Author's response to reviews

Title: Panobinostat synergizes with bortezomib to induce endoplasmic reticulum stress and ubiquitinated protein accumulation in renal cancer cells

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Author's response to reviews: see over
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Dr. Hayley Henderson
Executive Editor, BMC Urology

Dear Dr. Henderson,

Thank you very much for reviewing our original manuscript entitled “Panobinostat synergizes with bortezomib to induce endoplasmic reticulum stress and ubiquitinated protein accumulation in renal cancer cells,” which we submitted for publication in BMC Urology (MS 7405179971246999). We have revised the manuscript according to the reviewers’ comments and recommendations.

Thanking you for consideration and looking forward to your favorable response,

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Response to Reviewers

Referee 1
Major Revisions
1. The article could be corrected by native speakers.

Response
The revised manuscript has been checked by a native speaker of English.

2. Figures are too busy. Author should submit essential figures. The Figure 1, 2A and photos of colony assay in Figure 2B may be omitted, leading to be clearly expressed. The FACS data could be changed to bar graphs in Figure 3, leading to be clearly expressed.

Response
We agree that Figure 1 and the photos of the colony formation assay were not needed, so we have removed them and renumbered the figures accordingly. In the annexin-V assay we compared the degree of apoptosis by measuring the area under the curve, but we think the changes in the shape of the curves would also help one easily understand the degree of apoptosis. We also think the changes in the cell cycle are more informative than the number of the cells in the sub-G1 fraction alone. We would like to leave the presentation of the FACS data as it is.

3. When author present the data, author should explain it using suitable expression.

Response
We appreciate the reviewer’s suggestion and have changed the expressions pointed out in the Minor Revisions and Discretionary Revisions parts of the reviewer’s report.

4. Author should discuss clinical information such as efficacy and adverse events of the therapy with HDAC inhibitor and proteasome inhibitor for patients with malignant neoplasm in the section of DISCUSSION.

Response
The reviewer is correct. A description of the efficacy and adverse events of the combination is absolutely necessary because the combination has already been
examined clinically and if in the future it is applied to patients with advanced renal cancer, the initial doses given to the patients should be based on those used in the clinical trial rather than the doses we used in our in-vivo experiments. In the revised manuscript we have cited a paper reporting the most recent phase-II trial (ref 23) and have briefly described that trial and its results in the Discussion section (Lines 264–274).

5. An animal monitoring and tolerability should be described in Materials and Methods. Also, typical adverse events should be described if there are.

**Response**

The combination was tolerated (had no lethal side effects), but we didn’t perform a formal assessment of its tolerability. This may be a drawback of our study. What we showed is that the combination inhibited the growth of renal cancer cells in vivo as well as in vitro and thus may be effective in clinical settings. If it is applied to humans, the dose should be based on the results of the previous phase-II trials in patients with other malignancies, not on our experiments using mice. Therefore we feel it is of utmost important to describe the results of the previous trial in detail, and we have done so as described in our response to this reviewer’s comment 4. We have also added a description of the limitations of our in-vivo study to the Discussion section (Lines 274–278).

**Minor Revisions**

1. Line 25: Background: Inducing endoplasmic reticulum stress
   #Background: Inducing endoplasmic reticulum (ER) stress
2. Line 73: accumulation, and kills cancer cells effectively in vitro and in vivo.
   #accumulation, and kills cancer cells effectively in vitro and in vivo.
3. Line 149-150: Colony formation assay showed that the combination suppressed colony formation significantly (Figure 2B).
   #ex) Colony formation assay demonstrated the combination therapy significantly suppressed colony formation compared to control vehicle, panobinostat or bortezomib alone (Figure 2B, *p = 0.0495; **p = 0.0463).
   #combination therapy in vivo.
5. Line 156: group. The average tumor size at day 15 was 520 ± 175 mm\(^3\) (mean ± SE)
#group. The average tumor size at day 15 was 520 ± 175 mm$^3$ (mean ± SD)
#renal cancer growth both in vitro and in vivo.

