Author's response to reviews

Title: Jacarelhyperol A induced apoptosis in leukaemia cancer cell through inhibition the activity of BCL2 Proteins

Authors:

Shoude Zhang (sdzhang@ecust.edu.cn)
Jun Yin (Junyin@163.com)
Xia Li (powful_lixia@hotmail.com)
Jigang Zhang (jgzhang@sjtu.edu.cn)
Rongcai Yue (yrcowen@163.com)
Yanyan Diao (vydiao@ecust.edu.cn)
Honglin Li (hlli@ecust.edu.cn)
Hui Wang (huiwang@sibs.ac.cn)
Lei Shan (shanleicn@126.com)
Weidong Zhang (wdzhang@hotmail.com)

Version: 4 Date: 10 July 2014

Author's response to reviews:

Authors' Response
The authors are grateful to the reviewers and editors for their comments, which helped us to greatly improve our manuscript. This paper is revised exactly according to their comments.

For Editors:

We recommend that you ask a native English speaking colleague to help you copyedit the paper. If this is not possible, you may need to use a professional language editing service. For authors who wish to have the language in their manuscript edited by a native-English speaker with scientific expertise, BioMed Central recommends Edanz:

Response: Thanks for your suggestion. According to your suggestion, the English language has been edited by the Edanz.

For Referee 1:

Major Compulsory Revisions
1. There is no experimental proof that binding of Jac-A to bcl-xL etc is also the causative reason for apoptosis induction neither in in vitro nor in vivo experiments – confirmative Western blots (pull-downs) showing the modulation and/or binding properties of the bcl-2 family members +/- Jac-A are missing. In addition, and to underline the possible therapeutic preference for this novel compound, control experiments with already utilized drugs (such as e.g. ABT-737) should be performed to compare the observed effects: ABT-737 has anyway already been used for the modelling.
Response: Thanks for your suggestion. 1) To confirm that Jac-A binds to anti-apoptotic Bcl-2 family members and competes with binding of pro-apoptotic proteins, co-immunoprecipitation was performed to analyze if these interactions are disrupted by Jac-A. 2) Because of Jac-A is a natural product, we selected the natural BCL-2 inhibitor ##)-Gossypol as positive control in the FP experiment and flow cytometry. In addition, we selected doxorubicin as a positive control because it is common used in MTT. In the Western blots experiment and in vivo experiment, the results can be compared with the control group.

2) Previous fundamental work (the isolation and characterization of the compound) is stated but not cited.

Response: Thanks for your suggestion. The citation has been added.

3) Methods: apoptosis and cell cycle: there is no cell cycle data in this paper although the experimental features were (wrongly) described in the Meth & Mat section;

Response: Thanks for your reminding. This error has been revised.

4) Results: Fig.3: Ann/PI staining: it is not mentioned how many times this assay was performed and there is no graph showing the results including SD – I have to assume that this was a single experiment with an unusual buffer that might not work, since there is a specific Annexin-buffer normally used for the staining. The last plot says that Q1 and Q2 together should contain about 40% of cells but this is hard to assume from the shown plot.

Response: Thanks for your suggestion. We have done this experiment again with three times for every concentration. And the SD has been added into the results.

5) Fig 4B: Bcl-2 family proteins should be shown and the effects of a known inhibitor eg ABT 737 should be included as a control.

Response: Thanks for your suggestion. This experiment was performed with a method modified from other (J. Med. Chem. 2010, 53, 3465–3479). Our purpose of this experiment was to detect the expression change of Bcl-2 proteins after treatment with Jac-A compared to control. In pre-experiment, we used the ##)-Gossypol to inspect the reliability of this method and the data was not shown.

6) Fig 5C: according to the text the p value was <0.001; this should also apply for the 2mg/kg group? Fig.5D: p values?

Response: Thanks for your suggestion. Fig 5C are representatives of tumors harvested from dead nude mice bearing K562 cells from different groups treated with the vehicle or Jac-A, so there was no statistical significance. Fig.5D is the Kaplan-Meier survival plot of the K562-bearing nude mice and the statistical significance of differences in survival of the mice in different groups was determined by the log-rank test using the GraphPad Prism program (PNAS, 2007, 104, 8444–8448).

7) High weight in the high concentration treated group: there could be also other reasons for that beside mere well-being, please discuss! To which concentrations has Jac-A be tested in animals? What would be the effective dose in humans?
Could this be reached by any means? What are the expected side effects (extrapolated from ABT-737, e.g.? )? What are the side effects of a similar effective dose of ABT-737?

Response: Thanks for your suggestion. Reasons for High weight in the high concentration treated group have been discussed. Three concentrations of Jac-A (2, 10, or 50 mg/kg) be tested in animals. The effective dose of Jac-A in humans needs relative clinical research. We did not find any obviously side effects in this experiment. However, the resource plant of Jac-A, H. japonicum, is used as an animal feed in China because of its widespread growth and an 85% ethanol-treated water extract is documented in the Chinese Pharmacopoeia as an injection for the treatment of viral hepatitis, which demonstrating the safety of Jac-A. The effective dose of ABT-737 is far lower than Jac-A and ABT-737 is a synthesis compound, so it is not suitable for comparison.

