Author's response to reviews

Title: Enhancement of hypoxia-activated prodrug TH-302 anti-tumor activity by Chk1 inhibition

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Author's response to reviews: see over
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Krisha Mae Natan
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Dear Ms. Natan,

Thank you for forwarding the reviewer comments on the manuscript entitled: “Enhancement of Hypoxia-Targeted Drug TH-302 Anti-tumor Activity by Chk1 Inhibition”

We have addressed the comments in a revised manuscript which I enclose here, and also provide a point-by-point response below.

#1 Reviewer's report

Reviewer:Ester Hammond

Reviewer's report:
It is not clear to me if this is a revised manuscript or a first submission. I do not recall reviewing it previously.

Meng et al reply: Yes, this is a first submission.

Over all the manuscript provides a detailed and interesting report of the potential for combining the HAP TH302 with Chk1 inhibitors. I recommend the manuscript be accepted with few (text) changes. The introduction provides an up to date, concise description of both TH302 (the leading HAP in the field/clinal trail) and Chk1 inhibitors. However the combination of DNA damaging agent and Chk1 inhibition is far from novel. Indeed all the Chk1 clinical trials run to date have been in combination with a DNA damaging agent. There are also examples of Chk1 inhibitors modified for use in hypoxic conditions, form Sentinel Oncology and ACS Chem Biol. 2013 Jul 19;8(7):1451-9. doi: 10.1021/cb4001537, both should be referenced (although I realise that might be difficult for the Sentinel compound).

Meng et al reply: Both references have been added at lines 104, 105 and 535.
The efficacy of Chk1 activity in p53 null vs wild type cells is also not novel but nicely demonstrated here. The hypoxic conditions need further clarification. What does N2 mean? were the cells not provided with carbon dioxide?

Meng et al reply: Yes. There was also CO₂ included. This has been clarified with the information added at line 139 in the methods.

HT29 cells are not described until line 357, please move this to where they are first used.

Meng et al reply: The description of the HT29 cell line is added at line 161

I am surprised that the Chk1 inhibitors showed no toxicity alone and even more so that no increase in tail moment was seen. Some breaks would be expected during normal replication in the absence of Chk1, however it is likely that because this would be at a low level and only in S phase cells the comet assay may not have detected them. Perhaps acknowledge that a low level of damage would be expected. Overall, a nice study clearly demonstrating the effectiveness of TH302 in preclinical models.

Meng et al reply: Text addressing this point has been added at lines 319-320.

#2 Reviewer's report:
Some mini problems
1. Use the same term for same condition. e. g. “hypoxia (0.1% O₂)” and “hypoxia (N₂)”.

Meng et al reply: Both environmental states are defined as ‘hypoxia’, so we have to describe the particular oxygen concentration being employed in the two different hypoxic conditions employed in the study. Please note that we think this approach is superior to an alternative approach of describing the nitrogen environment as anoxia, as residual oxygen will still be present, admittedly at very low levels. Thus we have now clarified throughout the manuscript the specific hypoxia condition utilized in every instance.

2. Use the same units format. e. g. “24 h”, “24h” or “hr”.

Meng et al reply: These have been fixed.

3. Units should have a single space between the number and the unit. E. g. “0.1 μM” rather than “0.1μM”.

Meng et al reply: These have been fixed.
Specific points:
1. Fig. 1, Each cell line name needs to be given in each graph.
   
   **Meng et al reply:** This information has been added at line 739.

2. Fig. 2, Label the treatment time in each graph.
   
   **Meng et al reply:** This information has been added at lines 748-749.

3. Fig. 4, What are the different conditions between the upper and lower images in both (A) and (B)?
   
   **Meng et al reply:** The labels are included at the left side of each panel.

4. Fig. 5, Offering each cell line name in each graph.
   
   **Meng et al reply:** The information has been added to the Figure Legend at lines 769-771.

5. Fig. 6A, the AZD7762 concentration is described in both figure 6 legend and labels. Then, what do “the conditions” mean? The upper-right label should be corrected from “0.1 µM AZD7762” to “AZD7762” if TH-302 was treated by a series of concentrations.
   
   **Meng et al reply:** I have double-checked the graph label.

6. Fig. 6B, Previous reports showed that AZD7762 inhibits Chk1 autophosphorylation (S296) while phosphorylated Chk1(S345) by ATR/ATM increases. To ensure that Chk1 inhibition by AZD7762 is convincing, Chk1 (S296) should be detected by Western blot here.
   
   **Meng et al reply:** We have performed new experiments to address the reviewer’s comments. This new information has been added at lines 130, 355-364 and added the new Figure 6B. I also condensed Figure 6C since no additional information comparing 24 h and 48 h.

7. Fig. 7, Were the xenograft mice tumors pictures taken? If yes, it is better to
show them here. This data is essential for supporting the in vitro assays.

Meng et al reply: We did not take pictures of the isolated tumors, as this would have required sacrificing the animals and removing the tumors at a particular day in the study. Instead, we decided to continue to monitor the tumor growth of all the tumors on an individual animal basis to allow the characterization of the kinetics of the eventual tumor re-growth across all the experimental groups.

We thank the editor and both reviewers for their comments, as we believe the paper is significantly improved.

Sincerely yours,

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