

Reviewer 1:

The authors used H₂O₂ to treat a mouse embryonic hepatocyte. On top of that, the authors studied whether there is any protective effectiveness of Salvianolic acid B. Several pathways have been checked, including apoptosis, protein carbonyl content, ROS and lysosome membrane, ect. The hard work is appreciated. However, the review has several major concerns:

- 1) What is the innovation? That Salvianolic acid B can protect H₂O₂-induced cytotoxicity has been reported several times, such as PMID 24780446, PMID 25855584, ect.

Reply:

the innovation in this study is that Sal B protected cells from H₂O₂-induced cytotoxicity by increasing lysosomal protein LAMP1 level to keep lysosome membrane integrity. This is different from others reports .

YES, there are reports about Sal B protection on H₂O₂-induced cytotoxicity. however, these reports are different from ours. e.g, The PMID 25855584: reported that Sal B protected iPSC-derived neural stem cells (NSCs) by reducing expression of matrix metalloproteinase (MMP)-2 and -9, and phosphospecific signal transducer and activator of transcription 3 (p-STAT3).

The PMID 24780446: reported human bone marrow derived-endothelial progenitor cells (BM-EPCs) by modulating Akt/mTOR/4EBP1, p38 MAPK/ATF2, and ERK1/2 signaling pathways.

- 2) The mechanisms involved in this protection are various, involving multiple pathways. How could the authors draw the conclusion that stabilizing lysosomal membrane is the major reason or the cause? The authors merely observed that there is a correlation between drug treatment and lysosomal membrane stabilizing. But correlation obviously doesn't mean cause. The lost of lysosomal membrane permeability and/or the LMP and CatB/D Leakage could be a result of other mechanisms. The author need to find a specific drug to destroy lysosome membrane and perform experiments with this drug.

Reply: in another paper (cited in reference), we used positive drugs e.g bafilomycin A1(a specific inhibitor of V-ATPase activity), NH₄Cl (leading to lysosomal alkalization) and CA-074-Me (a CatB inhibitor) to verify The lost of lysosomal membrane permeability is one of main mechanisms of H₂O₂ induced cell cytotoxicity.

In this paper, we focus on the effects of Sal B on this pathway.

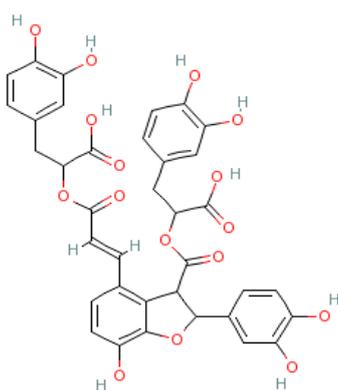
- 3) The experiments are only based on one cell line and there is no in vivo data. It is recommended to extend this study to include various cell lines. It is also recommended to include in vivo ROS models. Especially it is interesting to know that Guo et al. reported that Salvianolic acid B increase ROS levels in some human cells (PMID: 28000873).

Reply: Yes, we added CCl₄-induced acute liver injury mice to observe Sal B effects. The toxicity of CCl₄ is attributed to the reactive oxygen species (ROS) and free radicals produced during its metabolism. ROS involved in the pathological progress of liver diseases.

Actually, I can't explain Guo 's reporte about Sal B reversed MDR in HCT-8/VCR cells by increased intracellular ROS levels.

The Sal B molecular structure shows that there are some phenolic hydroxyl groups which

possess antioxidant effects.



the Salvianolic acid B structure

Other minors:

- 1) Salvianolic acid B is an important drug in the Traditional Chinese Medicine. It is recommended to introduce the background of Traditional Chinese medicine in treating liver diseases. References such as PMID 26006028, PMID 25292339 should be cited.

Reply: yes, these References had been added.

- 2) After Fig. 2, there is no mention about the concentration of SAB that is used. Are they all 10 μ M? Also, the authors need to explain why this concentration is used.

Reply: YES. After Fig2, the Sal B concentration of other experiments is 10 μ M. As shown in Fig 1, 10 μ M of the Sal B has obviously cell protection effect. On the other hand, Sal B concentration we prior used also 10 μ M(some Chinese papers). So we think this is a suitable concentration.

