EMBO Molecular Medicine

G-quadruplex-binding small molecules ameliorate C9orf72 FTD/ALS pathology in vitro and in vivo

Roberto Simone, Rubika Balendra, Thomas G. Moens, Elisavet Preza, Katherine M. Wilson, Amanda Heslegrave, Nathan S. Woodling, Teresa Niccoli, Javier Gilbert-Jaramillo, Samir Abdelkarim, Emma L. Clayton, Mica Clarke, Marie-Therese Konrad, Andrew J. Nicoll, Jamie Mitchell, Andrea Calvo, Adriano Chio, Henry Houlden, James M. Polke, Mohamed A. Ismail, Chad E. Stephens, Tam Vo, Abdelbasset A. Farahat, W. David Wilson, David W. Boykin, Henrik Zetterberg, Linda Partridge, Selina Wray, Gary Parkinson, Stephen Neidle, Rickie Patani, Pietro Fratta & Adrian M. Isaacs

Corresponding authors:
Stephen Neidle, UCL School of Pharmacy
Rickie Patani, UCL Institute of Neurology
Pietro Fratta, UCL Institute of Neurology
Adrian M. Isaacs, UCL Institute of Neurology

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 May 2017

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

You will see that the three referees find the study interesting and timely, with the main concern being similarly noted by both referees 1 and 3 who suggest performing supplementary analyses in flies to verify the compound effect on the eye phenotype and /or survival.

We would welcome the submission of a revised version for further consideration and depending on the nature of the revisions, this may be sent back to the referees for another round of review.

Please note that EMBO Molecular Medicine encourages a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Remarks):

In this manuscript, the authors identified unique small molecules that stabilize the G-quadruplex structure of C9ORF72 G4C2 repeat expansions. Using iPS-differentiated neurons and Drosophila that express G4C2 repeat expansions, the authors show the identified small molecules reduce both sense RNA foci and DPR proteins, two pathological features produced by G4C2 repeat expansions. The study is very interesting and timely as research teams in both academia and industry are deeply engaged in this area of preclinical space. Overall the manuscript is supported by compelling data with only minor comments noted. The specific points are list below.
1. In Figure 3D and E, the authors show compound 1273 decreases poly(GP) DPR protein in (G4C2)36 Drosophila model. Using this model, the authors should examine 1) whether compound 1273 can rescue the eye degeneration and impact lifespan; 2) whether compound 1273 influences RNA foci formation and/or c9 transcript levels.
2. It appears that the identified small molecules also bind to antisense G2C4 repeat expansions (Figure S1). The authors should examine whether these compounds can reduce antisense RNA foci in iPS-differentiated neurons or cell culture systems.

Referee #2 (Remarks):

Simone et al. report three structurally related compounds that reduce RNA foci and translation into poly-GP in iPSC and fly models. These compounds are the result of an in vitro screen of 138 preselected compounds for binding to the G4C2 RNA repeat and specifically stabilize the G-quartet structure. This exciting work goes all the way from compound screening, hard-core biochemistry to cellular and animals models. The compounds could be a lead for further optimization prior to clinical application in C9orf72 patients and should be interesting for the EMBO Mol Med readership.

The manuscript is well written and includes already all the major controls. Some small experiments could improve the manuscript further:

-The authors speculate that the compounds may either prevent binding of RBPs or facilitating degradation to the RNA. qPCR could be useful to distinguish between masking of the repeat RNA for FISH and actual degradation. How are antisense foci affected by the compounds given the preferential binding to sense RNA?
-Can DB1273 reduce repeat toxicity in the fly model? Does it have toxic side effects in flies at the used concentration?
-Figure S8 should be merged with Figure 2, because it contains key data.

Referee #3 (Remarks):

Simone et al., reported that G-quadruplex-binding small molecules obtained from aromatic diamidines ameliorated RNA foci formation and poly(GP) levels which were provoked by disease-associated G4C2 repeats in C9orf72. Three similar-structured compounds, DB1246, DB1247, and DB1273 were able to decrease the number of RNA foci in human iPSC-derived cortical/motor neurons which harbored mutant C9orf72. Moreover, the authors demonstrated that administration of those molecules reduced the expression levels of poly(GP) in a fly model of C9FTD/ALS. The presented data seems enough to convince the beneficial effects of the compounds at least on

silencing "aggregation formation" of both RNA and DPR(s). However, the manuscript did not contain the data of phenotypic change(s) or the molecular mechanism which weaken the impact of the manuscript. Since the authors have previously shown that the same fly model harboring mutant C9orf72 exhibited neurodegeneration and shorter survival, it would be necessary to investigate whether the compounds have beneficial effects on survival and/or neurodegeneration in the fly model.

