Author's response to reviews

Title: Samsoeum, a traditional herbal medicine, elicits apoptotic and autophagic cell death by inhibitingAkt/mTOR and activating the JNK pathway in cancer cells

Authors:

Aeyung Kim (aykim71@kiom.re.kr)
Nam-Hui Yim (nhyim23@kiom.re.kr)
Jin Yeul Ma (jyma@kiom.re.kr)

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Author's response to reviews: see over
Responses to Reviewer comments for Ms. ID.: 1989109049978832

We greatly appreciate the helpful comments of the reviewers that have led to changes to substantially improve the manuscript. In response to the comments of the reviewers, we performed additional experiments to clarify some results and provide new data and then added and edited some sentences in the revised manuscript to answer the comments. All of these changes are highlighted in Red color in the revised manuscript.

Responses to Editorial comments

Comments

- Please provide us with all of the original data for each of the Western blot figures presented in your manuscript. This can be uploaded as an additional file alongside your manuscript.

Response

- In response to the comment, we newly uploaded file including Western blot data (file name: western blot raw data.ppt).

Responses to specific comments from referee 1

Comments #1

- The authors concluded that SSE was not cytotoxic to normal cells because hepatocytes were resistant to SSE. I think, however, more cell types should be studied before making such a general conclusion. Furthermore, hepatocytes are rather distinct from carcinomas and melanomas. The authors should use more appropriate cells as a control. In addition, more discussion on why normal cells are resistant to SSE should be provided.

Response

- We intend to confirm whether SSE is safe to the normal hepatocytes because some herbal natural ingredients are hepatotoxic. To clearly deliver this point, we edited and added the following sentences in the Result section describing Fig. 1C (p.11),
“Some herbal remedies and dietary supplements have been reported to induce hepatotoxicity because the liver plays an essential role in transforming and clearing chemicals [23]. Therefore, we next examined the effect of SSE on the cell viability of normal hepatocytes. As shown in Figure 1C, normal hepatocytes were unaffected by SSE treatment even after incubation for 48 h at 50 µg/mL, suggesting that SSE is cytotoxic to cancer, but not to normal hepatocytes.”

In addition, to evaluate whether repeated administration of SSE elicits systemic toxicity, or not, serological and hematological parameters were evaluated in mice after SSE administration. Body weight and organ weight after SSE administration were also measured. As shown below, the administration of SSE at doses of 145 or 290 mg/kg did not affect weight gain by approximately 9% in mice (Table 1). Organ weight changes were not significantly different between SSE-administered group and control (Table 2). In addition, the ratio of GOT/GPT and BUN/CRE were not significantly altered in SSE-treated group compared to control, suggesting that SSE administration did not cause hepatic and renal damage (Table 3). The hematological parameters of SSE-treated mice were also similar to those of control mice (Table 4). These data collectively indicate that SSE has no adverse effect during treatment based on serological and hematological findings. In xenograft model, SSE administration at doses of 145 or 290 mg/kg significantly suppressed tumor growth (~80% inhibition compared to control (saline) group, HT1080 cells, 2×10^6 cell, s.c. injection on femoral region, administration for 15 days). These data are unpublished. So data for sub-acute toxicity are shown just for reference.

Table 1. Means of body weights of mice administrated with 145 mg/kg or 290 mg/kg of SSE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 10</th>
<th>Day 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>22.66 ± 0.46</td>
<td>23.54 ± 0.69</td>
<td>24.20 ± 0.79</td>
<td>24.85 ± 0.48</td>
</tr>
<tr>
<td>145 mg/kg</td>
<td>22.58 ± 0.31</td>
<td>23.53 ± 0.37</td>
<td>23.92 ± 0.42</td>
<td>24.67 ± 0.16</td>
</tr>
<tr>
<td>290 mg/kg</td>
<td>23.10 ± 0.55</td>
<td>23.74 ± 0.98</td>
<td>24.13 ± 0.85</td>
<td>24.98 ± 0.27</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.D. Each group of mice (female, athymic nude mice) (n=3) were orally administrated with 145 or 290 mg/kg of SSE daily and weighed body weight at 0, 5, 10, and 15 days.

Table 2. Organ weights of mice administrated with 145 mg/kg or 290 mg/kg of SSE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver</th>
<th>Heart</th>
<th>Lung</th>
<th>spleen</th>
<th>Kidney (L)</th>
<th>Kidney (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.15 ± 0.04</td>
<td>0.11 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td>0.15 ± 0.00</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>145 mg/kg</td>
<td>1.02 ± 0.05</td>
<td>0.11 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.12 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>290 mg/kg</td>
<td>1.04 ± 0.08</td>
<td>0.10 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.D. Each group of mice (n=3) were orally administrated with 145 or 290 mg/kg daily, sacrificed at 15 days, and weighed organs.
To demonstrate more clearly that both apoptosis and autophagy cause cancer cell death, examine if the treatment of 3-MA also partially inhibits cell death, and whether simultaneous addition of 3-MA and z-VAD-fmk fully inhibits it.

