nature portfolio

Peer Review Information

Journal: Nature Microbiology
Manuscript Title: Cryptosporidium PI(4)K inhibitor EDI048 is a gut-restricted parasiticidal agent to treat pediatric enteric cryptosporidiosis
Corresponding author name(s): Dr Ujjini Manjunatha

Editorial Notes:

This manuscript has been previously reviewed at another journal. This document only contains reviewer comments, rebuttal and decision letters for versions considered at Nature Microbiology. Mentions of prior referee reports have been redacted

Reviewer Comments & Decisions:

Decision Letter, initial version:

Message: 19th February 2024

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Dear Ujjini and Thierry,

Thank you for your patience while your manuscript "Rational design of a gastrointestinal-targeted parasiticidal agent to treat pediatric enteric cryptosporidiosis" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the of this email. You will see from their comments below that while they find your work of interest, some important points are raised. We are very interested in the possibility of publishing your study in Nature Microbiology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

In particular, you will see that referee #1 feels the choice of a 10mg/kg dose should be more clearly explained and that the clarity and interpretation of the clinical outcomes needs to be improved. The referee also suggests that in case data on clinical outcomes following cessation of treatment are available, it would be good to include these. Referee #2 asks for a comment on whether EDI048 also affects membrane biogenesis of the microgametes. Furthermore, this referee also asks to further discuss the possibility of resistance development in the Discussion section of the manuscript. Referee #3 asks to comment on whether compound 6 has activity against human PI (4) Kinase or has other off target safety risks. The referee also asks whether the compound will penetrate cells. The rest of the referees' reports are clear and the remaining issues should be straightforward to address.

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per display item, so if you need more space, you could move a Figure or Table to Supplementary Information.

Some reduction could be achieved by focusing any introductory material and moving it to the start of your opening 'bold' paragraph, whose function is to outline the background to your work, describe in a sentence your new observations, and explain your main conclusions. The discussion should also be limited. Methods should be described in a separate section following the discussion, we do not place a word limit on Methods.

Nature Microbiology titles should give a sense of the main new findings of a manuscript, and should not contain punctuation. Please keep in mind that we strongly discourage active verbs in titles, and that they should ideally fit within 90 characters each (including spaces).

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

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Figure legends must provide a brief description of the figure and the symbols used, within 350 words, including definitions of any error bars employed in the figures.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

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Please include a statement before the acknowledgements naming the author to whom correspondence and requests for materials should be addressed.

Finally, we require authors to include a statement of their individual contributions to the paper -- such as experimental work, project planning, data analysis, etc. -- immediately after the acknowledgements. The statement should be short, and refer to authors by their initials. For details please see the Authorship section of our joint Editorial policies at http://www.nature.com/authors/editorial_policies/authorship.html

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* ensure it complies with our format requirements for Letters as set out in our guide to authors at www.nature.com/nmicrobiol/info/gta/

* state in a cover note the length of the text, methods and legends; the number of references; number and estimated final size of figures and tables

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We hope to receive your revised paper within two months. If you cannot send it within this time, please let us know.

We look forward to hearing from you soon.

Yours sincerely,

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Reviewer Expertise:

Referee #1: Anti-parasitic drug discovery
Referee #2: Drug discovery, apicomplexan parasites
Referee #3: Cryptosporidium, pre-clinical drug development

Reviewers Comments:

Reviewer #1 (Remarks to the Author):

This manuscript, by Manjunatha et al, describes the characterization of EDI048, an inhibitor of the Cryptosporidium PI(4)K and is a clear and important extension of work previously published by the authors. The authors rightly note the importance and impact of human cryptosporidiosis, particularly in low-resource settings, and its effect on vulnerable

populations (people with AIDS and children under the age of 5). Moreover, there is a clear gap in the availability of effective treatments, with nitazoxanide being the only approved drug, which is sub-effective in the most vulnerable patients. The studies focus primarily on the hypothesis that Cryptosporidium parasites, residing within intracellular vacuoles of intestinal enterocytes, can be effectively treated by limiting exposure to the GI tract, which would provide advantages, particularly in terms of safety. This is a question that has challenged efforts in discovery of anti-cryptosporidials but is also of interest for considering treatment of other GI infections or GI-related conditions.

The studies benefit from the use of KDU731, a closely-related and equipotent analog (and predecessor) of EDI048 which displays uptake and systemic circulation; they convincingly show that circulation of KDU731 does not contribute the its anti-cryptosporidial efficacy in a well-established mouse model. The medicinal chemistry strategy employed to develop rapidly-metabolized analogs, leading to EDI048, is sensible and the characterization tracks with the hypothesis. The only confounding question here, as it relates to their hypothesis, is that compound 4 appears to have many of the desirable properties of EDI048, but without in vivo efficacy. This reviewer cannot comment on whether physicochemical or other properties of compound 4 would explain the result, but would be interested if the author have a hypothesis. The drug metabolism and PK studies performed are likewise well thought out and support the central hypothesis. It is welcome to see that the authors examined PK in both healthy calves and in calves with cryptosporidial diarrhea, as increased transit times would likely effect local exposure and this consideration will be critical for human dose prediction. One element of drug metabolism that is not addressed, but may be beyond the scope of the present study, is the consideration that in the target population of children under the age of 5 (and particularly under the age of 2) drug metabolism can be different than in adults, impacting exposure.

