organisms are required to examine this possibility, using more complex assays that do rely simply on the photomotor response.

The ligand-protein interactions of ML4-10 and the $GABA_A$ Flunitrazepam receptor were examined further by performing a dose-response radioligand binding assay for ML4-10, using concentrations from 3nM to 10μM (**Figure 2**). ML4-10 displayed a clear dose-response, similar to Diazepam (Valium, **Figure 2**). From this, the IC50, inhibitory constant (Ki) and Hill coefficient (nH) were calculated and compared to Diazepam (Valium, see below).

Pharmacological assessment of ML4-10 binding to GABAA Flunitrazepam receptor;

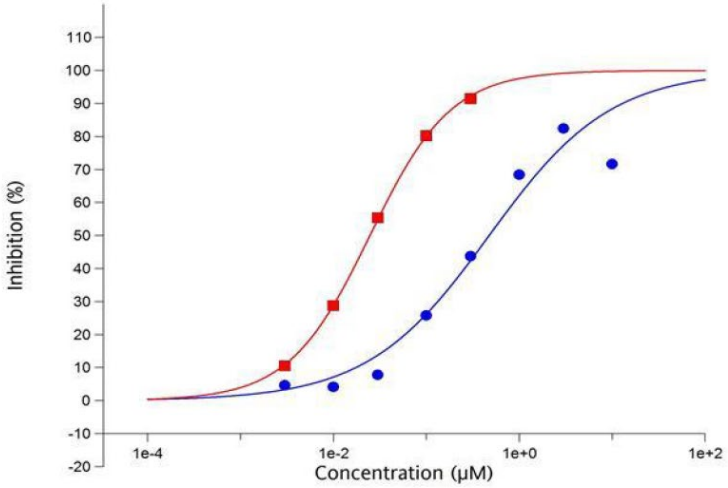
Receptor	Concentration	% inhibition	IC_{50}	Ki	nH
$GABA_A$ Flunitrazepam receptor	1μM	68%	0.47μM	0.39 μM	0.66

Comparison of ML4-10 to Diazepam (Valium)

Compound	IC_{50}	Ki	nH
Diazepam (Valium)	0.025 μM	0.020 μM	1.00
ML4-10	0.47μM	0.39 μM	0.66

These findings imply that the $GABA_A$ (Flunitrazepam) receptor is a target for ML4-10, although it is 20-fold less potent than Diazepam (Valium).

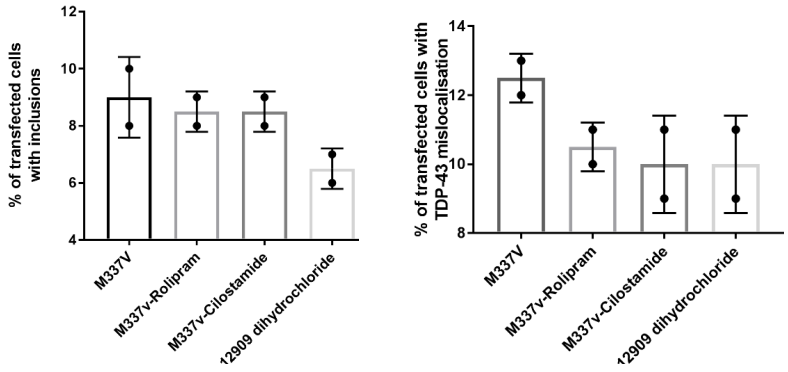
Figure 2. Dose response curve binding of ML4-10 to $GABA_A$ (Flunitrazepam) receptor
IC50 values were determined by a non-linear, least squares regression analysis using MathIQTM (ID Business Solutions Ltd., UK). The Ki values were calculated using the equation of Cheng and Prusoff (Cheng, Y., Prusoff, W.H., Biochem. Pharmacol. 22:3099-3108, 1973) using the observed IC50 of the tested compound, the concentration of radioligand employed in the assay, and the historical values for the KD of the ligand.



Safety profile: analysis of off-target effects

We then examined whether the other hits obtained in the Safety Screen 44 assay represent valid drug targets or non-specific targets. Hence, we used commercially available ligands for each of these targets to

Figure 3. Rolipram, cilostamide and GBR dihydrochoride are not protective in ALS models: ML4-10 binding to PDE3A, PDE4D2 and DAT represent non-specific off-target hits. Neuro-2a cells were transfected with TDP-43^{M337V} and were treated with 1.5 μm either rolipram, cilostamide and GBR dihydrochoride, 6 hr post-transfection. However, no significant differences was detected between TDP-43^{M337V} treated and untreated cells. 100 cells were analysed, n=2, values represent mean ± SD.

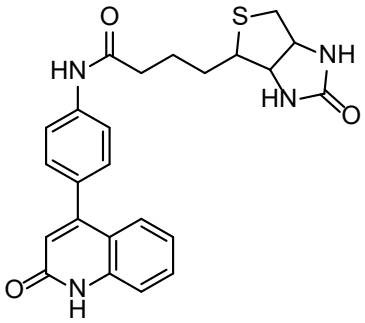


assess whether they were protective against TDP-43 pathology in cells expressing TDP-43^{M337V}: phosphodiesterases PDE3A (cilostamide) and PDE4D2 (rolipram) and dopamine transporter (DAT, GBR 12909 dihydrochoride). However, none of these compounds displayed significant protection against ALS-relevant phenotypes in these cellular assays (**Figure 3**). Thus, these findings suggest that the binding of ML4-10 to phosphodiesterases PDE3A and PDE4D2 and dopamine transporter represent off-target hits at 10uM concentration**.** This is not surprising as many early lead compounds commonly hit off target proteins, which may result in side effects. Thus, these studies reveal that in the medicinal chemistry studies we need to design novel analogues that maximise binding against the desired target and minimise against these off-target effects.

