Author's response to reviews

Title: Khz-cp (crude polysaccharide extract obtained from the fusion of Ganoderma lucidum and Polyporus umbellatus mycelia) induces apoptosis by increasing intracellular calcium levels and activating P38 and NADPH oxidase-dependent generation of reactive oxygen species in SNU-1 cells

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Author's response to reviews: see over
1. **Reviewer's report**

**Title:** Khz-cp (crude polysaccharide extract obtained from the fusion of Ganoderma lucidum and Polyporus umbellatus mycelia) induces apoptosis by increasing intracellular calcium levels and activating P38 and NADPH oxidase-dependent generation of reactive oxygen species in SNU-1 cells

**Version:** 9  
**Date:** 20 February 2014  
**Reviewer:** Guang Ji  
**Reviewer's report:**
Minor Essential Revisions

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests

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2. **Reviewer's report**

**Title:** Khz-cp (crude polysaccharide extract obtained from the fusion of Ganoderma lucidum and Polyporus umbellatus mycelia) induces apoptosis by increasing intracellular calcium levels and activating P38 and NADPH oxidase-dependent generation of reactive oxygen species in SNU-1 cells

**Version:** 9  
**Date:** 11 February 2014  
**Reviewer:** Sona Hudecova  
**Reviewer's report:**
Authors have to improve quality of figures and legends to figures shell be also better arranged.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
'I declare that I have no competing interests'
This manuscript “Khz-cp (crude polysaccharide extract obtained from the fusion of Ganoderma lucidum and Polyporus umbellatus mycelia) induces apoptosis by increasing intracellular calcium levels and activating P38 and NADPH oxidase-dependent generation of reactive oxygen species in SNU-1 cells” by Kim et al investigates the anti-cancer role of Khz-cp, a commonly used herb extract, on apoptosis and potential mechanisms in vitro. The authors claim that Khz-cp preferentially induces apoptosis in cancer cells and that the signaling mechanisms involve an increase in [Ca2+]i, P38 activation, and ROS generation via NADPH oxidase and mitochondria. It is quite an interesting piece of work, trying to figure out the mechanisms and potential targets of a drug is always a promising attempt. The authors have certainly done a great deal of work on this issue.

1. Is the question posed by the authors well defined? Yes
2. Are the methods appropriate and well described? Yes
3. Are the data sound? Mostly OK
4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes
5. Are the discussion and conclusions well balanced and adequately supported by the data? Mostly OK
6. Are limitations of the work clearly stated? Not sure
7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Yes
8. Do the title and abstract accurately convey what has been found? Yes
9. Is the writing acceptable? Yes

In exploring mechanisms involved, the authors considered all the factors comprehensively and the data were logically displayed. But several issues might need to be further clarified.
1. In the materials part, the supplier of G. lucidum and P. umbellatus mycelia powder need be indicated.

**Answer** Khz-cp were obtained from BrainGroup (Seoul, South Korea). Take different molecular weight polysaccharides solid amount, adding a certain amount of water dissolved polysaccharide solution was prepared. Polysaccharide solution with a pipette, slowly add the surrounding wall along the column, the sample thickness is less than 1 cm. After the addition of the sample solution, opening the piston and the sample into the column bed completely, and then close the piston, with the operation amount of the eluent at the sample under the residual wall washing, opening the piston, so that the filler layer above the small amount of solution retained into the column, and then suitably adjusting the flow rate and eluted.

**Mycelial content of Khz:**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Brown liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycelial content (%)</td>
<td>25.6</td>
</tr>
<tr>
<td>Germanium (mg/kg)</td>
<td>0.02 (ICP Std.)</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>99.1 (High-pressure heat drying)</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>0.1 (Dry ashing)</td>
</tr>
<tr>
<td>Crude fat (%)</td>
<td>0.5 (Acid hydrolysis)</td>
</tr>
<tr>
<td>Crude protein (%)</td>
<td>0.2 (Nitrogen coefficient: 6.25, <em>semi-micro</em> Kjeldahl method)</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>0.1</td>
</tr>
<tr>
<td>Calorie (Kcal)</td>
<td>5.7</td>
</tr>
<tr>
<td>Coli form</td>
<td>Negative</td>
</tr>
<tr>
<td>Bacterial count</td>
<td>0/mL</td>
</tr>
</tbody>
</table>

2. It seems that the description in the result part are not so in accordance with the Figures attached, such as Fig 3c

**Answer** I edited for your comments.

3. At the end of the result part, the authors said “…whether P38 activation was dependent on [Ca2+]i” was examined (Line 307), but this part of data were not present.

