Author's response to reviews

Title: Ardipusilloside I induces apoptosis via regulating Bcl-2 family proteins in human mucoepidermoid carcinoma Mc3 cells

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Author's response to reviews: see over
Dear editor Tom Rowles:

We must thank you and all other reviewers for the critical feedback. We feel lucky that our manuscript (MS: 1364305419733130) went to these reviewers as the valuable comments from them not only helped us with the improvement of our manuscript, but suggested some neat ideas for future studies. Please do forward our heartfelt thanks to these experts.

Based on the comments we received, careful modifications have been made to the manuscript. All changes were marked in red text. In addition, we also have a native English speakers double-checked the English for the revised R1 version. We hope the new manuscript will meet your magazine’s standard. Below you will find our point-by-point responses to the reviewers’ comments/questions.

Sincerely yours,
Xiao-Fang Xu
RESPONSE TO THE COMMENTS.

1. To reviewer Sukh Mahendra Singh

Reviewer's report:
This is an interesting piece of work regarding the apoptosis inducing effect of ardipusilloside I, an isolate from Ardisia pusilla, on a mucoepidermoid carcinoma cell line Mc3. The study shows the implication of caspase 3 and bcl2/bax in the anti-survival action of ardipusilloside I. While the work is in general well presented, the authors are advised to address the following issues (Major Compulsory Revisions), incorporation of which may help in improving the quality of the work

1. Introduction: The lacuna should be discussed in view of the earlier works of similar nature (see references below) to justify the logic of carrying out of the present investigation. Already some previous studies have reported regarding mechanisms involved as mentioned in point # 4 below. The novelty of the work is not clear in view of these previous studies with very similar or related observations. In the absence of a logical explanation to this point the originality of the study is not clear to a reader.

Response: Thank you very much for your literatures and constructive suggestions which improve our manuscript a lot. Major Revisions were performed in the “Introduction” and “Discussion” section of revised R1 version, in which we discussed the earlier works about Ardipusilloside I and emphasized the novelty result that Ardipusilloside I induces apoptosis by regulating caspases-3 and Bcl-2 family proteins level in mucoepidermoid carcinoma Mc3 cells.

2. Materials and methods:
(i) It should be clarified if the preparation of ardipusilloside I was free of endotoxin.

Response: Thanks. In the revised R1 version, we clarified the preparation of
ardipusilloside I was free of endotoxin.

(ii) The source of antibodies must be mentioned in the reagents section.

**Response:** Thanks for the valuable reminding. The source of antibodies was added in the reagents section.

(iii) In cell culture sub section what do the authors mean by ‘cell line Mc3 was established’.

**Response:** It has been corrected “cell line Mc3 was obtained and stored in our laboratory”. MEC-1 is a human mucoepidermoid carcinoma cell line derived from palatal salivary gland. Mc3 is a highly metastatic cell line selected by repeated in situ-transplants of MEC-1 cells into salivary gland of nude mice and obtained from lung metastatic nodes(Wu JZ, Si-Tu ZQ, Liu ZB, et al. Selection and characterization of a high metastatic cell clone from mucoepidermoid carcinoma cell line MEC1 derived from human salivary gland. J Fourth Mil Med Univ 1998;19(1):1–4. Article in Chinese).

(iv) Reference for MTT assay should be provided. This assay is mainly dependent on measurement of metabolic activity of cells while the authors use two terms interchangeably in the manuscript: viability and survival. The authors should rectify the same. In MTT assay the temperature of incubation needs to be mentioned. It will be desirable to use additional parameter of viable counting of cells.

**Response:** ①Reference for MTT assay was added in the manuscript. {No.12 BMC Complement Altern Med 2012, 12(1):69 The anticancer effect of saffron in two p53 isogenic colorectal cancer cell lines.} 
②Cell viability was chosen to describe the result of MTT assay. Meanwhile, fig 1 was rectified into the cell viability ratio on the basis of the same raw data.
③In MTT assay the temperature (at 37°C in a humidified atmosphere containing 5% CO2.) of incubation was added.
④Thank you for your constructive suggestions. In order to confirm the result of MTT
assay, we added the “S-phase fraction Analyses by Flowcytometry” section after “Cell Viability Assay” section, and fig3.