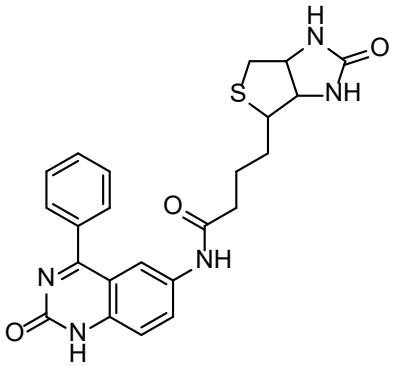
Using biotinylated versions of ML4-10 to identify the primary drug target

We have also synthesized biotinylated analogues of the lead quinazolinone ML4-10 as another strategy to identify its primary target This synthesis of five convergent steps required a hybrid methodology to be developed based on development of previously generated biotinylated compounds CM5-8 and ML4-11 (**Figure 4**) to produce a new compound, YLS1-021-4. All three biotinylated compounds were evaluated to select the compound that is preferable for target identification studies.

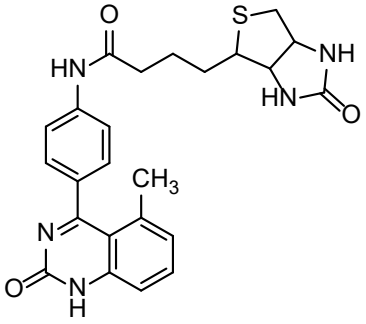
Figure 4 Compounds CM5-8, ML4-11 and YLS1-021 synthesised as biotinylated derivatives of ML4-10 and related analogues.



ID: CM5-8
MW: 448.16 g/mol
Biotinylated version of ML4-10 analogue



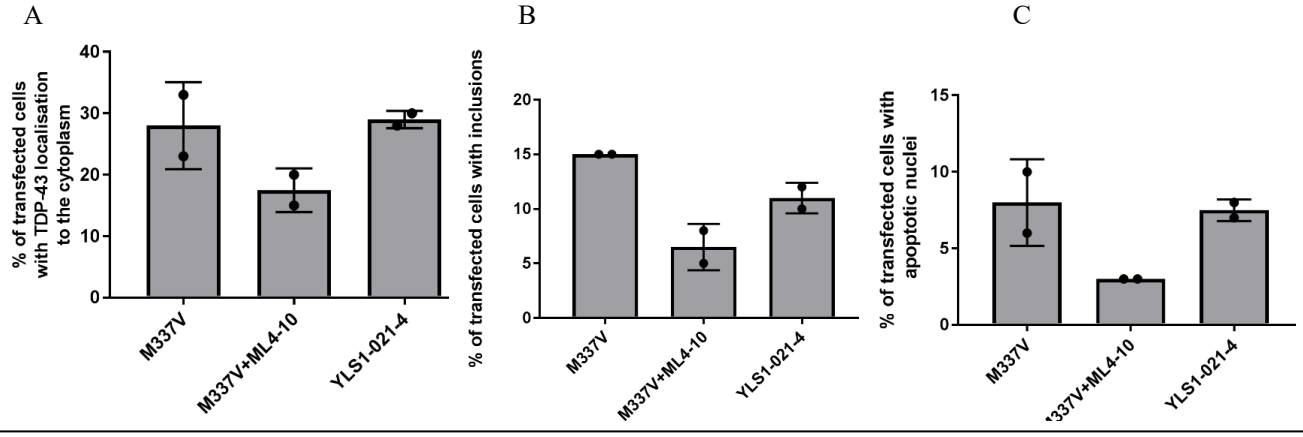
ID: ML4-11
MW: 436.51 g/mol
Biotinylated version of ML4-10 analogue



ID: YLS1-021-4
MW: 477.58 g/mol
Mass: 10.2 mg
Biotinylated version of ML4-10

The activity of biotinylated versions of ML4-10 were evaluated in Neuro-2a cells against inclusion formation, mislocalisation and apoptosis induced by mutant TDP-43^{M337V}. Neuro-2a cells were transfected with TDP-

Figure 5 Biotinylated version of ML4-10, YLS1-021-4, loses protective activity in ALS cellular models at 1.5 µm Neuro-2a cells were transfected with TDP-43^{M337V} and were treated with 1.5 µm ML4-10 or YLS1-021-4, 6 hr post-transfection. Whilst ML4-10 was protective, YLS1-021-4 did not show any protective effects against A) mislocalisation B) inclusion C) cell death. 100 cells were examined, n=2.



43^{M337V} and 6 hr post-transfection were treated with either 1.5 μm ML4-10 (positive control) or YLS1-021-4. At 72hr post-transfection cells were fixed and examined using fluorescent microscopy for GFP positive inclusions, TDP-43^{M337V} mislocalisation into the cytoplasm and condensed nuclei as a marker of apoptosis. Quantification of TDP-43^{M337V} expressing cells demonstrated that this biotinylated version of ML4-10 was not protective against mutant TDP-43^{M337V} YLS1-021-4 induced cellular defects in neuronal cells (**Figure 5**). Hence as YLS1-021-4 is not protective at 1.5 μm it cannot be used for target identification studies at this concentration.