The mechanistic work related to the interactions between EDI048 and PI4K is quite elegant, despite lacking a CpPI4K structure. While the use of homology modeling can be of limited use, the authors use this approach to formulate and test key binding hypotheses using point mutations and chimeric proteins that support the basis of EDI048 binding to CpPI4K and specificity over HsPI4K. Likewise, the kill kinetic studies are suitable, and underlie an advantage of EDI048 or other parasiticidal drugs would have over NTZ, which is only a static drug. While the conclusions are supported by the data, it is some confusing that EDI048 does not appear to display any dose response in the luciferase-based assay shown in Supplementary Fig 1, whereas KDU731 does.

Lastly, the authors look at the efficacy of EDI048 in a neonatal calf model, showing improvement in parasitological and clinical endpoints. While these are challenging studies, and the results do support the efficacy of EDI048, this reviewer does have some questions related to the performance and interpretation of this study. The soft-drug strategy does have the challenge of developing clear PK/PD relationships, so it is not clear whether the choice of 10mg/kg for this study was pragmatic, or based on any type of dose prediction or understanding of exposure. This is only potentially problematic in that the demonstrated efficacy could be an underestimate, and while further calf studies may be warranted to further the development of EDI048, this reviewer would not recommend them here. The most significant criticism this reviewer has for this study is the clarity and interpretation of clinical outcomes. It is not clear whether the clinical scores in Fig 3 represent composite

clinical scores or solely fecal consistency scores. This may not change the interpretation, but multiple clinical measures are mentioned and it is not clear how the authors handle those. The improvement in clinical outcomes could be more clearly addressed. It does seem as if there is a 2-3 day improvement in fecal consistency scores, but there is significant inter-animal variability. On line 290, the authors claim that by 72 hours, 5 of 7 animals showed no signs of diarrhea (extended data figure 5). While this is technically true, 4 of these 5 animals go on to experience bouts of mild-to-moderate diarrhea throughout the remainder of the treatment window. More appropriate is the cited metric of days of severe or mild-to-moderate diarrhea suffered. If the authors have data on clinical outcomes following cessation of treatment, it would be worth including these. They should also explain the absence of data points in the clinical measures for untreated calves, and how that was taken into consideration for data analysis. In the discussion, the authors state that EDI048 treatment resulted in rapid resolution of diarrhea in this study, but it would be more appropriate to say that treatment resulted in significant improvement of diarrheal symptoms.

Overall, despite some critique, this is a highly original and meritorious manuscript with implications not just for the development of needed anticryptosporidial agents, but also treatment of GI disease generally. The key hypothesis that local exposure is necessary and sufficient for treatment of Cryptosporidium infection with EDI048 is well-supported by the data. Beyond the thoughtful and interesting pharmacological strategy described, this also represents perhaps the most advanced candidate for treatment of cryptosporidiosis.

Minor comments:
• Line 135: The sentence is likely intended to be "While the detailed structure activity relationship…"
• Line 385: should read "potent paraciticidal activity"
• While the stated statistical methods appear appropriate, he authors should review figure legends to ensure definition of error bars, as this is not done uniformly.

Reviewer #2 (Remarks to the Author):

This is a well designed and comprehensive study on the design and evaluation of a new drug to treat cryptosporidiosis. The study is unique in that the lead molecule was specifically designed to have low systemic exposure in order to target the parasite in the intestine (soft-drug strategy). Low systemic exposure also greatly improves the safety profile of the drug, especially important as it will be used in young children.
Although the complete SAR is not shown, the authors demonstrate the optimization process with a few examples. The lead was tested in vivo and it was shown to be highly efficacious against the parasite with minimal systemic exposure. Extensive ADME and PKPD analysis is presented, and using mutants in the Cryptosporidium PI(4)K they were able to demonstrate that the selectivity of the drug over the human enzyme resides in the conserved residues in the ATP binding pocket. EDI048 is cidal against the parasite and targets meronts. The compound was also highly effective in the calf model of cryptosporidiosis. In oral toxicity studies, the NOAEL was at the highest dose (1000mg/kg/day). The development of this compound is very exciting and the data

suggest that the compound has a good chance of soon providing a safe and effective treatment for cryptosporidiosis, a significant advance for the field and for infectous disease medicine.
Two minor comments:
1. It would be interesting to know if EDI048 also affects membrane biogenesis of the microgametes. Could the authors comment on that?
2. One concern with a drug that targets a single enzyme in a parasite is the possibility of the development of resistant parasites over time. While EDI048 does target conserved residues in CpPI(4)K, there never has been this kind of selective pressure on the parasite. Could the authors comment on this possibility in the discussion?

Reviewer #3 (Remarks to the Author):

Very comprehensive overview of a soft-drug approach to target Cryptsporidium PI(4) kinase. The work is extremely important and the paper is well-written. Addressing a few points would improve the paper
1. The authors in the discussion, discussed the unknown of pulmonary cryptosporidium briefly, and then basically stated that most of the morbidity in LMIC children was from GI cryptosporidium, which is probably true. But they should probably state its unclear whether the parasite cycles back and forth from the pulmonary reservoir to the GI tract. Thus, treating with a soft-drug might not stop such a cycle.

