Author's response to reviews

Title: Identification of a potent herbal molecule for the treatment of breast cancer

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Author's response to reviews: see over
Dear Editor,

We are resubmitting our manuscript entitled “Identification of a potent herbal molecule for the treatment of breast cancer (MS 1296378060212447)” after making the necessary modifications as per the reviewers’ and editors’ comments for your kind perusal. We have also addressed all the queries raised by the reviewer’s in the following few pages (pages 2, 3 and 4 in this document). Please do not hesitate to contact me if you have any question.

Thanking You

Sincerely yours

Damodaran Chendil, PhD
Reviewer: Addanki Pratap Kumar

1) **Conclusions can be strengthened by presenting quantification of the western blotting data as there is difference in the kinetics between cells.**

As per the reviewer’s comment, bar graphs representing the relative density of each of the bands when compared to β-actin have been included in the figures 4 and 5 so as to strengthen the manuscript.

2) **In page 12, authors conclude that pAkt expression was not significantly down regulated in MDA 231 cells suggesting PDBD does not target Akt. However the figure provided shows approximately 50% reduction at 24h (Figure 4). Therefore quantification of the data with statistical analysis will strengthen the manuscript.**

Answer: We agree with the reviewer, however we wanted to clarify that although we saw a pAkt downregulation at 24h post treatment in MDA 231 cells, its downstream events like NF-κB, Bcl-2 were downregulated at 3 or 6h onwards, hence we concluded that inhibition of the pro-survival signaling in MDA 231 cells were in an Akt-independent manner. As per the reviewer’s suggestion, quantification bar graphs have been included (Fig 4&5) and supporting explanation in the results and discussion has also been included (page 8 and 11).

3) **Results should be presented with more clarity.**

As suggested by the reviewer, the necessary modifications that includes a detailed explanation of the percentages in the parentheses (page 7) in results and discussion about the effect of PDBD on MCF-10A cells (page 12) has been included.

4) **What is the affect of PDBD on apoptosis in MCF-10A cells? It is surprising that the compound induced 100% apoptosis in 24h. Is necrosis part of this? This needs clarification.**

In figure 2B the apoptosis assay data of MCF-10A has been included which shows 30% apoptosis at the highest concentration, however, in most of the BCa cells almost 100% apoptosis was observed. We conducted Annexin V-FITC/PI staining which reveals early apoptotic cells (Annexin V-FITC positive) and late stage apoptotic and dead cells (double positive- Annexin V-FITC and PI positive staining) and a combination of these two showed almost 100% apoptosis in the BCa cells.

5) **In figure 5 non-phosphorylated IκB-α is shown to be increased with PDBD treatment however western blot shows decrease at 24h which is contradictory. However at 12h time point there seems to be no significant change as presented. This needs to be clarified.**

Yes, we agree with the reviewer that at 24 h of PDBD treatment non-phosphorylated IκB levels are coming back to the basal level, however we observed an increase non-phosphorylated IκB levels for upto 12h as was seen by colorimetry and Western blotting.

6) **Figure legends are missing (e.g: figure 1 and 4).**

We appreciate the reviewer’s identification of this error; however we wanted to make clear that when we uploaded the file during our previous submission, some parts in the manuscript were inadvertently deleted during conversion into PDF.
Reviewer: Farrukh Afaq

1) **The authors should use normal mammary epithelial (184A1) cells instead of using immortalized mammary epithelial (MCF-10A) cells to determine the growth inhibitory effect of PDBD.**

We appreciate the reviewer’s comment, in our experience 184A1 cells are not suitable for flow cytometry experiments (it clogs), we have used MCF-10A cells, which is easy to handle in our experiments.

2) **The purity of PDBD is not discussed in the manuscript.**

As per the reviewer’s suggestion we have included the purity of PDBD (99.5%) which we used for our experiments. The information about isolation methods and purity of PDBD were highlighted in the manuscript, which is accepted for the publication in the Pharmaceutical Biology (Zhao et al, 2008).

3) **Are the dosages of PDBD used in cell culture studies pharmacologically achievable in the in vivo setting of treated experimental animal models?**

We are certain that the doses used in our *in vitro* (IC50 of 7.5µM in MCF-7 and 4µM in MDA 231) studies are achievable in animal models and this dose is sufficient to successfully regret the tumors in the study animals. Currently, we are performing animal experiments to determine the effect of PDBD on tumor xenografts, the data of which will be published separately as an extension of this current manuscript.

4) **The data in terms of time points shown in Table 1 do not correlate with that discussed in results section.**

As suggested by reviewer, we have verified our results and reframed the sentences in the results section pertaining to cell cycle (Page 8).

5) **It is also not clear that at this time point when all the cells were dead than how come the authors have analyzed the arrest of cells in different phases of the cell cycle.**

This is an interesting question, we wanted to clarify that, lesser dosage was used for cell cycle assays as the experiments had to be conducted for 48 h. For example, IC50 value for MCF-7 and MDA 231 cells were 7.5 µM and 4 µM, respectively, and we used only 3 µM and 2 µM respectively for cell cycle analysis.

6) **The manuscript in general is not properly prepared and presented.**

As suggested by the reviewer the entire manuscript has been thoroughly read and scrutinized for any necessary and relevant changes and the same has been included in the revised manuscript.
Reviewer: Shrikant Anant

1. *In Figure 4A, the authors have shown pAkt down regulation in breast cancer cells; however it would be better if they are able to show whether PDBD directly targets Akt or its upstream event, PI3 kinase.*

As suggested by the reviewer, the effect of PDBD on PI3K (one of the major upstream event of Akt) was determined and it was found that PDBD has no effect on either the expression (as was seen by Western blotting) or activity (as was seen by PI3K activation assay using colorimetry) of PI3K (Figure 4B).

2. *Figure 6D, an appropriate loading control such as Histone H3 for the nuclear extracts should be included.*

In the revised manuscript, Histone H3 has been used as the loading control for the nuclear extracts and included in the figure 6D.

3. *Figure 7C could be presented more clearly, the figure looks congested.*

As per the reviewer’s suggestion the figures in Figure 7 have been rearranged so as to make them look clearer.

4. *In the manuscript, some sentences could be reconstructed so as to make them more descriptive and also minor grammatical errors need correction.*

The manuscript was thoroughly scrutinized for grammatical and typographical errors and the necessary corrections have been made.