Major issues
1. As described above, the authors need to show whether the compounds have beneficial effects on the survival and/or eye degeneration observed in C9FTD/ALS fly model they used. The previous work from the same group has clearly shown the model had a neurodegeneration phenotype (Mizielinska et al., 2014).
2. In the discussion section authors described, "Decreased RAN translation could occur through impeding either assembly of the ribosomal pre-initiation complex (PIC), or subsequent PIC scanning of the RNA". Addressing this issue, the authors can enhance the quality of the manuscript which is required for the journal.
3. It is necessary to investigate whether the compounds can reduce the expression levels of other DPRs including poly(PR), poly(GR), and/or poly(GA).
4. It would be helpful if the authors describe more biological signature of the compounds, such as tissue distribution.

Minor issues
1. In Figure 1B, "DB-1273" should be "DB1273".
2. In P4 L12, the authors described, "three of these molecules had very similar chemical structures, differing by only two atoms, indicating a genuine structure-function relationship." I just wondered if there were no similar chemical structured observed in the other 135 molecules.
3. In Figure S3, there are two 'RNA-DB1247'. The last one seems to be RNA-DB1273.
4. It would be helpful if the authors add the result of RNA-DB1273 to Figure S4.

Mizielinska, S., Gronke, S., Niccoli, T., Ridler, C.E., Clayton, E.L., Devoy, A., Moens, T., Norona, F.E., Woollacott, I.O., Pietrzyk, J., et al. (2014). C9orf72 repeat expansions cause neurodegeneration in Drosophila through arginine-rich proteins. Science 345, 1192-1194.

1st Revision - authors' response 19 August 2017

We thank the reviewers for their helpful comments, which have considerably strengthened our findings; our point by point response is below. We are particularly excited that the one consistent request across the three reviewers – to investigate the effect of DB1273 on the survival of our (G4C2)36 flies – was performed and shows a significant improvement. We are also very excited that we were able to show that this treatment significantly reduces poly(GR), the toxic DPR in our flies. As far as we are aware, this is the first report of a potential therapeutic strategy for C9FTD/ALS that has shown a significant and quantitative reduction in the toxic DPR poly(GR). As such we feel the manuscript is now substantially improved and hope it is now acceptable for publication in EMBO Molecular Medicine.

Reviewer 1
1. In Figure 3D and E, the authors show compound 1273 decreases poly(GP) DPR protein in (G4C2)36 Drosophila model. Using this model, the authors should examine
1) whether compound 1273 can rescue the eye degeneration and impact lifespan;
Response
We treated our (G4C2)36 Drosophila with DB1273 and observed a significant increase in survival, indicating it has a beneficial functional effect in vivo, now shown in Fig. 3D. We thank the reviewer for raising this important point as these new findings considerably strengthen the manuscript.

2) whether compound 1273 influences RNA foci formation and/or c9 transcript levels.
Response

We agree that this is an important control. We performed quantitative real-time PCR and show that DB1273 does not influence C9orf72 transcript levels in our treated (G4C2)36 Drosophila, now described in the Fig. S11. This indicates the reduction in DPRs is a specific inhibition of RAN translation rather than a decrease in transcription of the transgene and further strengthens our findings.

2. It appears that the identified small molecules also bind to antisense G2C4 repeat expansions (Figure S1). The authors should examine whether these compounds can reduce antisense RNA foci in iPS-differentiated neurons or cell culture systems.
Response
We agree this is an interesting experiment. Unfortunately our antisense FISH protocol identified only low numbers of antisense foci in iPSC-neurons so we were unable to determine whether the small molecules had an effect. We feel that while interesting, this experiment is not essential for underpinning our main conclusions and can be addressed in future studies.

Reviewer 2
1. The authors speculate that the compounds may either prevent binding of RBPs or facilitating degradation to the RNA. qPCR could be useful to distinguish between masking of the repeat RNA for FISH and actual degradation. How are antisense foci affected by the compounds given the preferential binding to sense RNA?
Response
As stated above, our antisense FISH protocol identified only low numbers of antisense foci in iPSC-neurons so we were unable to determine whether the small molecules had an effect. The qPCR experiment would also be interesting, but due to our focus on the in vivo experiments we were not able to do this in time. We feel that these experiments, while interesting, are not essential for underpinning our main conclusions and can be addressed in future studies.

2. Can DB1273 reduce repeat toxicity in the fly model?
Response
We agree this is a key question. As described above, we now show that DB1273 can reduce toxicity in our fly model, now described in Fig. 3D, these data significantly enhance our findings.

3. Does it have toxic side effects in flies at the used concentration?
Response
We tested DB1273 in control flies at the used concentration and we do not observe toxicity, now described in Fig. S12.

4. Figure S8 should be merged with Figure 2, because it contains key data.
Response
We have now promoted Figure S8 to an expanded view figure (Fig. EV2) to make it more visible.

Reviewer 3
1. As described above, the authors need to show whether the compounds have beneficial effects on the survival and/or eye degeneration observed in C9FTD/ALS fly model they used. The previous work from the same group has clearly shown the model had a neurodegeneration phenotype (Mizielinska et al., 2014).
Response
We agree this is a key question. As described above, we now show that DB1273 can reduce toxicity in our fly model, now described in Fig. 3D, which significantly enhances our findings.