2. In HIV positive patients, it was felt that a biliary reservoir might explain the relapse after NTZ and paromomycin therapy.It's not clear if this will happen in young children. Will the soft drug appear in levels high enough in the biliary reservoir to address this issue?

3. It appears that compound 6 (metabolite) is predicted to circulate in higher levels than EDI048 in treated people. The text mentions that the compound is "inactive" and gives an activity for cryptosporidium. However, the question this reader was left with is does the compound 6 have activity against human PI (4) Kinase or other off target safety risks? Will the compound penetrate cells?

Author Rebuttal to Initial comments

Point by point response to editor's and Reviewers comments.

19th February 2024

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Ujjini and Thierry,

Thank you for your patience while your manuscript "Rational design of a gastrointestinal-targeted parasiticidal agent to treat pediatric enteric cryptosporidiosis" was under peer-review at Nature Microbiology. It has now been seen by 3 referees, whose expertise and comments you will find at the of this email. You will see from their comments below that while they find your work of interest, some important points are raised. We are very interested in the possibility of publishing your study in Nature Microbiology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

In particular, you will see that referee #1 feels the choice of a 10mg/kg dose should be more clearly explained and that the clarity and interpretation of the clinical outcomes needs to be improved.

We sincerely thank the referee #1 and the editor for their constructive and insightful comments. We have clarified the interpretation of the clinical outcomes and explained the choice of a 10mg/kg dose. Please refer to our response to referee #1 below and appropriate edits made to the manuscript.

The referee also suggests that in case data on clinical outcomes following cessation of treatment are available, it would be good to include these.

We have monitored the calves 7 days following the cessation of EDI048 treatment for both clinical and parasitological read-outs. As suggested by the referee #1 and the editor we have included that data in Extended Fig. 5c and made appropriate changes to the manuscript. Please see the detailed response to referee #1.

Referee #2 asks for a comment on whether EDI048 also affects membrane biogenesis of the microgametes.

We have not determined if EDI048 affects membrane biogenesis in microgametes and responded to referee #2 with details.

Furthermore, this referee also asks to further discuss the possibility of resistance development in the Discussion section of the manuscript.

Responded with details and made appropriate edits to the manuscript.

Referee #3 asks to comment on whether compound 6 has activity against human PI (4) Kinase or has other off target safety risks. The referee also asks whether the compound will penetrate cells.

*We have responded to referee #3 by directing to Table 1 (for human PI(4)K and permeability data) and Supplementary Data Table 1 (for off target safety risks) for the requested compound **6** data; and to further clarify we have added a statement to the manuscript.*

The rest of the referees' reports are clear and the remaining issues should be straightforward to address.
We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

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To accommodate the length limit for a Nature Microbiology Article and following the Nature Microbiology data presentation policy, we have made a few edits to manuscript:

- *Abstract condensed to ~150 words (added the condensed abstract below the original abstract in the version with track changes)*
- *Acknowledgement section has been shortened.*
- *Based on the requirements, further reduction to the manuscript can be made after in-principle approval.*
- *After the discussion added a line about "Correspondence and requests for materials"*
- *Added individual data points to Fig. 1a, 1d, 1e, 2e and Extended Data Fig. 3.*
- *Defined box-plot elements in the legends to the Extended Fig. 5b (lines 730-733) as "Data shown in **b** as a 'box and whiskers' plot; the box extends from the 25th to 75th percentiles, and whiskers with minimum to maximum showing all data points."*

Please note in the response, the line numbers "in red" correspond to the version of the manuscript submitted with track changes.

Some reduction could be achieved by focusing any introductory material and moving it to the start of your opening ‘bold’ paragraph, whose function is to outline the background to your work, describe in a sentence your new observations, and explain your main conclusions. The discussion should also be limited. Methods should be described in a separate section following the discussion, we do not place a word limit on Methods.

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* state in a cover note the length of the text, methods and legends; the number of references; number and estimated final size of figures and tables

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We hope to receive your revised paper within two months. If you cannot send it within this time, please let us know.

We look forward to hearing from you soon.

Nature Microbiology

Reviewer Expertise:

Referee #1: Anti-parasitic drug discovery
Referee #2: Drug discovery, apicomplexan parasites
Referee #3: Cryptosporidium, pre-clinical drug development

Reviewers Comments:

Point by point response to Reviewer #1

Reviewer #1 (Remarks to the Author):

This manuscript, by Manjunatha et al, describes the characterization of EDI048, an inhibitor of the Cryptosporidium PI(4)K and is a clear and important extension of work previously published by the authors. The authors rightly note the importance and impact of human cryptosporidiosis, particularly in low-resource settings, and its effect on vulnerable populations (people with AIDS and children under the age of 5). Moreover, there is a clear gap in the availability of effective treatments, with nitazoxanide being the only approved drug, which is sub-effective in the most vulnerable patients. The studies focus primarily on the hypothesis that Cryptosporidium parasites, residing within intracellular vacuoles of intestinal enterocytes, can be effectively treated by limiting exposure to the GI tract, which would provide advantages, particularly in terms of safety. This is a question that has challenged efforts in discovery of anti-cryptosporidials but is also of interest for considering treatment of other GI infections or GI-related conditions.