**Answer** I attached results for calcium and p38.
4. The authors declared that “...NADPH oxidase-derived ROS appear to trigger apoptosis via mitochondrial ROS.” (Line 279-280), but according to the data presented aforementioned, the evidence was not so adequate.

**Answer** I confirmed mitochondria ROS generation blocked by DPI (data not shown).

4. Minor essential revisions

I. Introduction - sentence: „Apoptosis is initiated by external signals....“ is not true. Apoptosis as programmed cell death process can be initiated also by intracellular pathways. I recommend to write 3-4 more sentences about principal pathways of apoptosis and then to pick up the way of external signals.

**Answer** I edited for your comments.

6. II. Materials and methods part Western blot analysis - density of SDS-PA gels is missing - 10 or 15% or gradient gels? Part Apoptosis assay- type of cytometer and microscope, as well as evaluation software are missing. There is some specification in the next method, but it should be preferable written in the apoptosis assay method description.

**Answer** I edited for your comments.

III. Results and Figures

1. Authors shell take more care of graphs and pictures, what can elevate the level of paper. Graphs are not of same dimensions and fonts in graphs and figures are also not united.

**Answer** Could you comments about edit figure detail? If you recommend about edit figure, I will be so happy.

2. Figure 1 - is senseless. Authors have used extract and not mixture of two purified chemical compounds. If the part A of Figure 2, would be Figure 1, also marking of pictures would be less complicated.

**Answer** I edited for your comments.
3. Figure 2 - part B - increase of population of PI positive cells is not apoptosis. PI, in contrast to annexin, flows into necrotic cells which have lesions in the cytoplasmic membrane. To see apoptosis, the peaks of annexin signal should be projected. Right interpretation is in part B - decreased apoptosis with increased necrosis in concentration-dependent manner. Part B shows cells treated with three different dilution of Khz-cp, but in the figure legend is described only 1:100 dilution.

Answer) I changed figure 2 – part B for annexinV&PI results. I showed time and dose dependently increase apoptosis cells by Khz-cp.

3. Figure 3 - part B - I suppose there should be α tubulin, not bubulin. It is not of fortunate choice to make references from one figure legend to another figure legend - and not only in this legend to figure 3. More reasonable would be to make a reference to materials and methods, or to describe it very briefly.

Answer) I edited for your comments.

4. Figure 4 - part G - Western blot picture - I suppose the exposition of cells should be in minutes, not in hours?

Answer) I edited for your comments.

5. Figure 5 - part B - description of lanes, does not fit with lanes.

Answer) I edited for your comments.

6. Figure 6 - it would be better to change marking of part C and D.

Answer) I could not good figure arrangement of part C and D. Could you recommend arrangement figure? If you recommend arrangement, I will be so happy.

7. Figure 7 - markers of columns in part D and E does not fit with columns. Probably shorter abbreviations for samples should be used (as C for control, Khz-cp only as K...?).

Answer) I edited for your comments.

IV. Discussion

Authors were so focused on argumentation of apoptotic effect of Khz-cp on transformed cells, that they have forgot to discuss, from my point of view, very important fact, that Khz-cp has not the same effect on non-transformed cells. Also the rise of cytoplasmic calcium concentration, what is according to scheme in figure 8, primary signal in the apoptotic cascade is not enough discussed. Is it from internal stores, or is it from enhanced calcium uptake? Calcium is very important general signal – how is it possible that tumor cells have another response as non-transformed cells? I think it is very important and interesting question.

Answer) I did ROS generation in non-transformed cells. In transformed cells,
ROS generation more increase than non-transformed cells (data not shown). But we had not been calcium increasing in non-transformed cells. So I think it had better make another research. Thank you for your comments.