2. In the discussion section authors described, "Decreased RAN translation could occur through impeding either assembly of the ribosomal pre-initiation complex (PIC), or subsequent PIC scanning of the RNA". Addressing this issue, the authors can enhance the quality of the manuscript which is required for the journal.
Response
We agree that this would be very interesting to determine, however, currently the mechanism(s) of RAN translation are not known. Therefore we feel it is beyond the scope of

this work to define this mechanism and how the small molecules specifically inhibit it. We have therefore removed this speculation from the discussion.

3. It is necessary to investigate whether the compounds can reduce the expression levels of other DPRs including poly(PR), poly(GR), and/or poly(GA).
Response
We agree that particularly measuring the levels of poly(GR) would strengthen our findings as poly(GR) is the toxic DPR in our flies and has widely been shown to be toxic. We therefore developed a sensitive and specific poly(GR) MSD immunoassay, now described in Figure EV4. We used this assay to show that poly(GR) is significantly reduced, in vivo, in Drosophila treated with DB1273, now described in Fig. 3E.

We note that even though poly(GR) is thought to be one of the toxic DPRs, we are not aware of any other study that has quantitatively shown a reduction in poly(GR) through any therapeutic approach, (largely because of the difficulty in detecting poly(GR)). We therefore feel that our demonstration of a reduction in poly(GR) is a step forward for the field and considerably strengthens our findings.

4. It would be helpful if the authors describe more biological signature of the compounds, such as tissue distribution.
Response
We have now investigated the tissue distribution of DB1273, now described in figure EV5. We show broad distribution in the gut with low but consistent presence in the central nervous system. We also discuss this finding further in the discussion

Minor issues
1. In Figure 1B, "DB-1273" should be "DB1273".
Response
We thank the reviewer for spotting this, we have now corrected it.

2. In P4 L12, the authors described, "three of these molecules had very similar chemical structures, differing by only two atoms, indicating a genuine structure-function relationship." I just wondered if there were no similar chemical structured observed in the other 135 molecules.
Response
We thank the reviewer for raising this point – no other compound had a similar structure, we therefore now state in the results section 'No other compounds in the compound set have similar features of two linked five-membered rings'.

3. In Figure S3, there are two 'RNA-DB1247'. The last one seems to be RNA-DB1273.
Response
Thanks again we have now corrected.

4. It would be helpful if the authors add the result of RNA-DB1273 to Figure S4.
Response
We were not able to perform fluorescence anisotropy on DB1273 due to its lower inherent fluorescence. We have clarified this by stating in the fluorescence anisotropy methods 'Florescence anisotropy was performed for DB1246 and DB1247 but was not possible for DB1273 due to its weaker fluorescence'.

2nd Editorial Decision 20 September 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now fully supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

- I could not find any details about how the iPSC were derived from and any ethical statement regarding patients. Can you please refer to our guidelines and provide the missing information?

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

The authors have addressed all the previous concerns and is markedly improved. The paper is now suitable for publication.

Referee #2 (Comments on Novelty/Model System for Author):

The authors fully addressed all the critical points and provide now in vivo data for the efficacy of the new compound, which correlates with the new poly-GR ELISA data. The DB1237 compound is an interesting candidate for further development to increase brain delivery in vivo. I fully recommend publication in EMM.

Referee #3 (Remarks for Author):

The authors adequately answered requested questions by performing additional experiments. The manuscript is now suitable for publication in EMBO Molecular Medicine.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Adrian Isaacs
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Manuscript Number:	EMM-2017-07850

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We performed a power calculation for the number of flies to include in order to detect an effect of the treatment, given the mean and standard deviation of the observed phenotype. Sample size for other experiments was chosen in accordance with other similar studies in the literature.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	As above
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	There were no a priori criteria for exclusion of samples or flies.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Yes, cells and flies were randomly assigned to groups throughout the study.
For animal studies, include a statement about randomization even if no randomization was used.	Flies were randomly assigned to treatment conditions for all experiments.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Yes, experimenters were blinded to experimental condition during the the analyses for cell studies and fly survival experiments.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For the fly survival study, the experimenter was blinded to the treatment condition.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used Graphpad Prism to assess whether datasets were normally distributed.
Is there an estimate of variation within each group of data?	Yes, we have given the SEM or SD for the data.
Is the variance similar between the groups that are being statistically compared?	Yes, unless stated, and in this case a non-parametric test was used.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All antibodies used are referenced within the Methods section, with details of catalog number for all commercially available antibodies.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	All cell lines used are detailed within the Methods section, and were regularly tested for Mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Detailed in Methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No live vertebrates were used in this study.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	No live vertebrates were used in this study. For fly studies, the guidelines, where relevant, have been complied with, and full details are given in the Methods section.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No