Author's response to reviews

Title: Targeting glycolysis by 3-Bromopyruvate improves Tamoxifen Cytotoxicity of Breast Cancer Cell 1 Lines.

Authors:

Yasmin M Attia 1 (yasmin.m.attia@gmail.com)
Hanan S EL-Abhar 2 (hanan.elabhar@pharma.cu.edu.eg)
Mahmoud M Al Marzabani 1 (mahmoud.almarzabani@nci.cu.edu.eg)
Samia A Shouman 1 (samia.shouman@nci.cu.edu.eg)

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Author's response to reviews: see over
Reviewer No 1

First of all, we do appreciate and thank very much your valuable comments and suggestions that will improve the quality of our manuscript. You will find our answers for each separate section for your professional revision. We hope that our answers are satisfactory, acceptable and waiting for your professional decision.

Reviewer's comments

1-As being the 2nd submission, the critics from the 1st set of reviewers as well as the “authors’ responses to the reviewers from first submission” shall have been included as a part of resubmission documents, but it is missing. Without the point-by-point response, evaluation upon the revised manuscript becomes difficult.

Author's response

1-Sorry, for this confusion, the first submission was related to adherence of the manuscript to the journal's instruction. The manuscript was not subjected to previous peer reviewing.

Reviewer's comments

2-In order to show the synergistic effects from TMA plus 3-BP are indeed ER-specific, it is suggested that other sets of breast cancer cell lines such as ER-negative or Triple negative shall be included as controls.

Author's response

2-We agree with your comment, although we are very sorry as we cannot respond to it, as we do not have other types of breast cancer cell lines. In addition, ER status, which characterizes the 2 cell lines used in this study, is the most important and primary determinant of treatment options through targeting ER functions by TAM. Therefore we used TAM as the actual drug used in this types of breast cancer and not in other types.
Reviewer's comments

3 – Instead of providing an overarching view pertaining to eliminate breast neoplasm, the sections of results and discussion were presented in a disjoint and fragmented manner. Perhaps, improved writing with inter-related issues along with connecting sentences as well as paragraphs would enhance the quality of the article.

Author's response

3-Thanks a lot for your suggestion, we checked and improved the two sections (results and discussion). Hoping that these changes are satisfactory. They are highlighted in yellow in the manuscript.

Reviewer's comments

4-It is unclear why the q PCR data shown in Figure 6E was more robust than the one from Western blotting shown in Figure 6G. Authors are suggested to provide some speculations and rationales.

Author's response

4-In accordance with reviewer suggestion we added some interpretation in the discussion section to clear the difference between the mRNA expression by q PCR and the protein content determined by western technique. These are highlighted in yellow.

In our study TAM, 3BP as well as their combination inhibited the expression and the protein content of HIF-1 α with concomitant inhibition of HK and VEGF. However, the expression levels of both HIF-1 α and HK of the combined treatment in both cell lines showed synergistic effect which did not appear in the protein level carried out by Western blotting. Such difference between mRNA expression and protein level may be due to several biological and methodological constraints that play a role when comparing mRNA to protein levels [46]. The most prominently influences the correlation between mRNA and protein are the translation efficiency or protein half-life. Individual protein half-lives range from several seconds to tens of hours [47], a more than 1000-fold range. Hence protein turnover is probably influencing the correlation between mRNA and protein
abundances to a greater degree. Minor effects are attributed translation initiation, start codon, stop codon and stop codon context [48] and [49].

Reviewer's comments

Other than the writing style is suggested to be improved, grammatical errors shall be also eliminated. For example, on line 17 of page 3, the sentence such as “by causing; induces ..., and inhibits…” shall be edited to “by causing; inducing ..., and inhibiting.“ …

Author's response

5-Revision of the whole manuscript are carried out as directed by the reviewer

Reviewer's comments

6-The labeling of individual figure panel is generally too small to be comfortably read. Larger and bolded fonts with clear labels would aid the readability. Likewise, in panels E and F of Figure 4 pertaining to Western blots, the bands corresponding to the full-length versus the cleaved caspase 7 shall be obviously labeled. Furthermore, shall Western blotting be similarly perform to caspase 3 and caspase 9, in addition to qPCR? It is the way that data about caspase 7 was presented (panels E and F of Figure 4)

Author's response

In agreement with reviewer comment we checked all the figures of the manuscript and made all the correction to be comfortably read.