Since YLS1-021-4 did not show similar protection as ML4-10 at the same concentration, the effect of YLS1-021-4 at higher concentrations was examined (15 μm). Cells were then analysed as above. However, quantification of TDP-43 GFP-positive cells demonstrated that this biotinylated version of ML4-10 was not protective against TDP-43^{M337V} (**Figure 6**) even at the higher concentrations. Hence because biotinylation has led to loss of biological protective activity even at higher concentrations, YLS1-021-4 cannot be used in target identification studies.

Figure 6 Biotinylated version of ML4-10, YLS1-021-4, loses protective activity in ALS cellular models at 15 μm Neuro-2a cells were transfected with TDP-43^{M337V} and were treated with 15 μm ML4-10 or YLS1-021-4, 6 hr post-transfection. ML4-10 significantly decreased, A) mislocalisation (*p < 0.05), B) inclusion formation (**p < 0.01) and C) cell death (*p < 0.05) however YLS1-021-4 did not show any protective in any assay. 100 cells were counted, n=3, values represent mean ± SD, n = 3, **p < 0.01, *p < 0.05.

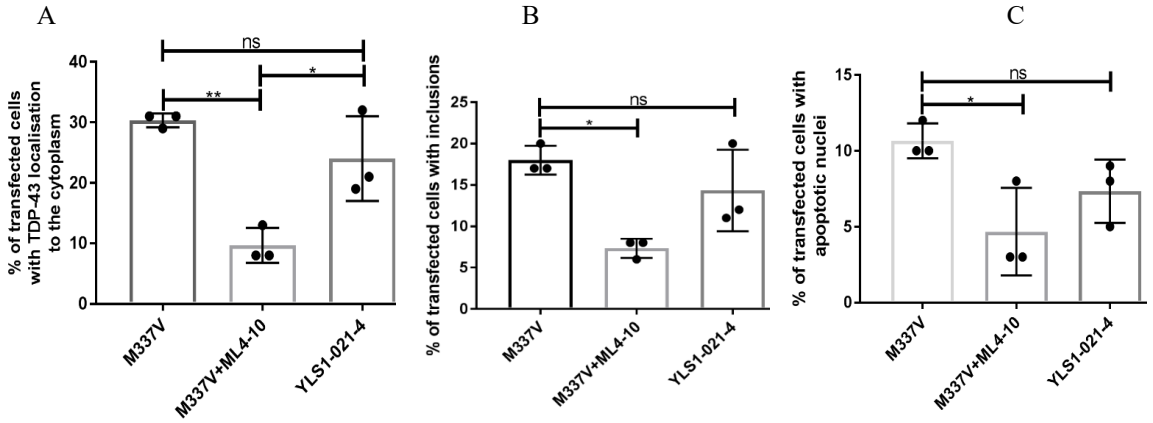
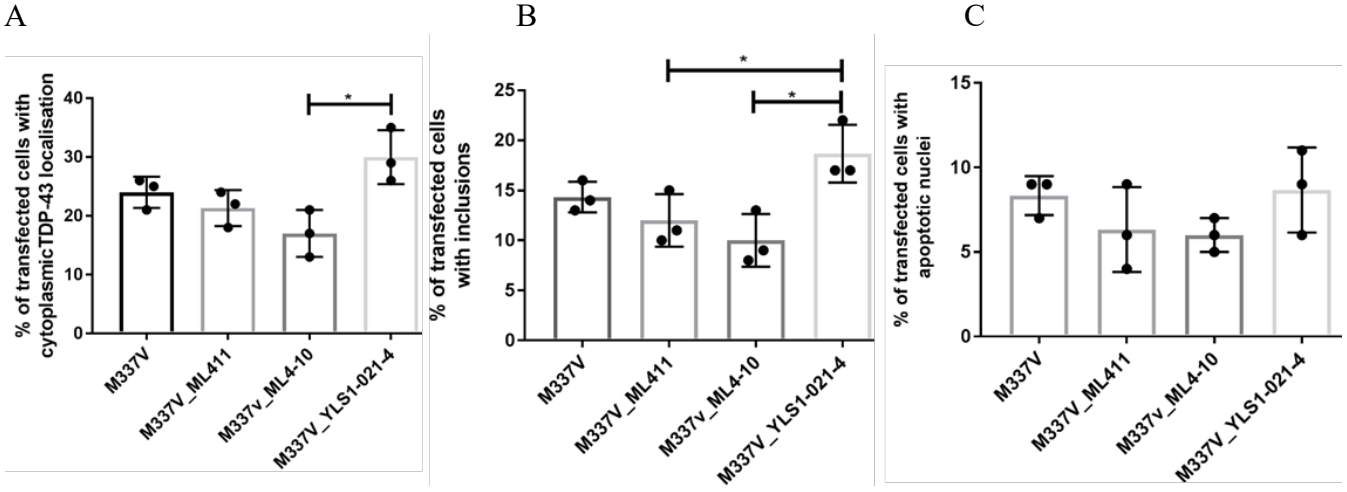


