Author’s response to reviews

Title: Boswellic acid sensitizes gastric cancer cells to Cisplatin-induced apoptosis via p53-mediated pathway.

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Author’s response to reviews:

A New Version of Manuscript ID # PHAT-D-20-00206 entitled "Boswellic acid sensitizes gastric cancer cells to Cisplatin-induced apoptosis via p53-mediated pathway".

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BMC Pharmacology and Toxicology

Dear Editorial Board

I would like to thank you and the reviewers for providing us with such opportunity in resubmitting our manuscript (ID # PHAT-D-20-00206) again for a possible publication in BMC Pharmacology and Toxicology.

We have carefully addressed the reviewers’ comments and I am sending herewith the revised manuscript as individual source file, together with the list of changes (Highlighted in the text), for re-evaluation by your respective journal.

We believe that the manuscript has been substantially improved and is now can be acceptable for publication in BMC Pharmacology and Toxicology.

I am looking forward to hearing from you good news and please do not hesitate to contact me if you need additional information.

Yours sincerely,

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Reviewer 1:

Paper is well described and designed. Authors should add the ethical committee agreement.

Response:

We appreciate the positive feedback from the reviewer on our submitted manuscript. We agree with the reviewer that the ethical committee agreement is important, however; the current study did not require such agreement. All used cell lines are commercially available and no patient samples were used.

Reviewer 2:

The present study describes the activity in vitro of the acetyl-keto-beta boswellic acid (AKBA) as a possible treatment for p53-mediated apoptosis of gastric cancer cells induced by cisplatin (CDDP) treatment.

The study is well designed and shows promising results of AKBA effect, consistent with an analogous proapoptotic activity of AKBA observed in previous studies. However, the description of technical features (e.g. cell seeding procedure, protein expression analysis, experiment repetition) and an unclear line of discussion, in terms of hypothesis generation, give rise to several concerns.

We are happy about the reviewer's comments regarding study design and findings. We are also thankful for the raised concerns that are very valuable. They are carefully addressed as follows:

Major revision

It is not clear how many experiments were performed by authors. Three replicates should be considered reliable only in case of repeated experiments. Western blot analysis and in particular protein normalization looks to be run on different blots. In this way it is hard to ascertain if GAPDH expression refers to the same sample loaded for the detection of targets of analysis.

Response:
We agree with the reviewer that the number of experiments should be clearly mentioned. Each experiment in the current study was performed in triplicates and repeated three times (replicates) independently with the same parameters. In each replicate, every test was carried out in duplicate. So the average of two duplicates represent one replicate and the average of three replicates represents one experiment that is eventually expressed on the graph as mean ± standard error (SE).

We also agree with the above statement "protein normalization looks to be run on different blots" and this is true but not for all. Proteins of p53 (MW 53), NFkB (MW 65) and GAPDH (MW 36) genes were analyzed on the same membrane due to the distinct differences in their molecular weight (MW) and thus can be easily detected unlike Akt (MW 60). In addition, different species of the primary antibodies were used such as anti-mouse for p53 and anti-rabbit for the rest of the proteins to avoid the overlap between detected bands due to their convergent molecular weights. The blot of GAPDH in each figure is a representative one and each protein was normalized with its own corresponding GAPDH.

Data referring to co-treatment (AKBA and CDDP) indicate a single concentration tested, it is not clear the rationale behind concentration choice.

Response:

We thank the reviewer for drawing our attention to this point, the details of concentration selection has been incorporated in the text of the manuscript. The following paragraph “The optimum concentrations were selected based on our findings from Figure 1 and 2 in which these concentrations showed significant difference compared to the controlled group. In addition, Liu et al and his team supported our selection when they found that IC50 of CDDP for NCI-N87 cell line is higher than AGS cells,18 hence NCI-N87 cells were treated with 50 μM and AGS 25 μM.” has been added to page 7 line 51.

Discussion of data righteously mentions previous studies showing AKBA activity as a possible therapeutic for GC. Since the first part of the discussion cites data in vitro in different cancer models, it should be more focused on features consistent with the P53 and Akt pathways.