Response
We have revised the expressions pointed out in comments 1 (Line 25) and 3 (Lines 148–150), but in comments 2, 4 and 6 there seem to be no changes made by the reviewer. Those expressions have therefore been left as they were. In comment 5 the reviewer suggests changing SE to SD, but 175 mm$^3$ is the SE rather than the SD.

Discretionary Revisions
1. When author describes significance of data, author should state the comparative group.
Line 162: The combination increased the annexin-V fluorescence intensity (up to 19.4-fold)
#ex) up to 19.4-fold compared to control vehicle.
Line 163-164: (Figure 4A) and also increased the number of the cells in the sub-G1 fraction (up to 70.5%)
#ex) 70.5% compared to control vehicle.

Response
As the reviewer advised, in the revised manuscript we have specified the comparison group (Lines 162–163). We have, however, used the idiomatically correct “compared with” rather than “compared to” because our purpose was not to state a similarity but to examine for differences or similarities. As for the cell cycle analysis, we did not change the expression because 70.5% is not a relative value compared with the control. Instead it means that 70.5% of the treated cells were in the sub-G1 fraction.

Referee 2
Major compulsory revisions
1. Please perform proper controls. In figure 2, when panobinostat and also bortezomib was not used (0 nm). Were experiments performed with DMSO to ensure the proper controls were performed.

Response
The reviewer is concerned about the lack of a proper control but, as shown in the
original Figure 2 (Fig. 1 in the revised manuscript), we have performed the experiments using proper controls: i.e., with 0 nM panobinostat and 0 nM bortezomib. The DMSO concentration was negligible in our experiments. The stock solutions (both panobinostat and bortezomib) were first diluted to 1/100 with medium, and a maximum of 13 µL (when cells were treated with 50 nM panobinostat and 15 nM bortezomib) of the diluted solutions was added to the 2 mL of medium in a culture plate well. The final concentration of DMSO was therefore at most only 0.0065%, which is negligible. Although adding 0.13 µL of DMSO to the 2 mL of control medium for normalization would be theoretically correct, using normal micropipettes to add less than 1 µL of a solution is technically quite difficult and trying to do so will cause an error.

2. Synergy describes the interaction of two entities to produce something that is greater than the sum while it is unclear from the data presented if the result is rather additive as figure 5 demonstrates induced ER stress and histone acetylation. I would suggest RT-PCR of the aforementioned select proteins to truly demonstrate synergy.

Response
The reviewer thinks that synergism is not clear from our western blotting results and suggests that we use RT-PCR to confirm synergism. While we agree that RT-PCR would demonstrate synergism more clearly because it is a quantitative rather than semiquantitative assay, we don’t think it is needed in the present study because the presented western blot data clearly shows the synergism of the combination.

We confirmed this by measuring the optical densities of the western blotting bands. The density of the band for each protein was semiquantified using public domain ImageJ software and normalized to density of the corresponding actin band. We also calculated what the density values would be if the combined effect was additive. These presumed values and the measured values are listed in the attached table, from which one can see that the densities of the bands obtained from cells treated with the combination therapy are higher than the presumed values. That is, the combined effect is synergistic.

Furthermore, we are afraid that acetylation and ubiquitination are both posttranscriptional modifications and therefore can’t be assessed using RT-PCR.

Minor Essential revisions
1. Please explain why the aforementioned concentrations of 50 nM of panobinostat and 10nM of bortezomib were used as the cell line Caki-1 demonstrated combination index
<1 for 25 nM and 10 nM for panobinostat and bortezomib, respectively.

**Response**
Our purpose is to establish a novel treatment against renal cancer, so we wanted to evaluate molecular changes produced at the concentrations that effectively inhibited renal cancer growth; that is, at therapeutic concentrations. Both 50 nM panobinostat + 10 nM bortezomib and 25 nM panobinostat + 10 nM bortezomib acted synergistically (i.e., with combination indexes < 1), but the latter combination killed only 20% of the cancer cells, whereas the former combination killed 70% of them. We therefore used 50 nM panobinostat + 10 nM bortezomib.