The studies benefit from the use of KDU731, a closely-related and equipotent analog (and predecessor) of EDI048 which displays uptake and systemic circulation; they convincingly show that circulation of KDU731 does not contribute the its anti-cryptosporidial efficacy in a well-established mouse model. The medicinal chemistry strategy employed to develop rapidly-metabolized analogs, leading to EDI048, is sensible and the characterization tracks with the hypothesis.

We appreciate the constructive and insightful comments of the reviewer #1.

The only confounding question here, as it relates to their hypothesis, is that compound 4 appears to have many of the desirable properties of EDI048, but without in vivo efficacy. This reviewer cannot comment on whether physicochemical or other properties of compound 4 would explain the result, but would be interested if the author have a hypothesis.

*Thank you for your comment. Compound **4** is a cyclic ester lactone with desirable soft drug properties but when compared to EDI048, it is less potent (~5-fold less active in both CpPI(4)K biochemical and C. parvum cellular assays) and shows a ~2-fold reduced solubility. Taken together these data may explain compound **4**'s poor efficacy in vivo. In addition, non-soft drug candidate like KDU731 benefits from enterohepatic recirculation to prolong GI exposure and*

enhance its efficacy in vivo. We thus believe that soft-drug candidates require superior in vitro potency (like EDI048) to compensate for the absence of enterohepatic recirculation and show mouse efficacy similar to KDU731. To clarify this point, we have updated the manuscript in lines ~166-167.

The drug metabolism and PK studies performed are likewise well thought out and support the central hypothesis. It is welcome to see that the authors examined PK in both healthy calves and in calves with cryptosporidial diarrhea, as increased transit times would likely effect local exposure and this consideration will be critical for human dose prediction. One element of drug metabolism that is not addressed, but may be beyond the scope of the present study, is the consideration that in the target population of children under the age of 5 (and particularly under the age of 2) drug metabolism can be different than in adults, impacting exposure.

We appreciate the reviewer's insightful comment. We agree that a refined PBPK (physiologically based pharmacokinetic) modeling is a relevant approach that would generate PK predictions in children ≤ 5 years of age. As EDI048 progresses into the clinic, we will build a PBPK model integrating EDI048 and establish the specific pediatric PK parameters. We agree with the reviewer that this is beyond the scope of this manuscript and therefore we did not address this topic in the revised version of the manuscript.

The mechanistic work related to the interactions between EDI048 and PI4K is quite elegant, despite lacking a CpPI4K structure. While the use of homology modeling can be of limited use, the authors use this approach to formulate and test key binding hypotheses using point mutations and chimeric proteins that support the basis of EDI048 binding to CpPI4K and specificity over HsPI4K. Likewise, the kill kinetic studies are suitable, and underlie an advantage of EDI048 or other parasiticidal drugs would have over NTZ, which is only a static drug. While the conclusions are supported by the data, it is some confusing that EDI048 does not appear to display any dose response in the luciferase-based assay shown in Supplementary Fig 1, whereas KDU731 does.

*Thank you for the positive feedback on the mechanistic work. The reviewer is right to point out that while KDU731 and EDI048 showed clear dose-response in vitro in the standard anti-parasitic assays against both C. parvum and C. hominis (Table 1), and in vivo in the mouse model (**Fig. 1f**), the 3-fold dilution data shown in Fig. 2f and Supplementary Data Fig. 1 may suggest that EDI048 does not show dose-response in this luciferase-based cidality assay. We believe if EDI048 were to be tested with 2-fold dilutions, a clear dose response could be achieved similar to KDU731. This is because EDI048 is very potent and shows a steep dose response curve in the cidality assay with maximum cidal effect observed at concentration as low*

as 27 nM but no killing at 3 nM. Note however that we do see an incomplete cidal effect of EDI048 at 9 nM for the 72-hours timepoint (red square, Fig. 2f), suggesting that higher resolution experiments in the range between 9 and 27 nm would be needed to reveal the expected dose response behavior of EDI048 in that assay. We have clarified this point in the legend of Supplementary Data Fig. 1 (lines 750-753).

Lastly, the authors look at the efficacy of EDI048 in a neonatal calf model, showing improvement in parasitological and clinical endpoints. While these are challenging studies, and the results do support the efficacy of EDI048, this reviewer does have some questions related to the performance and interpretation of this study. The soft-drug strategy does have the challenge of developing clear PK/PD relationships, so it is not clear whether the choice of 10mg/kg for this study was pragmatic, or based on any type of dose prediction or understanding of exposure. This is only potentially problematic in that the demonstrated efficacy could be an underestimate, and while further calf studies may be warranted to further the development of EDI048, this reviewer would not recommend them here.

Newborn calves are naturally susceptible to C. parvum infection, leading to profuse watery diarrhea. The main objective of the neonatal calf study was to establish proof-of-concept efficacy in presence of clinically relevant watery diarrheal symptoms for a GI-targeting drug candidate with limited systemic exposure. The choice of 10 mg/kg BID was indeed pragmatic and meant to enable a go/no-go decision for further development. We aimed to achieve statistically significant microbiological and clinical efficacy at this relatively high dose of ~900 mg EDI0489 per day for calves with average body weight of 45 kg. Considering significant physiological differences between calf and human (e.g. gastrointestinal volume, length and transit time) we do not anticipate deriving any relevant PK-PD parameters from the calves' study to anticipate human dose. Instead, we developed a Cryptosporidium Controlled Human Infection Model (CHIM; ClinicalTrials.gov Identifier: NCT05036668) in healthy adults and plan to assess preliminary PK/PD relationship in CHIM. Efficacy of EDI048 in CHIM with human cryptosporidiosis subjects will also help to demonstrating the prospect of benefit to initiate pediatric studies.

