Targeting *Toxoplasma gondii* CPSF3 as a new approach to control toxoplasmosis

Andrés Palencia, Alexandre Bougdour, Marie-Pierre Brenier-Pinchart, Bastien Touquet, Rose-Laurence Bertini, Cristina Sensi, Gabrielle Gay, Julien Vollaire, Véronique Josserand, Eric Easom, Yvonne R. Freund, Hervé Pelloux, Philip J. Rosenthal, Stephen Cusack and Mohamed-Ali Hakimi

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Transaction Report:

(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.)

Editor: Céline Carret

1st Editorial Decision 22 December 2016

Dear Ali,

Please find enclosed the reports on your manuscript. After many deliberations within the editorial office, but also including the referees, we finally decided to accept your article in EMBO Molecular Medicine pending the following final amendments:

Please provide a point-by-point response to the referees as a doc file. We will not ask you to test the drug on other apicomplexans as the other paper does that on malaria, that's enough in our view to validate the relevance and efficacy-I would like you to strongly mention this point. Please refer to the malaria paper accordingly, and make sure they do refer to yours as well. As in your letter from the 15/12/16, please highlight the differences between the studies and we would very much like if you could add the extended structural analysis you mention.

Please provide the final version of your article as soon as you can. Please note that while we require the above issues addressed, your paper is officially accepted today, 22 Dec. 2016.

Congratulations on your interesting and extremely well performed work, I do not get to accept papers after 1 round of review very often!

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

Referee #1 (Comments on Novelty/Model System):

This is a nice study demonstrating that some oxaborales can inhibit Toxoplasma and identifying the target of one active compound based on mutational studies. The work does not address a fundamental mechanism but rather is an applied study of identifying potential leads for new therapeutics. I did not find the class of inhibitors or target identified to be novel or compelling examples of new biology. As such, I feel this is better suited for a journal that specializes in the identification of new antibiotics.

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

In this study the authors identified Toxoplasma CPSF3, a protein that is involved in mRNA processing and highly conserved in eukaryotes as a novel, potential drug target for apicomplexan parasites.
They used Toxoplasma as a suitable model system to identify CPSF3 and using state of the art reverse genetic approaches convincingly showed also drug resistance mutations. The manuscript is of the highest technical quality and this reviewer couldn't identify any technical issues or probelms with interpretation of the data.
The only 2 points the authors should address is to test their identified inhibitor AN3661 also for other apicomplexan parasites (i.e. Eimeria, Neospora, Cryptosporidium, Plasmodium). The authors refer several times to a study submitted for Plasmodium falciparum (Sonoiki et al., submitted). It would be interesting to compare these two studies directly (are they submitted back-to-back)?

Another point to address is to test this inhibitor on persistent stages (tissue cycts), since this is the more medical relevant stage. Once chronically infected tissue cysts remain dormant and can reactivate. So far no useful drugs are available for this stage, which would be the im[portant one to target. Did the authors perform experiments to look at persistence?

Referee #2 (Remarks):

The authors convincingly demonstrate CPSF3 as the target for a highly active inhibitor AN3661 and using a combination of chemical mutagenesis and cas9/CRISPR strategies demonstrate resistance mechanism.
At this point the study is restricted to Toxoplasma and the relevance could be increased by including other apicomplexans (see comments above). Instead of measuring IC50 in different apicomplexans, the authors could also consider to use Toxoplasma as a model system and demonstrate that PfCPSF3 (for example) complements TgCPSF3 and can be inhibited by the same compound.

I think this study is best suited as short report and this reviewer fully supports publications.

Point-by-point response

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identification of new antibiotics.

Thank your for your positive comments. We agree with the reviewer on the point about the mechanism. Future biochemical and structural studies will be required to unveil the inhibition mechanism of AN3661, and in our opinion this is not the scope of this manuscript that focuses on the discovery of a novel drug target of *Toxoplasma*. We respectfully disagree on the comment regarding the novelty of our work. We extensively revised bibliography (prior to submission) and did it again now, and we did not find any study describing CPSF3 as a drug target with therapeutic interest. We are confident that, for the first time, CPSF3 is described as drug target in our manuscript.