2. In figure 2B, the colony formation assay demonstrates % number of colonies to be drastically different for combination treatment. When the cells were treated for 48 hrs, why was it necessary to wait 1–2 weeks for incubation? Why not a definite time point of 1 or 2 weeks.

**Response**
Because we wanted to evaluate how colony formation was affected by 48-hour treatment rather than by 1–2 week treatment. We examined the effect of 48-hour treatment on cell viability by MTS assay and on colony formation by colony formation assay. In the present study, assays were consistently done in the 48-hour time period.

**Discretionary Revisions**
1. The authors may comment on the mechanistic role of panobinostat and bortezomib on affected pathways as it has been suggested in lymphoma. For example, caspase pathway, stat 5 and stat 6 phosphorylation, and the hif1 pathway.

**Response**
Following the reviewer’s advice, we have cited the paper that reported inhibition of the AKT and NF-kB pathways by the combination and added some descriptions in the Discussion section (Lines 244–246).
### Table

Densitometry results for the western blotting bands.

<table>
<thead>
<tr>
<th></th>
<th>Caki-1</th>
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<tbody>
<tr>
<td></td>
<td>HSP70</td>
</tr>
<tr>
<td></td>
<td>GRP78</td>
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<tr>
<td></td>
<td>Ero1-Lα</td>
</tr>
<tr>
<td></td>
<td>ERP44</td>
</tr>
<tr>
<td></td>
<td>Ubiquitinated protein</td>
</tr>
<tr>
<td></td>
<td>Acetylated histone</td>
</tr>
<tr>
<td></td>
<td>Acetylated α-tubulin</td>
</tr>
<tr>
<td></td>
<td><strong>10 nM bortezomib</strong></td>
</tr>
<tr>
<td></td>
<td><strong>50 nM panobinostat</strong></td>
</tr>
</tbody>
</table>

|                  | ACHN                                                                 |
|                  | HSP70                     | 3079 | 4215  | 2551  | 6073  | 3687  |
|                  | GRP78                     | 0    | 0     | 0     | 2263  | 0     |
|                  | Ero1-Lα                   | 3200 | 2831  | 3174  | 5276  | 2805  |
|                  | ERP44                     | 9476 | 8956  | 11724 | 13488 | 11204 |
|                  | Ubiquitinated protein     | 8733 | 56799 | 12283 | 88998 | 60350 |
|                  | Acetylated histone        | 281  | 350   | 12470 | 28147 | 12538 |
|                  | Acetylated α-tubulin      | 2992 | 8601  | 9245  | 18543 | 14853 |
|                  | **10 nM bortezomib**      | −    | +     | −     | +     | Presumed value |
|                  | **50 nM panobinostat**    | −    | −     | +     | +     | Presumed value |

|                  | 769-P                                                                  |
|                  | HSP70                     | 2777 | 4039  | 2319  | 7541  | 3581  |
|                  | GRP78                     | 1266 | 1889  | 2867  | 3896  | 3490  |
|                  | Ero1-Lα                   | 2701 | 2656  | 2967  | 2959  | 2922  |
|                  | ERP44                     | 3150 | 4769  | 4423  | 6302  | 6042  |
|                  | Ubiquitinated protein     | 12238| 7635  | 5895  | 34979 | 1292  |
|                  | Acetylated histone        | 6109 | 2383  | 8625  | 9107  | 4900  |
|                  | Acetylated α-tubulin      | 563  | 955   | 680   | 5283  | 1071  |
|                  | **10 nM bortezomib**      | −    | +     | −     | +     | Presumed value |
|                  | **50 nM panobinostat**    | −    | −     | +     | +     | Presumed value |