In response to the comment, we examined the effect of 3-MA treatment on the cancer cell death. As shown below, 3-MA treatment partially inhibited SSE-induced cell death. Contrary to the expectation, simultaneous addition of 3-MA and z-VAD-fmk did not fully recover the cell viability. Instead, SP600125 (JNK inhibitor), which completely prevented the induction of Beclin-1, LC3-II, and Bax.

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### Table 3. Chemical analysis of serums obtained from mice administrated with 145 mg/kg or 290 mg/kg of SSE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GOT (IU/L)</th>
<th>GPT (IU/L)</th>
<th>BUN (mg/dL)</th>
<th>CRE (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56.8 ± 5.2</td>
<td>28.7 ± 8.1</td>
<td>22.1 ± 1.4</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>145 mg/kg</td>
<td>42.7 ± 4.6</td>
<td>22.0 ± 2.0</td>
<td>23.6 ± 1.2</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>290 mg/kg</td>
<td>48.4 ± 2.4</td>
<td>22.7 ± 2.3</td>
<td>23.0 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.D. Each group of mice (n=3) were orally administrated with 145 or 290 mg/kg daily, sacrificed at 15 days, and analyzed the levels of GOT, GPT, BUN, and CRE. GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; BUN, blood urea nitrogen; CRE, creatinine.

### Table 4. Hematological analysis of bloods obtained from mice administrated with 145 mg/kg or 290 mg/kg of SSE.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>control</th>
<th>145 mg/kg</th>
<th>290 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCP (×10^3 cells/µl)</td>
<td>2.0 ± 0.45</td>
<td>2.3 ± 0.15</td>
<td>2.1 ± 0.47</td>
</tr>
<tr>
<td>WBCB (×10^3 cells/µl)</td>
<td>2.1 ± 0.60</td>
<td>2.1 ± 0.13</td>
<td>2.2 ± 0.58</td>
</tr>
<tr>
<td>RBC (×10^6 cells/µl)</td>
<td>8.2 ± 0.20</td>
<td>8.8 ± 0.35</td>
<td>8.6 ± 0.47</td>
</tr>
<tr>
<td>Means HGB (g/dL)</td>
<td>13.8 ± 0.50</td>
<td>13.9 ± 0.61</td>
<td>13.7 ± 0.44</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>48.1 ± 1.55</td>
<td>49.6 ± 1.36</td>
<td>48.6 ± 1.55</td>
</tr>
<tr>
<td>MCV (IL)</td>
<td>57.3 ± 0.46</td>
<td>56.3 ± 0.90</td>
<td>56.7 ± 0.39</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>16.4 ± 0.10</td>
<td>15.7 ± 0.10</td>
<td>16.0 ± 0.46</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>28.6 ± 0.26</td>
<td>27.9 ± 0.53</td>
<td>28.2 ± 0.53</td>
</tr>
<tr>
<td>PLT (×10^4 cells/µl)</td>
<td>116.7 ± 2.4</td>
<td>108.4 ± 3.9</td>
<td>113.2 ± 3.6</td>
</tr>
<tr>
<td>% NEUT</td>
<td>38.7 ± 2.77</td>
<td>29.9 ± 2.10</td>
<td>19.7 ± 2.34</td>
</tr>
<tr>
<td>% LYM</td>
<td>53.5 ± 4.00</td>
<td>64.8 ± 3.2</td>
<td>71.9 ± 5.45</td>
</tr>
<tr>
<td>% MONO</td>
<td>1.9 ± 0.75</td>
<td>1.1 ± 0.42</td>
<td>1.4 ± 0.60</td>
</tr>
</tbody>
</table>

Data are presented as mean ± S.D. Each group of mice (n=3) were orally administrated with 145 or 290 mg/kg daily, sacrificed at 15 days, and analyzed hematologic parameters. CBC, complete blood cell count; WBCP, white blood cell count peroxidase method; WBCB, white blood cell count basophile method; RBC, red blood cell count; HGB, hemoglobin, HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; NEUT, neutrophil; LYM, lymphocyte; MONO, monocyte.

Comments #2
- Figure 5C: To demonstrate more clearly that both apoptosis and autophagy cause cancer cell death, examine if the treatment of 3-MA also partially inhibits cell death, and whether simultaneous addition of 3-MA and z-VAD-fmk fully inhibits it.

Response
- In response to the comment, we examined the effect of 3-MA treatment on the cancer cell death. As shown below, 3-MA treatment partially inhibited SSE-induced cell death. Contrary to the expectation, simultaneous addition of 3-MA and z-VAD-fmk did not fully recover the cell viability. Instead, SP600125 (JNK inhibitor), which completely prevented the induction of Beclin-1, LC3-II, and Bax.
and reduction of Bcl-2 by SSE treatment to the extent observed in untreated control cells (Fig. 5B), blocked cell death most efficiently. So, we emphasized this point in Fig. 5C.