Minor Essential Revisions

1. The modeling of the proposed binding is well done, however, fig description is incomplete and from the shown figure the reader cannot delineate the described mechanism.
Response: Thanks for your suggestion. The description has been updated.
2. first page results: BH3-bingding pocket - spelling error
Response: Thanks for your reminding. This error has been revised.
3. In the background section: the protein used for FP is mentioned as Flu-Bak-BH3, first line in methods mentions Bid-BH3 domain peptide # which one was used?
Response: Thanks for your reminding. It was Bid-BH3 and this error has been revised.
4. Fig 3: annotation of the lower right quadrant (Q4) is wrong (AnnV +/-Pi -)
Response: Thanks for your reminding. This error has been revised.
5. Fig 4A gives no essential information and is a cartoon only which can be Removed
Response: Thanks for your reminding. This cartoon has been removed.
6. The specific antibody clones utilized in western blots should be mentioned in the Materials section
Response: Thanks for your suggestion. The information of the specific antibody clones has been added.
7. Results: Fig 2a: residues are not numbered as described in the text, sub-pockets P4 and P5 are not shown?
Response: Thanks for your suggestion. It looks disorderly if all residues are numbered and we only labeled three key residues. Sub-pockets P4, and P5 binding pockets have been labelled with dash circles.
8. Fig.5A: no p-values given.
Response: Thanks for your suggestion. This information has been added.
For referee 2:

Major Compulsory Revisions

1. The authors used a fluorescence polarisation assay to show competitive binding of a BH3-peptide and Jac-A to anti-apoptotic Bcl-2 proteins. To confirm that Jac-A binds to anti-apoptotic Bcl-2 family members and competes with binding of pro-apoptotic proteins in vivo, co-immunoprecipitation of e.g. Mcl-1/Bak should be performed to analyze if these interactions are disrupted by Jac-A.

Response: Thanks for your suggestion. We have performed the co-immunoprecipitation experiment used the K562 cells. The results have been added into the manuscript with highlight.

2. To demonstrate involvement of the mitochondrial apoptosis pathway, the authors should analyze breakdown of the mitochondrial membrane potential and/or release of cytochrome c upon Jac-A treatment.

Response: Thanks for your suggestion. We have analyzed the release of cytochrome C upon Jac-A treatment. The results have been added into the manuscript with highlight.

3. The authors state that Jacarehyperol A induces apoptosis in leukemia cancer cells. However, analysis of Jac-A-induced apoptosis by Annexin/PI staining was done only for one of the cell lines (K526 cells in figure 3). For all the other cell lines only a proliferation assay is shown. Annexin/PI staining should at least also be done for the other leukemia cell lines. To conclude on a general role of Jacarehyperol A as a potential inducer of apoptosis it is also recommended to perform Annexin/PI staining with the solid cancer cell lines.

Response: Thanks for your suggestion. We have performed Annexin/PI staining with other leukemia cancer cells (HL60 and THP-1) and the results have added into supplement material. In addition, it is difficult for us to perform Annexin/PI staining with the solid cancer cell lines because the solid cancer cell lines was conserved too long to be used for test. However, we think it will be same with the results of in vitro cancer cell lines because the xenograft mice model was established by injected s.c. with K562 cells.

4. The authors showed caspase activation upon Jac-A treatment. To show that cell death induction by Jac-A depends on caspase activation they should also analyze whether inhibition of caspase activity abrogates Jac-A-induced cell death.

Response: Thanks for your suggestion. We have analyzed the ability of Jac-A-induced cell death after inhibiting the caspase activity using the caspase inhibitor Z-VAD-FMK. The results demonstrated that the inhibition of caspase activity really decreased the ability of Jac-A-induced cell death. These analyses have been added into the manuscript with highlight.

Minor Essential Revisions

1. In table 1 the affinity of gossypol to Bcl-xL, Bcl-2 and Mcl-1, determined by a fluorescence polarisation assay, is shown. However, gossypol is neither
mentioned in the legend nor main text. Furthermore, gossypol has been shown to induce caspase-dependent apoptosis in the absence of Bak and Bax by converting Bcl-2 from an inhibitor to an activator of apoptosis. The authors should discuss these results in consideration of Jac-A induced apoptosis.

Response: Thanks for your suggestion. We have mentioned the positive control gossypol in the legend and main text. Furthermore, the fluorescence polarisation assay was performed using the Bid BH3 domain peptide and previous research have proved that gossypol can disrupt the heterodimerization of Bax with Bcl-XL or Bcl-2 (A) and Bim with Bcl-XL (Life Sciences, 2007, 80, 767–774). However, recently research suggested that gossypol can induce caspase-dependent apoptosis in the absence of Bak and Bax by converting Bcl-2 from an inhibitor to an activator of apoptosis (FASEB J. 2006 , 20, 2147-2149). So we speculated that gossypol present different mechanism in the absence of Bak and Bax and in the present of Bak and Bax. In our research, we proved Jac-A can disrupt the heterodimerization of Bax with Bcl-XL or Bcl-2 and Bak with Mcl-1. About other mechanism of Jac-A needs further research in future.

2. In table 2 Doxorubicin was used as a positive control but neither mentioned in the legend nor main text. In addition it would be more appropriate and interesting to compare Jac-A with Obatoclax, a BH3 mimetic, which binds to a broad spectrum of Bcl-2 family members, including Bcl-2, Bcl-xL, and Mcl-1.

Response: Thanks for your suggestion. We have mentioned the positive control doxorubicin in the legend and main text. In addition, we selected doxorubicin as a positive control because it is common used in MTT and the anti-cancer activities of Obatoclax for most of cancer cells were not reported.

3. In figure 3 the “PI-AV+” quadrant Q4 is labeled “PI-AV-“.

Response: Thanks for your reminding. This mistake has been revised.

4. In figure 5 D labeling of the y-axis is confused.

Response: Thanks for your reminding. This mistake has been revised.

5. In figure 5D partitioning for the quadrants differs compared to figure 5A-D.

Response: Thanks for your suggestion. For figure 5D, we want to records the % survival after treatment by Jac-A, so we should start record from 0 day. However, for figure 5A and 5E, we want to record the tumor volume and body weight after treatment by Jac-A, so we should start record from before treatment (-1 day)