Figure 7 Biotinylated version of ML4-10, ML4-11, displays protective activity in ALS cellular models at 1.5 μm Neuro-2a cells were transfected with TDP-43^{M337V} and were treated with 1.5 μm ML4-10, ML4-11 or YLS1-021-4, 6 hr post-transfection. ML4-11 significantly decreased inclusion formation (*p < 0.05) compared to TDP-43^{M337V} treated with YLS-02-4 (B) although it was not protective against A) mislocalisation or C) cell death induced by TDP-43^{M337V}. 100 cells were examined, n=3, values represent mean ± SD,n = 3,*p < 0.05



Since YLS-021-4 was not protective, another previously synthesised biotinylated ML4-10 derivate, ML4-11, was examined in Neuro-2a cells alongside YLS1-021-4 and ML4-10 to determine if it was protective in the ALS cellular assays. Cells were transfected with TDP-43^{M337V} and at 6 hr post-transfection Neuro-2a cells were treated with either 1.5 µm ML4-10, ML4-11 or YLS1-021-4. At 72 hr post-transfection cells were fixed and examined for GFP-positive inclusions, TDP-43 mislocalisation and apoptotic nuclei as above. Quantification of TDP-43-positive cells demonstrated that YLS1-021-4 was not protective against mutant TDP-43^{M337V} induced cellular defects in neuronal cells, as above. However, ML4-11 was significantly protective against inclusion formation ($^{*}p < 0.05$) although it was not protective against mislocalisation induced or C) cell death. Hence Ml4-11 did display efficacy at 1.5uM, although it was not as protective as ML4-10 (**Figure 7).** However, we reasoned that this would still sufficient to detect interaction with its main binding partner. This more promising biotinylated analogue of quinazolinone ML4-10, ML4-11, was then used to undertake proteomic analysis to identify its major interacting partners.

Target identification

Proteomics analysis was then performed to identify the main target proteins interacting with ML4-11 in Neuro-2a cells, using biological replicates. Two control samples (lysis buffer and streptavidin beads) were also included with test samples - cell lysates incubated with streptavidin beads and 1.5 µm ML4-11. It is important to note that as cell lysates were used, these studies would therefore detect those proteins binding in the soluble cell fraction rather than the insoluble fraction, such as any membrane-bound proteins, including $GABA_A$ receptors. Samples were then reduced, alkylated and trypsin-digested to obtain tryptic digested peptides for mass spectrometry analysis.

From these studies, mass spectrometry identified 16 novel, unique proteins that were only present in the ML4-11 treated samples, and not the controls (**Table 1**). Interestingly, many of these targets are located in the nucleus, particularly the nucleous (**Figure 8**). This is an interesting finding given that nuclear processes are increasingly implicated as pathogenic events in ALS. To further confirm these findings, we performed immunocytochemistry using anti-streptavidin antibodies to examine the localisation of ML4-10 following treatment of cells (data not shown). We found that ML4-10 was localised primary within the nucleus, consistent with the normal localization of the novel protein targets below.

Table 1 Unique interacting partners of ML4-11 identified using proteomics

Gene	Protein
EEF2	Elongation factor 2
CNOT1	CCR4-NOT transcription complex subunit 1 -
NOP56	Nucleolar protein 56
RPN1	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 1
NDUFS1	NADH-ubiquinone oxidoreductase 75 kDa subunit
FKBP4	Peptidyl-prolyl cis-trans isomerase FKBP4
NONO	Non-POU domain-containing octamer-binding protein
RBM14	RNA-binding protein 14
DNAJC2	DnaJ homolog subfamily C member 2
GLS	Glutaminase kidney isoform
BAG3	BAG family molecular chaperone regulator 3
HNRPA2/B1	Heterogeneous nuclear ribonucleoproteins A2/B1
PHB1	Prohibitin 1
YWHAZ	14-3-3 protein zeta/delta
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1
SRSF7	Serine/arginine-rich splicing factor7

Gene Ontology enrichment analysis revealed that these proteins function primarily in five molecular processes (**Figure 9**): RNA binding, receptor signalling complex scaffold activity, translation regulator activity, transcription factor binding, and deaminase activity. This is significant given that the most prominent function was RNA binding, and dysregulation of multiple RNA binding proteins, including TDP-43 and FUS, is central to pathophysiology in ALS.

Figure 8 Cellular localization of proteomics hits: 16 novel interacting partners of ML4-11
Some proteins are present in multiple cellular locations; hence the total>100%

