Author’s response to reviews

Title: PAC down-regulates Estrogen Receptor alpha and suppresses Epithelial-to-Mesenchymal Transition in breast cancer cells

Authors:

Huda Al-Howail (alhowail@kfs.hrc.edu.sa)
Hana Hakami (hakami@kfs.hrc.edu.sa)
Basem Al-Otaibi (alotaibi@kfs.hrc.edu.sa)
Amer Al-Mazrou (almazrou@kfs.hrc.edu.sa)
Maha Daghestani (daghestani@kfs.hrc.edu.sa)
Ibrahim Al-Jammaz (aljammaz@kfs.hrc.edu.sa)
Huda Al-Khalaf (alkhalaf@kfs.hrc.edu.sa)
abdelilah aboussekhra (aboussekhra@kfs.hrc.edu.sa)

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Response to reviewer’s comments

We are grateful to the reviewers for their relevant and constructive comments and suggestions, which helped us to improve the quality of the manuscript. All the modifications are highlighted in red in the text.

Reviewer #1:

1. The PCA could inhibit the proliferation and suppress the epithelial-to-mesenchymal transition process in breast cancer cells, with higher efficiency on the TNBC subtype. I am wondering whether the author test the effect of PCA on cell cycle.
Response:

I believe that the reviewer is referring to PAC (not PCA)? If this is the case, we have previously shown that PAC, like curcumin, inhibits cell cycle at G2M with higher effect on ERα-negative cells (Al-Hujaily et al., 2011).

2. The statistical analysis results should be presented in Figure 1B, 2B, 3C, and 6B.

Response:

This has been done accordingly. For Figure 1B the variations were very small, therefore they were not taken into consideration in the previous version of the figure.

3. Figure 4C and Figure 6c should be replaced with highly qualified pictures.

Response:

These figures were replaced with higher quality figures.

Reviewer #2:

Only a couple of minor concerns have been noted for the authors to address.

1. Although reported previously, please show the chemical structure of PAC and curcumin in Figure 1.

Response:

The chemical structures of PAC and curcumin were added in Figure 1A.

2. More details about the HPLC stability test should be supplied to describe the data presented in Figure 1A. Which peak resembles the intact PAC and its degradation products?
Response:

Arrows were added in the figure to indicate the peaks corresponding to the intact molecules.

3. The two axis of the FACS plot should be labeled (Figure 2A and 3B).
Response:
Both axis were labeled in both figures

4. Based on the evidence herein that the cytotoxicity of PAC is ERα dependent, it will be worthwhile for the authors to additionally test if ERα inhibition by small molecular inhibitors such as 4-hydroxytamoxifen can further augment the cytotoxicity of PAC. This can be done by IC50 based Chou-Talalay analysis to test if PAC and 4-hydroxytamoxifen have synergistic anti-tumor activity.
Response:
Yes, I do agree with the respected reviewer, and we did this experiment but the results were not conclusive. Therefore, we are planning to repeat this experiment in a separate study to address the possible synergy between ERα inhibitors and PAC, or the possible role of PAC on tamoxifen resistant cells that are available.

5. The c-myc qRT-PCR data (Figure 4B) is inconsistent to the Western Blot data (Figure 4A). Please explain or consider repeating the experiment.
Response:
In fact, these experiments were repeated several times, and we got the same pattern of c-MYC mRNA up-regulation upon PAC treatment of MCF-7 cells. At the protein level only a slight increase was also observed at 24h of treatment, which corresponds to the incubation time of the RT-PCR experiment. This information was added in the results page 14. This difference in the response of c-MYC to PAC treatment at the mRNA and protein levels could be due to translational or post-translational regulatory process in MCF-7 cells but not in MDA-MB-231 cells. This has been added in the discussion page 20.
6. Please use normalized integral scale (e.g. 0,1,2,3,4…) to report qRT-PCR data (Figure 4B).

Response:

This has been done accordingly

7. Figure 4C needs to be labeled - which lane resembles PAC treated samples?

Response:

This has been done accordingly

8. Figure 7 is somehow difficult to interpret. First, it is not clear whether the authors were investigating the biology of tumor-associated fibroblast (if so, desmoplastic activation markers such as α-SMA and related increase in cytokine production should be tested) or the paracrine potential of tumor cells. Second, the preparation of conditioned media is not well controlled. The difference may simply arise from the reduced MDA-MB-231 cell number resulted from the cytotoxicity of the pulse treatment of PAC. Please explain carefully or simply take off this Figure from the manuscript.

Response:

In fact, in this experiment we assessed the potential of PAC in inhibiting the pro-carcinogenic paracrine effect of breast cancer cells on breast stromal fibroblasts as stated in the title of the corresponding paragraph and explained in the first 6 lines. However, the preparation of conditioned media was missing in the materials and methods section. It was added in the revised version, and it explains that the number of cells is taken into consideration when using SFCM.

9. Please replace 'ERa' with 'ERα' (ERalpha) throughout the manuscript.

Response:

This has been changed throughout the manuscript
Reviewer #3:

1. They stated that the above functions of PAC are mediated via down-regulation of estrogen receptor-alfa (ERα). However, the reviewer does not agree with this opinion.

Response:

This is a very important point. I would like to start by saying that we have seen better effects of PAC on ERα-negative cells than in ERα-positive cells, but we did not say that these effects are due to ERα-downregulation only for the induction of apoptosis. As example our conclusion in page 17: “Together, these results indicate that PAC suppresses the EMT process in breast cancer cells both ERα-positive and -negative, with a more potent effect on ERα–cells”.

Why PAC may induce apoptosis through ERα downregulation?

1. PAC down-regulates ERα in both ERα-negative and ERα-positive cells

2. When ERα was down-regulated by specific shRNA in ERα-positive cells, the sensitivity of these cells to PAC became similar to that of ERα-negative cells (Figure 2 and 3).

3. We have previously shown that the expression of the ERα coding gene ESR1 in ERα-breast cancer cells (MDA-MB-231) increased their resistance to PAC (Al-Hujaily et al., 2011).

Together, these results clearly show that ERα inhibits the pro-apoptotic effect of PAC. Since PAC down-regulates ERα, we assumed that the pro-apoptotic effect of PAC is mediated through down-regulation of ERα. Since ERα-negative cells express only low levels of ERα the effect on PAC is stronger than ERα-positive cells wherein ERα is highly expressed and it’s down-regulation in these cells is not enough to affect the downstream effector genes such as c-MYC and cyclinD1, as shown in figure 4A. This figure also shows that the level of ERα still remains high even after 72 h of PAC treatment (higher than the basal level of ERα in MDA cells). Therefore, for clarification some sentences were changed or added in the abstract and the discussion sections.
2. If PAC functions through down-regulation of ERα, ERα negative cell lines should be less sensitive to PAC.

Response:

I strongly believe that “if PAC functions through down-regulation of ERα, ERα negative cell lines should be less resistant to PAC” because the level of the factor that mediates resistance (ERα) is low, and consequently the cytotoxic agent (PAC), which down-regulates ERα, will have higher effect.

References