In caspase 3 we used ELISA to determine activity, in caspase 9 we used PCR both are assessed quantitative. While in caspase 7 the gel gave 2 bands (activation of the procaspase) which difficult to calculate as presented in many manuscripts.

Reviewer's comments

7-For data presented in panel B of Figure 5, please discuss or speculate why 3-BP didn’t noticeably impair the angiogenic activities referred from VEGF, in T47D cell line.
7-In consistent with reviewer comment we add (References were referred that T47D is more aggressive that MCF-7 so it explained why the drugs and their combination gave better results on MCF-7).

The combined treatment produced significant decrease in VEGF compared to either drug alone, the effect was synergistic in MCF7, while it was additive in T47D. This difference in drug interaction between the two cell lines could be attributed to the aggressive nature of T47D compared to MCF7. According to their biological functions, the proteins involved in cell growth stimulation, anti-apoptosis mechanisms and carcinogenesis are more strongly expressed in T47D than in MCF7[54].

8-The 3rd symbol in panel E of Figure 6 was mistakenly labeled. 20 uM 3-BP + 20uM TAM was incorrect and it shall be changed to 20 uM 3-BP (without + 20uM TAM). One small free-standing figure panel placed below Figure 7G is a repeat of Figure 7G and thus it shall be eliminate.

8-Thanks again for your professional review. Correction was made in the manuscript

9-Even though the main text mentioned Figure 10 and it is supposed to show the effect of TAM and BP on VEGF, the data (figure) is not presented in elsewhere of the current manuscript

9- Sorry for this mistake. The figure will be added with the manuscript
10- In vivo studies utilizing tumors developed from Ehrlich Carcinoma in mice appeared to be a confusing approach. It is quite puzzling that why the same breast cancer lines (MCF7 and T47D) used throughout the whole project were not chosen to be developed as a xenograft model, because the latter approach shall better mirror the in vitro evidence.

**Author's response**

10-We fully agree the reviewer suggestion, but due to the unavailability of xenograft mice in our institute or any research center, we used the available one as we were eager to see the effect of this combination in vivo.

**Reviewer's comments**

11-In a parallel in vivo study in mice, it might be important to assess the “background” cytotoxicity attributed from TAM plus 3-BP in the cancer-free system. To this aim, un-diseased mice shall be treated with vehicle agents, TMA alone, 3-BP alone and TMA plus 3-BP and then be evaluated for their health biometrics (parameters). This set of experiments shall serve as an important control to prove the negligible cytotoxicity is associated with the combinational agents (TMA and 3-BP).

**Author's response**

11-In this work, we used only the model that candidate for TAM intake (only breast cancer patients). Although, it will be important to study this cytotoxicity of this combination normal animals in another work.

**Reviewer No 2**

The authors of the manuscript appreciate very much reviewer no 2 for spending his valuable time in conducting this professional review of our manuscript. I hope that my answers are satisfactory, acceptable and waiting for your professional decision expecting your stimulating cooperation.

**Reviewer's comments**
1-Results section, the writing needs to be greatly changed and improved. Each result section needs to have a title that delivers a major conclusion, not describe what to do like a legend title. In addition, should write results smoothly and briefly bring the rational. Results section needs to do a major revision.

**Author's response**

1-Thank you for your suggestion, we made the required changes to improve the quality of the manuscript. It is highlighted in yellow in the manuscript. I hope that our changes are satisfactory, acceptable and waiting for your professional decision expecting your stimulating cooperation.

**Reviewer's comments**

2-Figure 2. The doses for both drugs used were pretty high, but the combination effects were not very strong

**Author's response**

We agree the reviewer comment about the concentrations of the two compounds are somewhat high and the effect on cytotoxicity is not excellent. But because the two compounds are considered less toxic compared to other chemotherapy. Another important effect of the two compounds is they have anti-invasion metastasis and anti-angiogenesis.