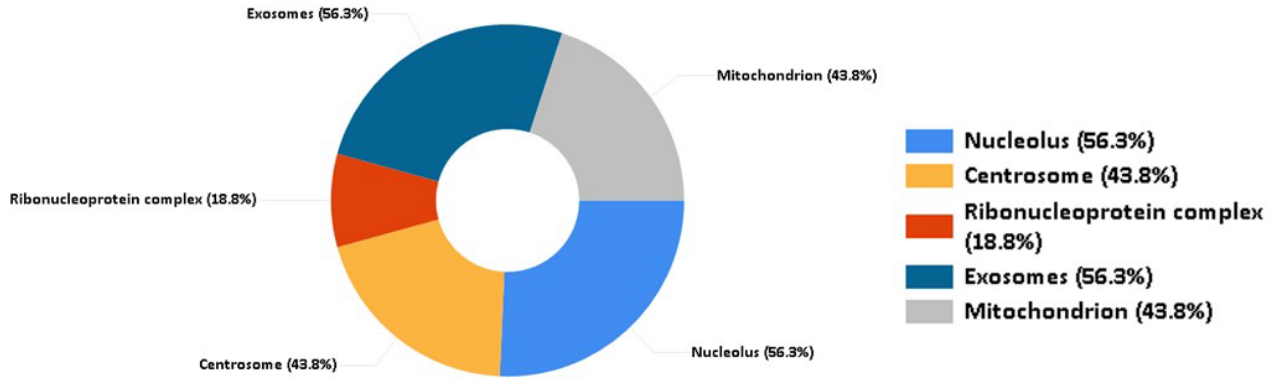
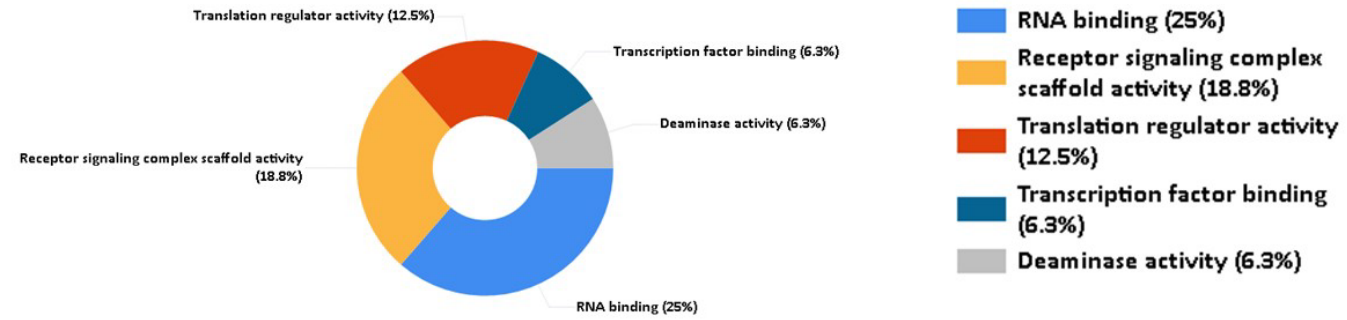


Figure 9 Molecular functions of proteomics hits: 16 novel interacting partners of ML4-11



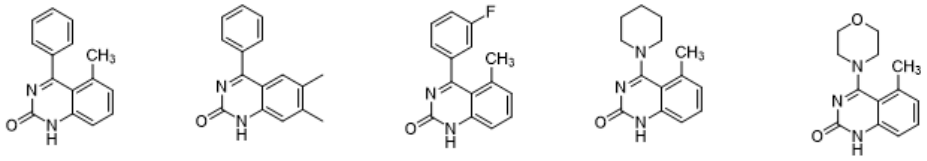
Prioritization of hits

In summary we have obtained one promising membrane target of our lead compound ML4-10: $GABA_A$, and 16 promising targets from the soluble fraction. We are currently prioritizing these hits to identify the primary target. For this purpose, we are performing proteomics again of both membrane and soluble fractions with competition studies to assess which of these targets binds the most strongly to ML4-10. This will enable us to identify the primary target of our lead quinazoline, ML4-10.

Specific Aim 2: Investigation of Delivery and Formulation Methods for ML4-10 and analogues

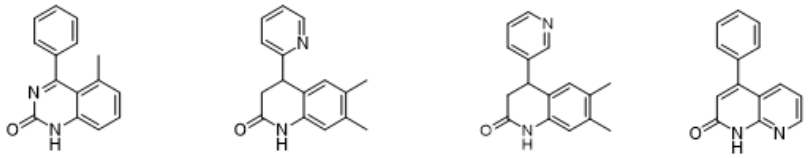
Major Task 1: Synthesis and evaluation of ML4-10 analogues

We have used StardropTM software to analyse 139 analogues of quinazolines, quinolinones previously made in the Abbott laboratory. We were able to use these oral CNS scoring results to identify the best 8 compounds out of 139. These 8 analogues are being further investigated for zebrafish tracking analysis (**Table 2 and Table 3**). Furthermore, we have also identified non-quinazolinone compounds that should be targeted for the synthesis of the corresponding quinazolinone analogue (**Table 3**).



Compound Name	ML4-10	ML1-2	RC10-1	RC10-4	RC10-3
Oral CNS Scoring Profile	**0.3187**	**0.3038**	**0.2398**	**0.2049**	**0.153**
Oxidative stress	0.695	0.756	0.894	0.909	0.805
Tracking Results	**1.466**	**1.139 (n=2)**	**1.212 (n=2)**	**0.971 (n=1)**	**0.854 (n=3)**
LogS	2.918	2.583	1.852	3.037	3.552
LogS @ pH7.4	2.918	2.583	1.852	1.069	0.9525
LogD	2.335	2.763	2.648	2.168	1.805
LogP	2.335	2.763	2.648	2.067	0.8795
MW	236.3	250.3	254.3	243.3	245.3
HBD	1	1	1	1	1
HBA	3	3	3	4	5
TPSA	45.75	45.75	45.75	48.99	58.22

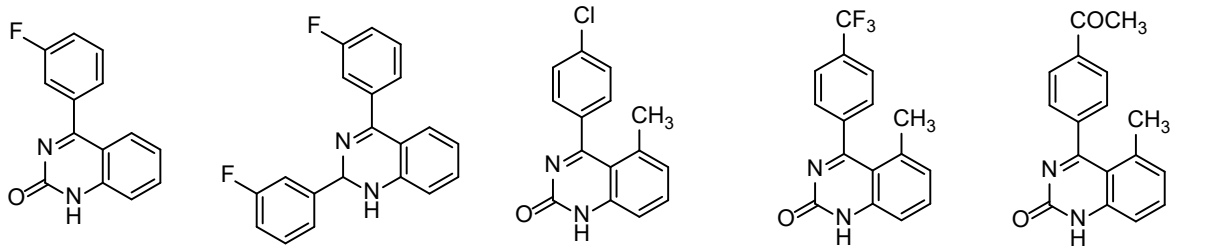
Table 2 Quinazolinone library compounds identified for further zebrafish testing.