Response:

We thank the reviewer for bringing up this point that will be a great addition to the discussion and thus strengthen our findings. Although some models were mentioned in the submitted manuscript but similarities and differences were not explained in depth. In the revised form of the manuscript, additional references were incorporated and more emphasis was put on the similarities and differences between these studies and the current one. The following statements were added as indicated below:

“As many drugs that affect multiple pathways, boswellic acid acetate was also found to induce apoptosis via p53-independent pathway as Xia L et al and his team showed in myeloid leukemia cell.23. They demonstrated that the levels of apoptosis–related proteins Bcl-2, Bax, and Bcl-XL were not modulated by boswellic acid acetate, however; it induced Bid cleavage and decreased mitochondrial membrane potential without production of hydrogen peroxide. By using general caspase inhibitor (Z-VAD-FMK) and a specific caspase-8 inhibitor II (Z-IETD-FMK), boswellic acid acetate–induced apoptosis was blocked, suggesting that such mechanism was through activation of caspase-8 and not p53. Such variation in boswellic acid acetate action might be due to the differences in genetic background of both cancer types as well as the activities of these tested proteins.23” on page 9 line 5

“supporting our results where CDDP reduced Akt expression in the tested cells” on page 10 paragraph 2
“Altogether, suggesting that AKBA can act on different molecular mechanisms and pathways accountable for inducing cell apoptosis in different cancer cell types”.14 on page 10 paragraph 3

Point by point revision

1. Page 3 lines 13-15: mention of "other substrate" is too generic, please rephrase.

Response:

We thank the reviewer for drawing our attention to this point. The substrates’ names has been mentioned in order to elaborate more on the phrase. Therefore, the following statement “through activation of other substrates” was changed to “through activation of other substrates such as mTOR and Cyclin D” on page 3 lines 13-15

2. Page 3 line 32: reference 14 refers specifically to an in vitro data on pancreatic cell lines, not to other oncological conditions. Please provide consistent references or modify the sentence accordingly.

Response:

The reviewer’s point was well taken and was changed the text in the manuscript from and the appropriate references has been added. Therefore, the following statement “types of cancer including breast, colorectal, gastric, pancreatic and intestinal cancers14” was replaced by “brain, colon, prostate and pancreas14,15” on page 3 lines 32-33

3. Page 3 line 43: it is worth mentioning, also in the discussion paragraph that apoptosis of M. leukemia cell line was p53 independent (see Xia L et al. PMID: 15767547).

Response:

Thank you for suggesting the above reference, which will be a good addition to the current references. The data from the suggested reference was incorporated as follows: “As many drugs that affect multiple pathways, boswellic acid acetate was also found to induce apoptosis via p53-independent pathway as Xia L et al and his team showed in myeloid leukemia cell.23. They demonstrated that the levels of apoptosis-related proteins Bcl-2, Bax, and Bcl-XL were not modulated by boswellic acid acetate, however; it induced Bid cleavage and decreased mitochondrial membrane potential without production of hydrogen peroxide. By using general caspase inhibitor (Z-VAD-FMK) and a specific caspase-8 inhibitor II (Z-IETD-FMK), boswellic acid acetate–induced apoptosis was blocked, suggesting that such mechanism was through activation of caspase-8 and not p53. Such variation in boswellic acid acetate action might be due to the differences in genetic background of both cancer types as well as the activities of these tested proteins.23” under the discussion section on page 9 line 5.


Response:

We thank the reviewer for directing us on the written language in these lines, it was revised and changed from “Antibodies of Anti-rabbit Akt, NFkB, GAPDH and PARP and Anti-mouse p53, and p53-siRNA, Goat anti- Rabbit IgG and anti-Mouse IgG (HRP conjugation) were obtained were cell
signaling (Beverly, MA, USA)” to the following statement “Primary antibodies of rabbit monoclonal anti-Akt, -NfkB, -GAPDH, & -PARP and mouse monoclonal anti-p53 in addition to secondary antibodies (Gout anti-rabbit and -mouse IgG (HRP conjugated) were obtained from cell signaling technology (Beverly, MA, USA). P53 siRNA was also purchased from the same company” on page 4 lines 19-25

5. Page 4 lines 48: please provide a rational for treatment concentration by adding related references.

Response:

The reviewer’s point was well taken and the related references were cited under the reference section to support the used concentrations of CDDP and AKBA. They are listed below:


6. Page 4, cell culture paragraph: please specify cell-seeding feature such as number/plastic used, whether well plates or dishes, this would further clear the reader about total protein yield.

Response:

We thank the reviewer's guidance in this aspect and therefore, the seeding features “Seeding density for AGS was 8000 cells/mm while for NCI-N87 was 15000 cells/mm in an appropriate 24-well plate or 6-well plate” was added on page 4 lines 38 to 42.

7. Page 5 western blot paragraph: please specify the amount of protein loaded.

Response:

We thank the reviewer for the useful comment and the amount of loaded protein was specified as “Fifty microgram” on page 5 line 19.
8. Page 5 lines 12: please provide lysis buffer composition, or kit reference whether the case.