The key findings supporting the novelty of our study are:

1) AN3661 inhibits *T. gondii* growth in fibroblasts at low micromolar concentrations, and its potency is comparable to the clinically relevant drug pyrimethamine.
2) AN3661 when orally administered to mice, controls otherwise lethal infections with comparable, if not better, efficacy to sulfadiazine.
3) Mice treated with AN3661 developed protective immunity to subsequent *Toxoplasma* infections. Serologic analysis confirmed enriched levels of anti-*Toxoplasma* specific antibodies.
4) Using forward genetics, parasite lines resistant to AN3661 were selected and all had the mutations in CPSF3, a homolog of mammalian CPSF-73, which is involved in mRNA processing.
5) Using structural modelling we found that the mutations clustered at the putative catalytic site of *T. gondii* CPSF3 and when introduced by CRISPR/Cas9 editing in wild type parasites those conferred resistance to AN3661 both *in vitro* and *in vivo*.

We believe that our findings are of sufficient novelty and will be of general interest to the EMBO Molecular Medicine readership, as they show *Tg*CPSF3 as a promising target that for the generation of new drugs against toxoplasmosis.

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We fully agree with the reviewer on this point. Indeed, a concomitant study by our collaborators (Sonoiki et al, pending final acceptance in Nat. Comm.), describes the *in vitro* activity of AN3661 against *Plasmodium* and its *in vivo* efficacy in a murine malaria model.

Surprisingly, and despite the different selection strategies used to obtain resistant mutants to AN3661, we found that the mutations clustered to the same region of the catalytic site of CPSF3 of *Plasmodium* and *Toxoplasma*. Indeed, two out of the three residues mutated in *Toxoplasma* resistant strains are identical to those found in *Plasmodium* strains. Concretely, *Toxoplasma* CPSF3 residues Y483 and E545 are equivalent to the *Plasmodium* residues Y408 and D470, respectively. Similar to our study, knock-in of mutations in *Plasmodium* recapitulated resistance to AN3661 *in vitro*. We think these identical mutations and the agreement on the findings strength our conclusion that AN3661 acts on CPSF3 and suggests a common inhibition mechanism.

In addition to the above findings, we provide additional key findings in our manuscript with *Toxoplasma*:

1) **Animal studies that validated the drug-target *in vivo* by using the murine model of toxoplasmosis**. These experiments were done not only using wild-type parasites, but also a strain resistant to AN3661 (TgCPSF3^{E545K}, which bears just a single mutation). When mice were infected with this strain and treated with AN3661, they did not survive *Toxoplasma* infection, similarly to untreated mice. This experiment provides the unique evidence for AN3661 working as an active compound *in vivo* on *Toxoplasma* through the inhibition of CPSF3 activity.

2) **Extended structural analysis to investigate the role of TgCPSF3 mutations on the resistance to AN3661**. This analysis shows that rather than clashing with AN3661, the mutations Y483N and Y328C distort the geometry of the drug binding pocket with consequent loss of contacts between the protein and AN3661 that would decrease its binding affinity. The mutation E545K has an indirect effect on the drug binding pocket that is mediated by Y483, again likely via the perturbation of the drug binding pocket (please see **Figure 3defg**). Given that the CPSF3 mutations in *Plasmodium* (Y406, D470) are equivalent in *Toxoplasma*, it is plausible that they share the same mechanism of resistance, and hence the analysis of the resistance mechanism that we provide in this manuscript would be useful for the design of future CPSF3 inhibitors against any of these parasites.

3) **Development of immunity to toxoplasmosis after treatment by AN3661.** *In vivo* studies showing that animals that were healed of toxoplasmosis by AN3661 could survive, without further drug treatment, new lethal infections of two virulent *Toxoplasma* strains (RH and GT1), whereas mice that were never treated with AN3661 succumbed. Interestingly, serologic analyses revealed enriched levels of anti-*Toxoplasma* specific antibodies in treated animals, strongly suggesting that animals treated with AN3661 developed protective immunity to toxoplasmosis. These promising results could have future implications for vaccine development or other preventive therapies.

Another point to address is to test this inhibitor on persistent stages (tissue cycts), since this is the more medical relevant stage. Once chronically infected tissue cysts remain dormant and can reactivate. So far no useful drugs are available for this stage, which would be the important one to target. Did the authors perform experiments to look at persistence?