**Minor comments #1**
- “Methods” section describes that the “S.E.” was used for the statistical analyses, whereas the “S.D.” was used in the figures.

**Response**
- We corrected “S.E.” in the method section into “S.D.”.

**Minor comments #2**
- p13, line2-line9: It would be better that this part explaining the behaviors of LC3 on autophagy is moved to the previous paragraph which refers to the images of RFP-LC3.

**Response**
- In response to comment, we moved indicated part to the previous paragraph.

**Minor comments #3**
- Figure 3B: It is difficult to judge chromatin condensation and nuclear fragmentation from the images.

**Response**
- In response to comment, Fig. 3B images were changed and converted to gray-scale. SSE-treated cells displayed chromatin condensation. We corrected sentences in the Result section describing Fig. 3B (p.12),

“After DAPI staining, AGS and B16F10 cells treated with SSE (50 µg/mL) for 24 h exhibited chromatin condensation (Figure 3B, white arrows)”
**Minor comments #4**
- Figure 3C: In B16F10 cells, the RFP-LC3 signal looks punctuated even without the treatment of SSE.

**Response**
- In response to the comment, we corrected sentences in the Result section describing Fig. 3C (p.12-13),

“As shown in Figure 3C, in AGS cells, RFP-LC3 was evenly diffused throughout the cytoplasm in control cells, whereas SSE-treated cells displayed a punctuate pattern of RFP-LC3 fluorescence, indicating the association of RFP-LC3 with the autophagosomal membrane. In B16F10 cells, SSE treatment remarkably increased punctuate pattern of RFP-LC3 fluorescence”

**Minor comments #5**
- Figure 8 should be cited in the text and needs a legend that includes the meaning of the colors.

**Response**
- Thank you for this comment. In response the comment, we modified Fig. 8 to clearly show the mechanism of anti-cancer effect by SSE. Overall explanation for the anti-cancer mechanism of SSE was described in Results and Discussion section and ‘Figure 8’ was cited in p17.
Responses to specific comments from referee 2

Comments #1
- Method, Cell lines, last sentence "What do you mean here? Coating with PBS? or something else?"
  Please clarify.

Response
- Regarding hepatocyte culture, we used plates after coating with 10% gelatin diluted in PBS and described it in the original manuscript as follows (p6, Method, Cell line):

  “hepatocytes were seeded on the culture plate coated with 10% gelatin/phosphate buffered saline (PBS), and incubated at 37°C in a humidified 5% CO2 incubator”

Comments #2
- Method Cell Viability and cell death. Why not determined the ICs e.g. IC50 for each chemical agents and herbal extract. Better to calculate that.

Response
- In response to the comment, we calculated the IC50 by MTT assay after treatment with SSE at doses 10~1000 µg/mL for 24 h. Values are as follows.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1080</td>
<td>25.1</td>
</tr>
<tr>
<td>AGS</td>
<td>42.1</td>
</tr>
<tr>
<td>A431</td>
<td>344.8</td>
</tr>
<tr>
<td>B16F10</td>
<td>388.9</td>
</tr>
</tbody>
</table>

These results are added in the Result section describing Fig. 1A and 1B (p11),

“As shown in Figure 1A and 1B, SSE reduced cell viability and caused cell death in proportion to concentration, whereas the relative concentration of DMSO (0.01%) had little influence on cell proliferation (HT1080, IC50=25.1 µg/mL; AGS, IC50=42.1 µg/mL; A431, IC50=344.8 µg/mL; B16F10, IC50=388.9 µg/mL)”

Minor comments #1
- Method, Statistical analysis. SigmaPlot do not belongs to SPSS, please correct the typographical mistake.
Response
- We used Sigma Plot 8.0 software which is maintained by SPSS Inc. (Chicago, IL, USA). According to your comment, we corrected sentence as follows (p10-11, Method, Statistical analysis),

“Statistical significance was analyzed by the two-tailed Student’s \( t \)-test in Sigma Plot 8.0 software (SPSS Inc., Chicago, IL) and a \( P \) value of less than 0.05 was considered statistically significant”

Quality of written English
- Needs some language corrections before being published

Response
- The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see:
Again, we greatly appreciate all of your great comments.

Sincerely yours,

Jin Yeul Ma, Ph. D

Center for Herbal Medicine Improvement Research,
Korea Institute of Oriental Medicine (KIOM),
483 Expo-ro, Yuseong-gu,
Daejeon 305-811,
Republic of Korea
Phone: +82-42-868-9466
Fax: +82-42-868-9573
E.mail: jyma@kiom.re.kr