Response:

We thank the reviewer for drawing our attention to this missing information. The lysis buffer composition “(50 mM Hepes, 150 mM NaCl, 1 mM EGTA, 10 mM Sodium Pyrophosphate (Nappi), 1.5 mM MgCl2, 100 mM NaF, 10 % Glycerol, 1 % Triton X-100)” was incorporated on page 5 line 12.

9. Page 6 line 14: Statistical analysis was performed on three independent replicates. Is this a single experiment (i.e. 3 replicates=three wells)? Were three independent repetition of the experiment run as standard quality procedures request?

Response:

We thank the reviewer for the important comment and; as mentioned above; each experiment in the current study was performed in triplicates and repeated three times (replicates) independently with the same parameters. In each replicate, every test was carried out in duplicate. So the average of two duplicates represent one replicate and the average of three replicates represents one experiment that is eventually expressed on the graph as mean ± standard error (SE).

10. Page 6 line 53: "normalize to total protein content" would be preferable instead of "precisely measure". In this respect, it is not clear how author consider GAPDH reference: from the original blots available in supplements, it is not detectable whether bands corresponding to targets and reference (i.e. GAPDH) were analyzed on the same membrane. In addition, a lane with protein ruler present in each blot should be reported for clear band weight report.

Response:

According to the reviewer’s advice, the statement "normalize to total protein content" was changed to "precisely measure" on page 6 line 53.

Regarding the GAPDH concern, proteins of p53 (MW 53), NFkB (MW 65) and GAPDH (MW 36) genes were analyzed on the same membrane due to the distinct differences in their molecular weight (MW) and thus can be easily detected unlike Akt (MW 60). In addition, different species of the primary antibodies were used such as anti-mouse for p53 and anti-rabbit for the rest of the proteins to avoid the overlap between detected bands due to their convergent molecular weights. The blot of GAPDH in each figure is a representative one and each protein was normalized with its own corresponding GAPDH.

11. Figure 3 reports combined treatment CDDP and AKBA in one column each (AGS 25 and 25 uM; NCl-N87 50 and 50 uM, respectively. Please justify the choice of concentration. In addition I would kindly suggest to render y axis to consistent through all graphs shown.

Response:

We thank the reviewer for drawing our attention to this point. The details of the selected and tested concentrations were incorporated in the text of the manuscript as mentioned in point 5. The Y axis in all graphs are modified to make them consistent throughout as suggested by the reviewer.
12. Reporting p values in figure might be redundant since they are already stated in figure legends.

Response:

We thank the reviewer for bringing up such observation and we agree that it is better to modify p values representation within the figures. For that reason, all the p values were modified in the figures.

13. Please provide a version of figures with quality feature that can allow reading, especially for ICC analysis.

Response:

We thank the reviewer for commenting on the quality of the figures. The figures’ resolution was enhanced and the new version of figures will be submitted along with the revised manuscript.

14. Discussion of the data would benefit by describing consistencies and differences with the other models mentioned focusing more on gastric cancer and the shared features with other oncological diseases.

Response:

We thank the reviewer for bringing up this point that will be a great addition to the discussion and thus strengthen our findings. Although some models were mentioned in the submitted manuscript but similarities and differences were not pointed out very clearly. In the revised form of the manuscript, additional references were incorporated and more emphasis was put on the similarities and differences between these studies and the current one.

“As many drugs that affect multiple pathways, boswellic acid acetate was also found to induce apoptosis via p53-independent pathway as Xia L et al and his team showed in myeloid leukemia cell.23. They demonstrated that the levels of apoptosis-related proteins Bcl-2, Bax, and Bcl-XL were not modulated by boswellic acid acetate, however; it induced Bid cleavage and decreased mitochondrial membrane potential without production of hydrogen peroxide. By using general caspase inhibitor (Z-VAD-FMK) and a specific caspase-8 inhibitor II (Z-IETD-FMK), boswellic acid acetate–induced apoptosis was blocked, suggesting that such mechanism was through activation of caspase-8 and not p53. Such variation in boswellic acid acetate action might be due to the differences in genetic background of both cancer types as well as the activities of these tested proteins.23” on page 9 line 5

“Altogether, suggesting that AKBA can act on different molecular mechanisms and pathways accountable for inducing cell apoptosis in different cancer cell types. Examples of these targets at the cellular molecular level includes but not restricted to kinases, growth factors, transcription factors, enzymes and receptors”.14 on page 10 lines 1-6