The most significant criticism this reviewer has for this study is the clarity and interpretation of clinical outcomes. It is not clear whether the clinical scores in Fig 3 represent composite clinical scores or solely fecal consistency scores. This may not change the interpretation, but multiple clinical measures are mentioned and it is not clear how the authors handle those.

Thank you for highlighting the need for clarifying the nature of the various clinical endpoints measured in the study. The clinical read-out data in the Fig. 3c solely represent fecal

consistency scores. This has been mentioned in the Y-axis of both graphs in Fig. 3c as "Clinical read-out (fecal consistency score)" and AUC (fecal consistency scores); also mentioned in the results section in lines 304-305 citing Fig. 3c. To provide further clarity we have made the following changes,

- *Updated the title of Fig. 3c from "Clinical readout" to "Clinical readout (fecal consistency score)"*
- *Changed the Fig. 3c legend from "….improved clinical scores of diarrhea" to "….improved clinical scores of diarrhea (fecal consistency scores)" in lines 662 and 663*

*In this study, infected calves showed diarrheal symptoms as measured by fecal consistency scores, the primary symptom for cryptosporidiosis. However, and in contrast with our previous report for KDU731 (Manjunatha et al. A Cryptosporidium PI(4)K inhibitor is a drug candidate for cryptosporidiosis. Nature 546, 376–380 (2017)), the neonatal calves enrolled in the EDI048 study did not develop severe impact on other clinical symptoms such as mentation, dehydration, or appetite scores. Thus, the effect of EDI048 compared to untreated calves was evaluated on fecal consistency scores only. To clarify this, we added **a** couple of lines (lines 704-710) in the methods section.*

The improvement in clinical outcomes could be more clearly addressed. It does seem as if there is a 2-3 day improvement in fecal consistency scores, but there is significant inter-animal variability.

Considering the anticipated significant inter-animal variability in the neonatal calf studies, we had 7 calves per group, and analysis have been performed using different methods to ensure statistical significance. Firstly, plotting data on a per day basis, holistically comparing all untreated and treated calves, there is a statistically significant improvement in fecal consistency scores within 2 days of EDI048 treatment (Fig. 3c, left). Secondly, analyzing individual calf data as AUC over days of treatment and comparing the two groups also showed a statistically significant improvement in fecal consistency scores with EDI048 treatment (Fig. 3c, right). These analyses fully account for inter-animal variability and demonstrate the compound efficacy in this study. For more specifics on addressing overall improvement in clinical outcomes and missing data points, please see specific responses below.

On line 290, the authors claim that by 72 hours, 5 of 7 animals showed no signs of diarrhea (extended data figure 5). While this is technically true, 4 of these 5 animals go on to experience bouts of mild-to-moderate diarrhea throughout the remainder of the treatment window. More appropriate is the cited metric of days of severe or mild-to-moderate diarrhea suffered.

We thank the reviewer for his suggestion and agree it is more appropriate to cite metric of days of severe or mild-to-moderate diarrhea suffered than emphasizing the difference on a specific day. We have deleted part of the sentence (lines 307-308) which states, "and by 72 hours five of seven calves showed no signs of diarrhea compared to one of seven calves in the control group (Extended Data Fig. 5b).", and we have retained the sentence on the metrics of days of severe or moderate-severe diarrhea (now Extended Data Fig. 5b) (lines 309-311). Further, as we will be providing the excel sheet raw data for fecal consistency score as a Supplementary information we have deleted "old Extended Data Fig. 5b", and also added new panel to Extended Fig. 5 showing the data following cessation of treatment (please see reviewer's next comment).

If the authors have data on clinical outcomes following cessation of treatment, it would be worth including these.

We have monitored the calves 7 days following the cessation of EDI048 treatment for both clinical and parasitological read-outs. We did not observe recrudescence of infection or clinical symptoms in both treated and untreated groups. As suggested by the reviewer and the editor we have included that data in new Extended Fig. 5c and made the following updates to the manuscript.

- *Added a new figure "Extended Data Fig. 5c" and updated the figure legend.*
- *Added the following sentence to results section "No recrudescence of infection was observed up to 7 days following cessation of treatment (Extended Data Fig. 5c)." lines 310-311.*
- *Added the following sentence to discussion "Furthermore, no recrudescence in infection was observed even after the cessation of EDI048 treatment (Extended Data Fig. 5c)." lines 385-386.*

They should also explain the absence of data points in the clinical measures for untreated calves, and how that was taken into consideration for data analysis.

As the reviewer mentioned above, calf efficacy studies are technically challenging. The reason we sample every 12 hrs is to be certain we don't go for a 24 hrs period without a sample. Typically, sample collection is done by manual manipulation of the calf rectum with fingers to enforce defecation with sufficient sample volume to enable reliable scoring. This sample collection is especially difficult in heavily infected calves as their GI tracts are hypermotile due to diarrhea and sometimes they are just empty with no additional stool to produce at anticipated fixed time points. Hence, we have more samples missing in untreated calves.