- 3) Discussion is out of focus. From the reviewer's perspective, there is no discussion. Most of the current Discussion content can be put into the Introduction. And the others are merely repeat the results.

Reply: Discussion has been revised

Reviewer 2:

The paper is very interesting but needs to be edited for language. It contains numerous grammar mistakes. Moreover, whereas the authors target the effect of Sal B on the protection of the integrity of the lysosomal membrane by upregulating the expression of LAMP1, via reduction of cathepsin B/D leakage into the cytosol, and protects hepatocytes from apoptosis. However, the validation of these interesting results in vitro should be performed either in vivo or with clinical samples.

Reply: Yes, in vivo experiments was supplement, which is about CCl4-induced acute liver injury mice to observe Sal B effects

Reviewer 3:

In this contribution, the authors tried to demonstrate that the compound Sal B upregulated the expression of LAMP1, thereby protecting the integrity of the lysosomal membrane through reduced cathepsin B/D leakage into the cytosol. The authors show that this had a protective effect on hepatocytes. The study in this respect is interesting however revision is needed before publication. Please address the following questions and comments:

- 1) The main concern is the novelty of the use of Sal B instead of NAC. Hornick JR et al (J Exp Clin Cancer Res. 2012 May 2; 31:41. doi: 10.1186/1756-9966-31-41) have reported involvement of LMP and oxidative stress which is protected from by NAC. How the findings regarding Sal B (in light of the findings involving NAC) are novel should be explained. The experimental details/set up are not clear and more information needs to be added to materials and methods/legends.

Reply: Yes, in the paper, NAC decreased ROS by H₂O₂ but not α -tocopherol, so we know antioxidants sometime display different effects, whether Sal B involved in LMP and oxidative stress in hepatocytes still need to be research.

The experimental details were revised

- 2) Figure 1B shows that 5 μ M of Sal B is sufficient to reverse the effect of H₂O₂ on cell viability and that concentrations > 5 μ M don't result in a further increase in cell viability. In light of this, it is unclear why 10 μ M (and not 5 μ M) Sal B was used for subsequent experiments.

Reply: Yes, Figure 1B shows that 5 μ M of Sal B is sufficient to reverse the effect of H₂O₂ on cell viability. But 10 μ M Sal B is used in the following experiments. the reason is, on one hand, we think the 5 μ M Sal B is a beginning concentration, to get better repeated results, we choice the 10 μ M Sal B. on the other hand, our previous experiments (papers published in Chinese) used to 10 μ M Sal B concentration.

- 3) In figure 1B, duration of H₂O₂ treatment is 2 hours, whereas in Figure 2, the duration is 8 hours. The reason for these different time periods of treatment is unclear.

Reply: sorry, this is a writing mistake. In the beginning, we used time points to get better results, and then all following experiments the H₂O₂ concentration is 500 μ M H₂O₂ and the time is 2 h.

- 4) Based on the above discrepancies, it is unclear how long the cells were treated with H₂O₂ for subsequent experiments such as Figure 3, 4 and so on.

Reply: in all experiments, the H₂O₂ concentration is 500 μ M H₂O₂ and the time is 2 h.

Other comments:

- 1) The last two sentences in the section titled "Sal B Protects the Lysosome Membrane" state "The intensity of green fluorescence gradually decreased with the increasing duration of H₂O₂ treatment. The fluorescent intensity increased with the Sal B pretreatment. NAC used here as a positive antioxidant and showed similar result as Sal B (Fig 4D-E)". Figures 4D-E don't show any data pertaining to different durations of H₂O₂ treatment.

Reply: the mean is that The fluorescent intensity of Sal B and NAC is different from H₂O₂ treatment. The describe was revised.

- 2) The discussion is too brief. As mentioned in Point 1, novelty of the study compared to state of the art findings of NAC should be described. Applications of the results should be discussed, for eg. Therapeutics, etc. What advantages Sal B has over NAC, if any should be mentioned.

Reply: revised

- 3) For a better relevance to application, it would be useful to include some results from human hepatocytes since most cells and models show variability between species.

Reply: Yes, the best cell line is human hepatocyte cell line, but there is no human hepatocyte cell line in China market by now in my knowledge.