Compound Name	ML4-10	C655 MIPS0019655	C656 MIPS0019656	CM5-6
Oral CNS Scoring Profile	**0.3187**	**0.4817**	**0.4813**	**0.313**
Oxidative stress	0.695	0.985	0.826	0.829
Zebrafish Tracking Results	1.466			
LogS	2.918	2.777	2.777	2.975
LogS @ pH7.4	2.918	2.777	2.777	2.975
LogD	2.335	2.349	2.349	1.598
LogP	2.335	2.349	2.349	1.598
MW	236.3	252.3	252.3	222.2
HBD	1	1	1	1
HBA	3	3	3	3
TPSA	45.75	41.99	41.99	45.75

Table 3 Non-quinazolinone library compounds identified for quinazolinone compound synthesis.

It should be pointed out that the Stardrop™ modelling and prediction figures correlated well with multiparameter optimisation or MPO scoring that we also use. MPO was described by Wagner et al. in 2016 (https://doi.org/10.1021/acschemneuro.6b00029) which is based on molecular weight, cLogP, cLogD, total polar surface area, number of hydrogen bond donors and pKA. We prefer compounds with MPO scores > 4 and where possible > 5.5 for synthesis. Using MPO parameter and Stardrop™ oral CNS scoring prediction, we have designed 25 analogues. This led to several analogues being synthesised as a priority (YLS1-25, YLS1-26, NB1-17, NB1-20 and NB1-38 in **Figure 10**). The other 20 analogues will be synthesised in the coming months.



ID: YLS1-026-3
MW: 240.24 g/mol
Mass: 8.68 mg
tPSA: 41.46
CLogP: 2.04
MPO score: 5.5

ID: YLS1-025-4
MW: 240.24 g/mol
Mass: 8.68 mg
tPSA: 41.46
CLogP: 2.04
MPO score: 5.5

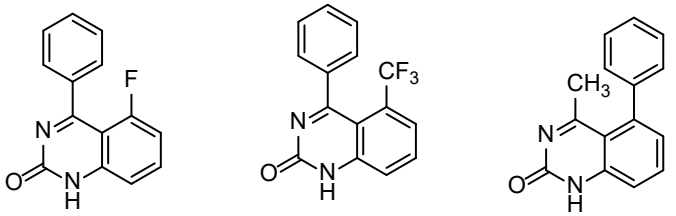
NB1-17
MW: 270.72 g/mol
Mass: 6.27 mg
tPSA: 41.46
CLogP: 3.11
MPO score: 4.5

NB1-20
MW: 304.27 g/mol
Mass: 5.02 mg
tPSA: 41.46
CLogP: 3.30
MPO score: 4.5

NB1-38
MW: 278.31 g/mol
Mass: 6.30 mg
tPSA: 58.53
CLogP: 1.92
MPO score: 5

Figure 10: Quinazolinone compounds of YLS1-26, YLS1-25, NB1-17, NB1-20, NB1-38.

We are interested in seeking out alternative groups at the 5-position of the quinazolinone structure that is equivalent or preferred to the methyl group of ML4-10. We have identified three commercially available anthranilic acids that result in a fluoro, trifluoro and alcohol at the 5-position and a fourth acid that gives a phenylpyridopyrimidinone (5-methyl analogue but with an additional nitrogen moiety within the ring). Two of these 5-substituted compounds have been prepared to date as YLS-45 and YLS1-46 (**Figure 11**), noting that each of these requires an independent four or five-step synthetic strategy.



ID: YLS1-045-3
MW: 240.24 g/mol
Mass: 10.05 mg
tPSA: 41.46
CLogP: 2.13
MPO score: 5.0

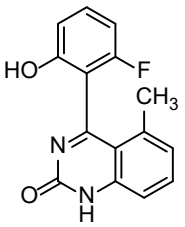
ID: YLS1-046-5
MW: 290.25 g/mol
Mass: 6.52 mg
tPSA: 45.75
CLogP: 3.23
MPO score: 4.5

ID: YLS1-071-3
MW: 236.2740 g/mol
Mass: 5.54 mg
tPSA: 41.46
CLogP: 2.15
MPO score: 5.0

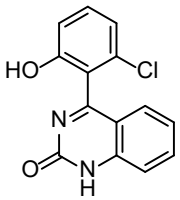
Figure 11: Quinazolinone compounds of YLS1-45, YLS1-46 and YLS1-71.

Another priority has been to explore the bioactivity of different compounds as a potential source of novel intellectual property. To this end, we have successfully developed a methodology to produce the ML4-10 analogue with the phenyl and methyl substituents reversed to produce YLS1-71 (**Figure 11**). Again, this compound has required a tailored synthetic strategy (four steps) to be developed from a 4-bromoindoline-2,3-dione precursor.