**Reviewer's comments**

3-Figure 4. For panel A-D should show western blot data. For Panel E, it looks Caspase-7 decreased in the combination treatment. For Panel F, the increase of Caspase-7 was not significant.

**Author's response**

3-Concerning figure 4 panel A-B, show caspase 3 activity using ELISA method, Panel C and D show expression level of caspase 9 using qPCR. Only panel E and F show cleavage (activated) caspase-7. There was no quantitative data for cleavage caspase from pro caspase as presented in other manuscripts.
4- Figure 6, all western blot data were not clear. Although there were quantification analyses, the western blot bands were not really changed between control and drug treatment.

Author's response

We improved the quality of the figures. For quantitation the mean intensity of each band (mean pixel) was compared with intensity of B Actin and then calculate relative to the control using software Scion Image Beta 4.0.2 (Scion Co., MD, U.S.A).

Reviewer's comments

5-Figure 7, Panel F, western blot data were not clearly changed. For Panel G and H, 3-BP treatment increased MMP-9 levels, but quantification showed decrease.

Author's response

In according with the reviewer comments, we improved the quality of figures, and checked our data using software.

Reviewer's comments

6- Figure 8. Should show tumor growth curve, or at least final tumor picture. And stain CD31 to show vasculature effects of drug treatment. In addition, why choose Ehrlish 346 carcinoma, instead of MCF7 and T47D cell lines, for xenograft study? In vitro and in vivo cell lines were not consistent.

Author's response

6-Sorry we did not have tumor growth because we took only tumor volume just before treatment and at the end of the treatment. We will take in consideration in other works.

We used only the available antibody of VEGF.

Regarding the use of Ehrlish 346 carcinoma, due to the unavailable of xenograft animal in whole country.
Reviewer's comments

In the manuscript, please try to keep consistency when the hour is short for “h” or “hrs”, still “20μM” or “20 μM”.

Author's response

Thanks we made the correction.

Reviewer's comment

8-There are two G panels in the figure 7. Please remove one

Author's response

Sorry, Correction was made in the figure.

Reviewer's comments

9-For title, please consider changing to ‘Targeting glycolysis by 3-bromopyruvate…’ to better deliver the drug effect.

Author's response

Thanks a lot, corrected title was highlight in green.

Reviewer No 3

The authors of the manuscript acknowledge very much the reviewer no 3 for his valuable comments and suggestions that improve the quality of the manuscript.

We appreciate your valuable time in reading the manuscript. I hope that my answers are satisfactory, acceptable and waiting for your professional decision expecting your stimulating cooperation.

Reviewer's comment

The authors should explain clearly the rationale of conducting each experiments. For example, before describing the effects of TAM/3-BP on oxidative stress markers (MDA, rGSH), they should explain briefly why they conduct this experiments
In agreement with the reviewer suggestion we added rational of conducting each experiments

Reviewer's comment

2. On page 10, the authors examined the expression of VEGA-A with title "the effect of 3-BP, TAM on their combination on angiogenesis". However, it might be overstated as they only examined the expression of VEGF but not angiogenesis.

To examine angiogenesis, the authors should perform either co-culture experiment using HUVEC cells or culturing HUVEC cells using the conditioned media from the treated breast cancer cell lines;

Author's response

We agree the reviewer comment and changed the title as it is required

Reviewer's comment

3. The western blot of Figure 4E, F is sub-optimal. To examine apoptosis, the authors should perform flow cytometer using annexin V/PI assay or PI-sub G1 peak assay;

Author's response

We improved the quality of the fig. we apologize as we do not have flow cytometry

Reviewer's comment

-Figure 10 cannot be found.

Author's response

We apologize for this and we added the fig.

Reviewer's comment

Minor essential revision:

1. The format of references are not consistent.

2. There are several typos and grammatical mistakes

Author's response
We made revision in editing for the whole manuscript.