(v) DNA fragmentation subsection the authors refer to both attached and detached cells: what was the difference?

Response: That’s a good question. Thank you. In our study, we found that a small part of cells were detached from the plates at the early stage of Ardipusilloside I treatment. In order to collect all those cells to compare the DNA fragmentation, we harvested both attached and detached cells for test.

(vi) Flowcytometry: cells were treated for ‘certain period of time’ what does this mean? (vii) Statistical analysis: Students’ t test check spelling.

Response: Sorry for those typo errors. It has been changed into “48h” and “Students’ t test”

3. Results:

(i) The method of calculating IC 50 should be included in materials and method section. From Fig 1a IC 50 being 9.94 μg/ml as mentioned in results in not matching. It may be rechecked. The authors than need to give the logic of using a dose of 10 μg/ml of ardipusilloside I for in vitro treatment of cells.

Response: Thanks a lot for your critical reading of the manuscript. Fig 1a was the result of 48h treatment with various concentrations of Ardipusilloside I. And all the other tests with 10.0μg/ml Ardipusilloside I were for 48h, which was rechecked in the revised manuscript (R1 version).

① The method of calculating IC 50 was added into the materials and method section.
② The IC 50 were calculated using SPSS version 16.0, which was based on the logic statistics. However, the curves of Fig 1 were drew by software GraphPad Prism based on the raw data. So there is a difference between the IC 50 calculated by SPSS version 16.0 and Fig 1. The IC 50 9.94μg/ml was a statistics data and the Fig 1 is for the trend performance.
The MTT assay revealed that Ardipusilloside I affected the viability of Mc3 cells in a dose- and time-dependent manner, and the IC50 of Ardipusilloside I in the present study was approximately 9.98μg/ml at 48 h of treatment. With a higher does or more time treatment, most of the cells were disintegrated and necrosis. So, we chose 10.0 μg/ml (which was approximate to the IC50 9.98 μg/ml) and 48h for this study, which was suit for the mechanism study.

(ii) In Fig. 1 a deviation bar should be included. In both Fig 1 a & b indication for significant differences needs to be included.

Response: Thanks for your suggestions. Cell viability was chosen to describe the result of MTT assay. Meanwhile, this figure was rectified into the cell viability ratio on the basis of the same raw data with deviation bar.

(iii) Fig 2. c & d should be on same scale.

Response: Thanks. In order to keep TEM Figure on same scale, another two figures with the magnification of 6000 were chosen.

(iv) What do the authors mean by the terms/statements like: ‘apoptotic rate’; ‘10 μg/ml of ardipusilloside I was sufficient to induce’; ‘while caspase 3 and bax protein was activated’.

Response: Sorry to confused you by those Chinglish.

1 ‘apoptotic rate’ was changed into “apoptotic cell ratio”
2 ‘10 μg/ml of ardipusilloside I was sufficient to induce’ was changed into “10 μg/ml of ardipusilloside I induce apoptosis significantly”
3 ‘while caspase 3 and bax protein was activated” was changed into “while caspase-3 and Bax protein was increased”

(v) Fig 3. The DNA ladder shown in lane 3 should be improved.

Response: Thanks. We repeated the DNA fragmentation detection again and again.

In the R1 version, we uploaded another figure of DNA ladder.
(vi) It may not be appropriate to state that the study investigates the anti cancer activity of ardipusilloside I merely based on in vitro investigations on MC3 cells. It will be better to use ‘inhibitory or apoptosis inducing action of ardipusilloside I’.

Response: We totally agree with your comment. “Apoptosis inducing action of Ardipusilloside I” was used in the R1 version.

4. Discussion: Discussion should highlight the novelty in wake of following previously reported mechanisms of the anticancer action of ardipusilloside I, which at present are not included in bibliography.