In the data set, it is important to note that there is never more than a 24 hr period without a sample. Thus, there is at least one fecal consistency score per calf per day for comparisons. Furthermore, since there are 7 calves per group (Untreated or EDI048 treated), on any given day there are at least 11 (out of 14) fecal consistency readings per group available for direct comparison.

Additionally, data has been analyzed in 2 different ways (i.e., analyzing data on a per day basis and plotting AUC over days on a per calf basis) as described above for careful assessment of missing data and account for inter-animal variability. As evident from in Fig. 3c, there is a statistically clear differences between the groups when analyzed in either of the above mentioned two ways further underlying significance of EDI048 in improving fecal consistency scores in calves.

In the discussion, the authors state that EDI048 treatment resulted in rapid resolution of diarrhea in this study, but it would be more appropriate to say that treatment resulted in significant improvement of diarrheal symptoms.

Thank you for the suggestion, based on reviewers' recommendation we updated the same in lines 384-385.

Overall, despite some critique, this is a highly original and meritorious manuscript with implications not just for the development of needed anticryptosporidial agents, but also treatment of GI disease generally. The key hypothesis that local exposure is necessary and sufficient for treatment of Cryptosporidium infection with EDI048 is well-supported by the data. Beyond the thoughtful and interesting pharmacological strategy described, this also represents perhaps the most advanced candidate for treatment of cryptosporidiosis.

We appreciate the thoughtful assessment and critical feedback from the reviewer.

Minor comments:
• Line 135: The sentence is likely intended to be "While the detailed structure activity relationship…"

Suggestion has been incorporated.

• Line 385: should read "potent paraciticidal activity"

Corrected, thank you.

• While the stated statistical methods appear appropriate, the authors should review figure legends to ensure definition of error bars, as this is not done uniformly.

Thanks for the comment. Appropriate edits are done in figure legends line 606 (Fig. 1a), line 616 (Fig. 1e), line 665 (Fig. 3b and c), and footnote to Table 1.

Point by point response to Reviewer #2

Reviewer #2 (Remarks to the Author):

This is a well designed and comprehensive study on the design and evaluation of a new drug to treat cryptosporidiosis. The study is unique in that the lead molecule was specifically designed to have low systemic exposure in order to target the parasite in the intestine (soft-drug strategy). Low systemic exposure also greatly improves the safety profile of the drug, especially important as it will be used in young children.
Although the complete SAR is not shown, the authors demonstrate the optimization process with a few examples. The lead was tested in vivo and it was shown to be highly efficacious against the parasite with minimal systemic exposure. Extensive ADME and PKPD analysis is presented, and using mutants in the Cryptosporidium PI(4)K they were able to demonstrate that the selectivity of the drug over the human enzyme resides in the conserved residues in the ATP binding pocket. EDI048 is cidal against the parasite and targets meronts. The compound was also highly effective in the calf model of cryptosporidiosis. In oral toxicity studies, the NOAEL was at the highest dose (1000mg/kg/day). The development of this compound is very exciting and the data suggest that the compound has a good chance of soon providing a safe and effective treatment for cryptosporidiosis, a significant advance for the field and for infectious disease medicine.

We are grateful for the valuable suggestions and feedback from the reviewer #2.

Two minor comments:
1. It would be interesting to know if EDI048 also affects membrane biogenesis of the microgametes. Could the authors comment on that?

We have not determined if EDI048 affects membrane biogenesis in microgametes. However, in related apicomplexan parasites, Plasmodium species that cause malaria, PI(4)K inhibitors have been shown to inhibit gamete formation and reduce gametocyte viability (McNamara, C., et al. Targeting Plasmodium PI(4)K to eliminate malaria. Nature 504, 248–253 (2013)). Additionally PI(4)K gene is expressed throughout the intracellular life stages in Cryptosporidium parvum. Based on these data, we would speculate that EDI048 is likely to affect viability of microgametes, possibly through inhibition of membrane biogenesis and that this should warrant further studies outside the scope of this report.

2. One concern with a drug that targets a single enzyme in a parasite is the possibility of the development of resistant parasites over time. While EDI048 does target conserved residues in

CpPI(4)K, there never has been this kind of selective pressure on the parasite. Could the authors comment on this possibility in the discussion?

EDI048 inhibits an essential enzyme by binding to the highly conserved ATP binding pocket leading to a parasiticidal activity. We have tested a few field isolates of C. parvum and also to C. hominis, all are equally sensitive to EDI048, suggesting there is no pre-existing PI(4)K mutation in the field. Emergence of resistance and underlying mechanism of drug resistance in Cryptosporidium is least understood due to lack of robust in vitro continuous culture system. However, the first report of naturally emerging Cryptosporidium drug resistance was observed with CpMetRS inhibitor (Hasan et al 2021), where resistance emerged during drug treatment in neonatal calf model. No such emergence of resistance during or up to 7 days after treatment was observed with EDI048, thus the overall risk for drug resistance to EDI048 seems relatively lower. However, further monitoring in CHIM and other clinical studies will be required to further assess this risk. We thank the reviewer for the suggestion and have added the following statement to the discussion (lines ~386-389) "Unlike the CpMetRS inhibitor, resistance did not emerge during treatment with EDI048. Therefore, based on the limited data available, the perceived risk of resistance to EDI048 appears to be relatively low. Nonetheless this risk should be further assessed during EDI048 clinical development."