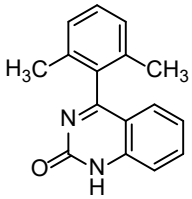
It has been hypothesised that the 5-methyl substituent is essential to result in the rotation of the pendant phenyl ring and improved physiochemical properties. It was thus proposed that the 5-methyl group may not be required if there is an appropriate substitution on the pendant phenyl group. This idea has been investigated by the addition of disubstituted boronic acids or esters to the quinazolinone scaffold using Suzuki coupling reactions (**Figure 12**). Two of these compounds retain the 5-methyl substituent due to the presence of a relatively small fluoro group but the larger chloro/alcohol/dimethyl substituted pendant rings were completed without a 5-substituted quinazoline.



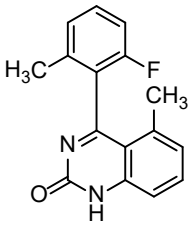
ID: YLS1-049-2
MW: 270.2634 g/mol
Mass: 9.30 mg
tPSA: 41.46
CLogP: 2.74
MPO score: 4.5



ID: YLS1-058-8
MW: 272.6880 g/mol
Mass: 10.36 mg
tPSA: 61.69
CLogP: 1.74
MPO score: 5.5



ID: YLS1-064-3
MW: 250.3010 g/mol
Mass: 5.00 mg
tPSA: 41.46
CLogP: 2.26
MPO score: 5.5



ID: YLS1-067-6
MW: 268.2914 g/mol
Mass: 9.25 mg
tPSA: 41.46
CLogP: 2.74
MPO score: 5.0

Figure 12: Disubstituted quinazolinone compounds of YLS1-49, YLS1-58, YLS1-64 and YLS1-67.

Alongside the synthesis of analogues of ML4-10, we are working on an alternative approach for MND target identification studies in case the proteomic studies with the biotinylated analogues of quinazolinone ML4-10 do not work as expected. To this end, we are exploring Liu and co-workers' recent investigation using dual-modifier labelling (*ACS Pharmacol. Transl. Sci.* 2022, 5, 859−871). Using this strategy, we have designed 5 initial analogues of ML4-10 (**Figure 13**). These analogues will be prepared and tested in competition with ML4-10 and will provide information about which areas of the ML4-10 scaffold will be tolerated by the dual modifier functionality, specifically the top aromatic ring or the quinazolinone moieties. Once these analogues are synthesised and tested, the next step will be to incorporate the bigger dual modifier in the most tolerated position of ML4-10.

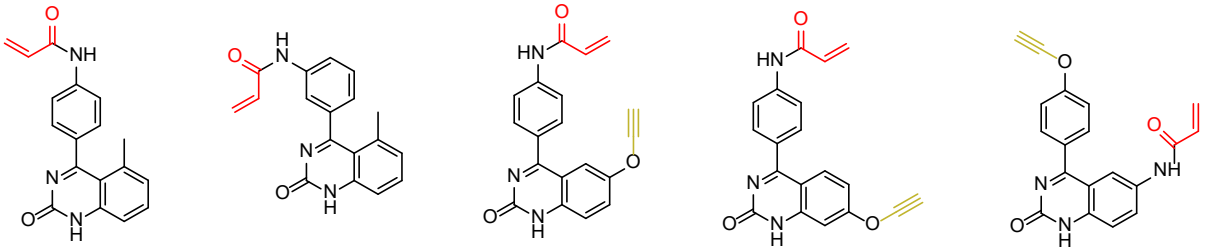


Figure 13. Proposed ML4-10 analogues to explore the identification of a target for NMD.

The chemistry team have also undertaken some work on the shelf stability of ML4-10 as a solid compound and potential alternate formulation options for *in vivo* work. Proton NMR spectroscopy was used to compare a sample of ML4-10 in deuterated dimethyl sulfoxide (d_6-DMSO) from when it was synthesised in February 2021 against the same sample stored as a solid for approximately 24 months and studied in March 2023. The near identical spectra, with an absence of any increased impurity peaks, indicate that the compound is stable as a solid at room temperature in ambient light from natural and artificial sources (**Figure 14**).

^{1}H NMR (DMSO-d6)