   Xing Y, Lou L, Chen X, Ye Q, Xu Y, Xie C, Jiang J, Liu W


5. English: There are several grammatical and typing errors for which the manuscript essentially needs an extensive revision.

Response: The manuscript has been reviewed by an English-speaking scientist and the language edited accordingly.

2. To reviewer Jia-Jun Liu
Review report:

Specific recommendations:

1. In the abstract, the authors should declare their objective of this study, or is the word “Background” is typo error? Also, the molecular structure of ardipusilloside I should be provided in the introduction.

Response: Thank you for your constructive suggestions.

① We rewrote the “Background” part of abstract as following: Ardisia pusilla A. DC, of the family Myrsinaceae, is a traditional Chinese medicine named Jiu Jie Long with a variety of pharmacological functions including anti-cancer activities. We have purified a natural triterpenoid saponin, Ardipusilloside I, from Ardisia pusilla and shown that Ardipusilloside I exhibits inhibitory activities in human mucoepidermoid carcinoma Mc3 cells. In this study, we investigated the underlying mechanisms of proliferation inhibition of Ardipusilloside I in Mc3 cells.

② Also, we added a figure to show the chemical structure of Ardipusilloside I in the introduction.

2. Only one cancer cell line (Mc3) was used in the study, the authors need to demonstrate that the compound specifically induces apoptosis or cell death in more (two or three) cancer cell lines, but not normal tissues or cells.

Response: In our previously study, we showed that Ardipusilloside I inhibits the proliferation of Mc3 cells in a dose-and time-dependent manner. The aim of this study is to investigate the underlying mechanisms of proliferation inhibition of Ardipusilloside I in Mc3 cells. Still, we thank you for your constructive suggestion, which brings us neat ideas for future studies.

3. Some species specific serum esterases can break down small molecules really fast, so try different formulations, serum concentrations, species of serum, or even try replacing serum with a synthetic replacement. Alternatively, the authors might apply the compound to the cells in the absence of serum for some period of time, then replace the serum after the cells have had a chance to absorb it. However, keep in
mind the importance of controls when doing these sorts of modifications to experimental design.

Response: We totally agree with the reviewer’s comment and thank you for your kind advice.

4. The MTT assay is used mainly describes the mitochondrial functional capacity alone, as a tool of cell proliferation is not enough. It is important the authors to show data using proliferation markers (either 3TdR incorporation in tumoral cells, or flow cytometric assessment of S-phase fraction based on BrdU labeling, or immunocytochemical staining for Ki-67 or PCNA proteins)

Response: Thank you for your constructive suggestions. In order to confirm the result of MTT assay, we added the “S-phase fraction Analyses by Flowcytometry” section after “Cell Viability Assay” section, and fig3.

5. In figure 3, the electrophoresis results showed just like “smear” line, and no typical “DNA Ladder” was found. Also, the expression of bax and bcl-2 should be detected before 24 h (before apoptosis occurred).

Response: Thanks. We repeated the DNA fragmentation detection again and again. In the R1 version, we uploaded another figure of DNA ladder.

3. To reviewer Bungorn Sripa

1. The consistency of using "A" or "a" of ardupusilloside I in the sentences.

Response: Thank you very much. In the revised R1 version, "A" of ardupusilloside I was used to keep the consistency for the whole manuscript.

2. page 5, line 4: should be "200 x g".

Response: Sorry for the error. We changed this typing err into "200 x g".
3. page 8, line 11: should be "after exposure to ardipusilloside I".

**Response:** Thanks. We changed it into "after exposure to ardipusilloside I".

4. page 9, line 12 and page 10 line 10: should be "underlying".

**Response:** Sorry for the error. They were changed into "underlying".

5. Figure legends:

   - Fig 1 should be "5.0, 7.5,"
   - Fig 2 should put "a" "b" and bar size or magnification on these Fig.
   - Fig 4 should put "panel c".

**Response:** Thanks for your carefully reading. All the errors were corrected following the suggestion.