Point by point response to Reviewer #3

Reviewer #3 (Remarks to the Author):

Very comprehensive overview of a soft-drug approach to target Cryptsporidium PI(4) kinase. The work is extremely important and the paper is well-written. Addressing a few points would improve the paper

1. The authors in the discussion, discussed the unknown of pulmonary cryptosporidium briefly, and then basically stated that most of the morbidity in LMIC children was from GI cryptosporidium, which is probably true. But they should probably state its unclear whether the parasite cycles back and forth from the pulmonary reservoir to the GI tract. Thus, treating with a soft-drug might not stop such a cycle.

We thank the reviewer#3 for the insightful comments and feedback. We have addressed the reviewer's above comment by adding the following sentence in the discussion (lines 393-394) "and may have limited efficacy if the parasite cycles between extra-GI and GI sites."

2. In HIV positive patients, it was felt that a biliary reservoir might explain the relapse after NTZ and paromomycin therapy. It's not clear if this will happen in young children. Will the soft drug appear in levels high enough in the biliary reservoir to address this issue?

We have not analyzed levels of EDI048 in the biliary system. However, if the drug levels in the biliary system is a reflection of systemic circulation, levels of soft drug are unlikely to be high enough for anti-parasitic activity. Biliary cryptosporidiosis is well-recognized in severely immune-compromised patients, especially in those with CD4+ T-cell < 50 cells / µl. We agree with the reviewer that it is not clear if this happens in pediatric cryptosporidiosis patients.

3. It appears that compound 6 (metabolite) is predicted to circulate in higher levels than EDI048 in treated people. The text mentions that the compound is "inactive" and gives an activity for cryptosporidium. However, the question this reader was left with is does the compound 6 have activity against human PI (4) Kinase or other off target safety risks? Will the compound penetrate cells?

*In vitro anti-Cryptosporidium activity, biochemical CpPI(4)K activity, human PI(4) kinase selectivity and permeability data for compound **6** are summarized in Table 1. And also, the off-target safety profile of compound **6** with a panel of human recombinant receptors and pharmacologically relevant proteases/kinases is summarized in Supplementary Data Table 1. Overall, compound **6** is inactive against C. parvum (EC_{50} > 20 µM), less active against HsPI(4)K*

*(IC_{50} = 0.484 μM), and inactive against a broad panel of receptors profiled (majority of them being >30 μM). The permeability of compound **6** is low as measured in MDCK-MDR1 cells (A-B = 0.56 x 10^{-6} cms^{-1}) indicating this compound is unlikely to penetrate cells. To make this point clear in the manuscript, we have added the following statement in lines ~174-175.*

*"Compound **6** has a low permeability and no significant off-target safety liability risks (Table 1 and Supplementary Data Table 1)."*

Decision Letter, first revision:

Message: 7th June 2024

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Manju,

Thank you for your patience and your understanding that we consulted an additional X-ray crystallography referee to comment on your study "Rational design of a gastrointestinal-targeted parasiticidal agent to treat pediatric enteric cryptosporidiosis". The manuscript has now been see by such an expert (referee #4) and the comments are at the end of this email. You will see that while they find your work of interest, some important points are raised. We are very interested in the possibility of publishing your study in Nature Microbiology, but would like to consider your response to these concerns in the form of a revised manuscript before we make a final decision on publication.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

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Some reduction could be achieved by focusing any introductory material and moving it to the start of your opening 'bold' paragraph, whose function is to outline the background to your work, describe in a sentence your new observations, and explain your main conclusions. The discussion should also be limited. Methods should be described in a separate section following the discussion, we do not place a word limit on Methods.

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Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

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If your paper is accepted for publication, we will edit your display items electronically so they conform to our house style and will reproduce clearly in print. If necessary, we will re-

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Figure legends must provide a brief description of the figure and the symbols used, within 350 words, including definitions of any error bars employed in the figures.

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We hope to receive your revised paper within two weeks. If you cannot send it within this time, please let us know.

We look forward to hearing from you soon.

Yours sincerely,

****

Reviewer Expertise:

Referee #4: X-ray crystallography

Reviewers Comments:

Reviewer #4 (Remarks to the Author):

The revised manuscript "Rational design of a gastrointestinal-targeted parasiticidal agent to treat pediatric enteric cryptosporidiosis," by Manjunatha and colleagues, reveals interesting results on the development and testing of the potential drug EDI048 and could promote the further development of this clinical candidate and/or other new selective inhibitors against pediatric enteric cryptosporidiosis.

The manuscript is well written, full of convincing methods and, in my opinion, deserves a publication.

I have been asked to focus on the structural data, which is why I am reducing my report to this specific point.

The analysis of the crystallographic data of the HsCpPI(4)K - HsRab11a chimera complex is generally acceptable, although there are a few comments that need to be addressed.