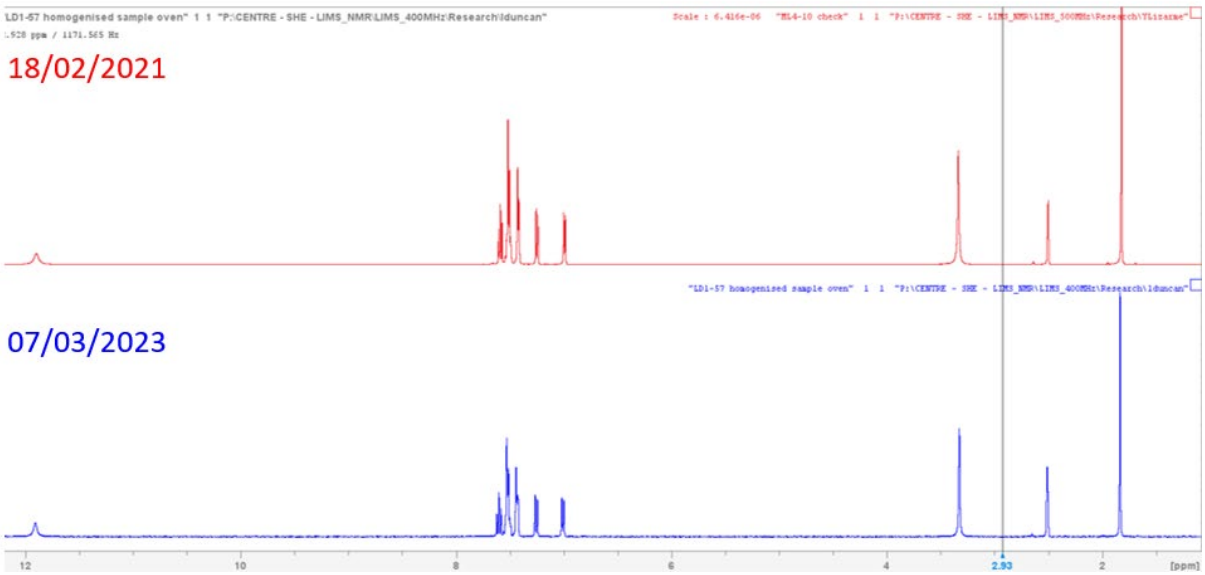
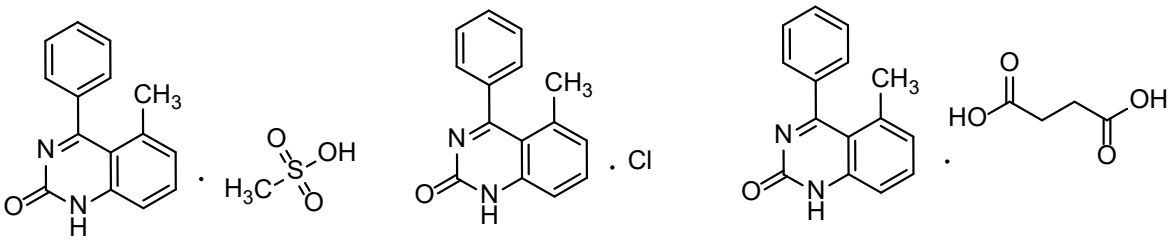


Figure 14. Proton NMR spectra obtained of newly synthesised ML4-10 (18/2/2021) compared with the same sample after approx. 24 months (7/3/2023).

Three salt forms (mesylate, hydrochloride and succinate) of ML4-10 were also prepared (**Figure 15**). Initial formulation studies of ML4-10 salts were carried out using a DMSO stock solution at both room temperature and at 45°C, with the addition of various amounts of PEG-400 and 0.9% NaCl solution. Further work was then carried out using a 50:50 DMSO and PEG-400 solution at 50 °C (**Table 4**). Optimum conditions of the ML4-10 mesylate salt were found to be 5:55:40 of DMSO:PEG-400:0.9%NaCl solution, which results in a significant reduction of DMSO required in the formulation of ML4-10 for *in vivo* studies (previously 20% now 5%). These results are thus expected to inform the formulation of later analogues for future *in vivo* studies.



Mesylate salt 56 mg Hydrochloride salt 54 mg Succinate salt 56 mg

Figure 15. Initial formulation exploration of salt forms of ML4-10.

Compound	Stock (DMSO/PEG400)	Solubility observation at RT	Solubility observation at 50°C
ML4-10 (original)	10 mg/200µL 0.212 M	Insoluble at RT	Insoluble after heating to 50°C
ML4-10 Mesylate	10 mg/200µL 0.150 M	Insoluble at RT	**Soluble after heating to 50°C**
ML4-10 Hydrochloride	10 mg/200µL 0.188 M	Insoluble at RT	Partially soluble after heating to 50°C

Compound	Stock (DMSO/PEG400)	PEG-400	0.9% NaCl solution	Solubility	DMSO%
ML4-10 Mesylate	10 µL	40 µL	50 µL	No	5%
ML4-10 Mesylate	**10 µL**	**50 µL**	**40 µL**	**Yes**	**5%**

Table 4. Formulation studies of ML4-10 salts using a DMSO/PEG stock solution.

Biological evaluation of ML4-10 analogues including salt and prodrug forms

We tested the following analogues in our panel of assays in ALS cellular models: NB1-17, NB1-38, YLS-10-26, YLS-10-46, NB1-20, YLS1-025-4, YLS1-045-3, YLS1-049-2, YLS1-058-8, YLS1-064-3, YLS1-067-6, YLS1-071-3. However, we did not detect any improvement in protective activity for these compounds, and several compounds displayed less activity than ML4-10. Hence we have not yet examined these compounds in primary neurons. Nevertheless, these findings provide significant information to aid in designing new compounds in the medicinal chemistry studies, and synthesis of further analogues is in progress.

IMPACT

We have identified the $GABA_A$ Flunitrazepam receptor as a target of our lead quinazolinone compound, ML4-10. Three off-target binding proteins were also identified, and future analogues of ML4-10 can now be tested for binding to these proteins. We have also identified 16 additional targets of ML4-10, nine of which are localised in the nucleus. This is a significant and interesting finding given the nucleus is increasingly implicated as a major cellular site of pathology in ALS. Studies are underway to prioritize these hits and determine which of these proteins are the primary drug target of ML4-10. In addition, we have shown that Diazepam (valium) is protective in our cellular ALS models, implying that it may have potential to be repurposed as a novel therapeutic in ALS.

Changes/Problems

None

Products

None

Participants & Other Collaborating Organizations

Professor Julie Atkin, Macquarie University, Australia

A/Prof Belinda Abbott, La Trobe University, Australia

A/Prof Angela Laird, Macquarie University, Australia

Special Reporting Requirements

None