This is a very good point, and we acknowledge the suggestion. In our experiments with *Toxoplasma* type II strains (which cause chronic infection and form cysts containing bradyzoites in mice deep-tissues) we showed that AN3661 has excellent efficacy to reduce parasitemia to undetectable levels after a 7-Day oral treatment. However, despite the presumed activity against tachyzoites (as we are showing in HFF cells and the acute infection murine model), we have no evidence of AN3661 acting on bradyzoites-containing cysts.

Chronic toxoplasmosis is associated with tissue-localized cysts, primarily in the brain of both mice and human. Unfortunately, treatment of chronic toxoplasmosis is hampered by the poor drug brain penetration to achieve therapeutic concentrations. Thus, the combined administration of sulfadiazine and pyrimethamine has shown efficacy against acute toxoplasmosis but failed against chronic cerebral toxoplasmosis (Faucher B et al., 2011; J Antimicrob Chemother. 2011 Jul; 66(7):1654-6). To properly address this question one should test first the AN3661 bioavailability in the brain, and then monitor AN3661 efficiency against cysts in chronically infected mice. Such experiment remains extremely challenging and incorporating it into this manuscript will not be possible in a reasonably short timeline.

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At this point the study is restricted to Toxoplasma and the relevance could be increased by including other apicomplexans (see comments above). Instead of measuring IC50 in different apicomplexans, the authors could also consider to use Toxoplasma as a model system and demonstrate that PfCPSF3 (for example) complements TgCPSF3 and can be inhibited by the same compound.

We did not perform this experiment because AN3661 was tested directly against *Plasmodium*, which indeed showed *in vitro* activity and *in vivo* efficacy (Sonoiki et al, pending final acceptance in Nat. Comm). However, for the purpose of supporting CPSF3 as the target of AN3661 in *Toxoplasma*, we performed an experiment equivalent to the one suggested by the referee. We introduced a copy of TgCPSF3^{E545K} (resistant to AN3661) into *Toxoplasma* wild type parasite. The resultant parasite line, containing two CPSF3 copies, wild type and mutant TgCPSF3^{E545K}, recapitulated resistance at a AN3661 concentration >5-fold its IC50 value and efficiently restored parasite growth (see Figure EV3). This experiment further confirmed CPSF3 as the target of AN3661.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: HAKIMI Mohamed-ali
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2016-07370

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 $\chi 2$ 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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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?	The sample size was determined empirically. The CBA mice infected by the type I RH strain has no chance to survive infection by a single parasite. Therefore our assays conducted with 10^3 parasites corresponds to an extrem experimental treatment and the effect of currative treatment can be revealed without ambiguity from three independent experiments thar were performed each with 3 mice per condition.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Assays developped by the community working on Toxoplasma use a total of n = 6 per group of two independent experiments has been shown sufficient to give representative data.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	Randomization of animals was applied for each assay using animal model for toxoplasmosis to reduce bias in mice selection and outcome assessment.
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was applied to determine the effect of the parasite strains of drug treatment on mice survival to reduce bias in mice selection and outcome assessment.
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.	Figure 4A: significance was tested using Log-rank (Mantel-Cox) test (***, p-value = 0.0009) and Gehan-Breslow-Wilcoxon test (p-value = 0.0015). Figure 4B (lower panel), significance was assessed by unpaired Student's t-test (*P = 0.020).
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

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).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	Animal studies were performed with female CBA/JRj mice purchased from Janvier (Le Genest-Dt-Isle, France); they were 7–9 week-old. Aminal housed under a 12-h light cycle with water and food ad libitum in an approved animal experimentation establisment (C38 516 10 006, 2016/05/10).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal procedures were validated by the Animal Care Committee of the University of Grenoble and the French Ministry of Higher Education and Scientific Research in accordance with the guidelines established by the French Decree (Decree N 2013-118 of February 1st, 2013) on the protection of animals used for scientists purposes following European Directive 2010/63/EU. The reference number is APAFIS#3938-20160204155261 2v2.
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.	The scientific manuscript has satisfied, and is in compliance with the ARRIVE guidelines.

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 accession codes for deposited data. See author guidelines, under '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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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

23. 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.	NA