- The first crucial question is: How can the authors reliably determine the direction (during insertion into the electron density) of the EDI048 ligand? Based on the map (in the rcsb validation report), I would say they can't. If this cannot be clearly determined, which is essential, they might want to delete Figure 2b (close-up) or offer two alternative variants. This also raises the question of how exactly line 223-230 can be written. A contact analysis together with a suitable SI-figure would also be very good for the reader (e.g. with ligplot or similar tools). But perhaps the presentation of the data is simply not sufficient here.

- It would be important for the readers to check the validity of the ligand electron density and thus of the ligand binding by applying e.g. simulated annealing omitted maps. I recommend some views of these maps in an SI Figure.

- The analysis clearly has too many RSRZ outliers (almost 10%). R free vs. R work are also very far spread (greater than 4%) but this is not a very significant problem.

- Please exclude the inappropriate term …high-resolution… in line 223.

Author Rebuttal, first revision:

Point by point response to Referee #4 (X-ray crystallography)

Reviewer #4 (Remarks to the Author):

The revised manuscript "Rational design of a gastrointestinal-targeted parasiticidal agent to treat pediatric enteric cryptosporidiosis," by Manjunatha and colleagues, reveals interesting results on the development and testing of the potential drug EDI048 and could promote the further development of this clinical candidate and/or other new selective inhibitors against pediatric enteric cryptosporidiosis.

The manuscript is well written, full of convincing methods and, in my opinion, deserves a publication.

I have been asked to focus on the structural data, which is why I am reducing my report to this specific point.

We sincerely appreciate the thoughtful assessment and insightful comments from the reviewer.

The analysis of the crystallographic data of the HsCpPI(4)K - HsRab11a chimera complex is generally acceptable, although there are a few comments that need to be addressed.

- The first crucial question is: How can the authors reliably determine the direction (during insertion into the electron density) of the EDI048 ligand? Based on the map (in the rcsb validation report), I would say they can't. If this cannot be clearly determined, which is essential, they might want to delete Figure 2b (close-up) or offer two alternative variants. This also raises the question of how exactly line 223-230 can be written. A contact analysis together with a suitable SI-figure would also be very good for the reader (e.g. with ligplot or similar tools). But perhaps the presentation of the data is simply not sufficient here.

Response: We thank the reviewer for the critical comments and insightful questions. As reviewer alluded to, the presentation of the data was simply not sufficient, the electron density figures generated in the deposition (RCSB validation report) are poorly angled and do not show how well the density envelop conforms to the shape of the ligand. As the reviewer suggested, to interpret the fit of the electron density map more easily we have included simulated annealing omitted maps (2mFo-DFc and mFo-DFc) as an Extended Data Figure 3e. We have also included a ligand interaction plot from MOE (Chemical Computing Group) to further elucidate the binding mode of ligand as Extended Data Figure 3d. We believe these additional information and data provided in Extended Data Figure 3d and 3e clarify the concerns raised by the reviewer and enable the continued inclusion of Fig2b and the description in lines 223-230.

With the addition of Extended Data Figure 3d and 3e, we have made the following edits to the manuscript

- *cited Extended Data Figure 3d and 3e on page 6, lines 225-226.*
- *updated legend to the Extended Data Figure 3 on page 18, lines 669-670*
- *updated the method section on page 13, lines 451-453*

- It would be important for the readers to check the validity of the ligand electron density and thus of the ligand binding by applying e.g. simulated annealing omitted maps. I recommend some views of these maps in an SI Figure.

Response: A simulated annealing omitted (2mFo-DFc and mFo-DFc) electron density map is included as an Extended Data Figure 3e (see detailed response to Q1 above).

- The analysis clearly has too many RSRZ outliers (almost 10%). R free vs. R work are also very far spread (greater than 4%) but this is not a very significant problem.

Response: The experimental data has been reprocessed at 3.0 Angstrom resolution. This has improved statistics, including the RSRZ, which now has a more typical value of 1.4%. This has made no significant difference to the structure, or the interactions and conclusions drawn from the structure. The new RCSB validation report is enclosed and Extended Data Table 3 has been updated (page 25).

- Please exclude the inappropriate term …high-resolution… in line 223.

Suggestion has been incorporated, word "high-resolution" deleted (page 6, line 223)

Decision Letter, second revision:

Message: Our ref: NMICROBIOL-24010163B

10th July 2024

Dear Dr. Manjunatha,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Microbiology manuscript, "Rational design of a gastrointestinal-targeted parasiticidal agent to treat pediatric enteric cryptosporidiosis" (NMICROBIOL-24010163B). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

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In recognition of the time and expertise our reviewers provide to Nature Microbiology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "Rational design of a gastrointestinal-targeted parasiticidal agent to treat pediatric enteric cryptosporidiosis". For those reviewers who give their assent, we will be publishing their names alongside the published article.

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If you have any further questions, please feel free to contact me.

Reviewer #4:
Remarks to the Author:
I think the authors have implemented the suggestions and the short structural part of the mansucript is now sufficiently described.

Final Decision Letter:

Message: 15th August 2024

Dear Manju and Thierry,

I am pleased to accept your Article "Cryptosporidium PI(4)K inhibitor EDI048 is a gut-restricted parasiticidal agent to treat pediatric enteric cryptosporidiosis" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Microbiology style. We look particularly carefully at the titles of all papers to ensure that they are relatively brief and understandable.

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