Author's response to reviews

Title: Anti-tumor activity of phenethyl isothiocyanate in HER2 positive breast cancer models

Authors:

Parul Gupta (parul.gupta@ttuhsc.edu)
Sanjay K Srivastava (sanjay.srivastava@ttuhsc.edu)

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Author's response to reviews: see over
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Dear Dr. Tree-Booker
Editor
BMC Medicine

Sub: Revision of manuscript MS: 5774420436473992

We are thankful to you for considering our manuscript entitled, “HER2 expression sensitizes breast cancer cells to apoptosis by PEITC in vitro and in vivo”, and sending it out for peer review. We are also thankful to the reviewers for a candid review of our manuscript. The comments/suggestions by the reviewers make our manuscript scientifically more strong. We performed few more experiments to complement our responses and hope that the additional data will answer the reviewer’s comments and make the manuscript much better. As per reviewer’s suggestion, the title of the manuscript has been changed to: “Anti-tumor activity of phenethyl isothiocyanate in HER2 positive breast cancer models”. The new data has been incorporated and highlighted in the revised manuscript. Following are the point-by-point response to the reviewer’s specific comments:

Reviewer #1

1. Full name of PEITC should be provided in the manuscript.

Response: We are thankful to the reviewer for critically reviewing our manuscript. The full form of PEITC has been provided in the manuscript.

2. A list of abbreviations or full name of each abbreviation mentioned first time should be provided.

Response: As suggested, a list of abbreviations has been included in the manuscript before the abstract.

3. English, grammar and punctuation should be improved.

Response: As suggested, we have fixed the manuscript for grammar and punctuation.

4. Source and purity of PEITC and doxorubicin should be provided.

Response: PEITC and doxorubicin were purchased from Sigma Aldrich and the percentage purity specified were 99% and 98-102% respectively. The source and purity information has been included in the manuscript under material and methods section.
5. The site of cell injection in mice should be provided.

Response: The tumor cells were injected subcutaneously into the right flank of each mouse. This information has been included in the revised manuscript.

6. Giving PEITC every day by gavage to mice may cause GI irritation and damage, an alternate route (such as mixing in feed, in drinking water etc.) should have been considered.

Response: PEITC was given to each mouse by oral gavage every day and the treatment protocol was approved by our IACUC. We did not observe any GI irritation or damage as the lab staff is well trained. The advantage of oral gavage is to deliver the exact dose and avoid intra-variability due to unequal dose uptake by each mouse. However, we agree with the reviewer and were planning to administer PEITC in the diet in the near future in another project.

7. Method for measuring tumor mass and calculating tumor volume should be provided.

Response: We apologize for not providing the formula for calculating tumor volume. In the revised manuscript, we have provided the information.

8. An in vivo experiment with a combination of PEITC and doxorubicin should have been done.

Response: The focus of our present study was to evaluate the effect of PEITC on HER2 overexpressing breast cancer cells. Preliminary studies on the combination of PEITC and doxorubicin were performed to test and show the efficacy of PEITC with doxorubicin in vitro. We apologize for our inability to provide in vivo data at this time, as it will take at least two more months to perform the experiment. However, we assure the reviewer that the combined effect of PEITC and doxorubicin in vivo will be evaluated in detail in near future.

9. Bands in Western blots should be quantitated. Variations in replicates should be indicated. Data should be analyzed statistically.

Response: Due to lack of space we were unable to provide the quantitation data. However, as suggested by the reviewer, Western blot bands have now been quantitated, variations in replicates provided and the difference between control and treatment compared statistically.

10. A potential target for PEITC should be discussed.

Response: Our in depth studies suggest that PEITC inhibits the growth of breast cancer cells by suppressing HER2, indicating HER2 as a target of PEITC. In a time-dependent study, we observed a clear correlation between the down regulation of HER2 and the cleavage of caspase 3 indicating that apoptosis followed HER2 degradation. All the information has been now discussed in the revised manuscript.
Reviewer #2

1. The fundamental problem in this study is that the in vitro studies showed PEITC had potent anti-proliferative activity against the parent MCF-7 and MB231 cell lines which are HER2-negative or silenced, which clearly does not support that HER2 is an important target for PEITC. Moreover, the authors reported pretty high HER2 expression in the parent cell lines.

Response: We are thankful to the reviewer for a candid review of our manuscript. MCF-7 and MDA-MB-231 cells do have constitutive levels of HER2 as shown by our western blots. Several published studies have also shown the constitutive expression of HER2 in MCF-7 and MB231 cells. Please see the references below. PEITC was found to exert anti-proliferative effects in MCF-7 and MB-231 cells by down regulating HER2. The effect of PEITC was enhanced in the same cell lines when HER2 was overexpressed indicating it as a target.


2. Some experiments are not well designed. In the experiments related to Fig 3 and 4, the vector control cell lines should be used in the experiments for comparison with the HER2-overexpressing cell lines. In addition, at least two colonies of the paired control and manipulated cell lines should be used for function assays.

Response: The HER2 overexpressing cells were obtained from two different labs and we didn’t had vector transfected cells at the time we performed these studies. Based on reviewer’s suggestion, we requested the two investigators to provide us vector control cells. We were only able to obtain vector transfected MB-231 cells. Dr. Fei Huang informed us that they don’t have vector transfected cells and advised us to use MCF-7 as control cells. We therefore treated vector transfected, non-transfected and HER2 (HH) transfected cells with PEITC and compared the levels of HER2 and cleavage of PARP. We did not observe any significant difference between MDA-MB-231 vector transfected cells and non-transfected control cells in response to PEITC treatment. The new data (Fig 5A) has been incorporated in the revised manuscript. We thus provided evidence that the results obtained from vector transfected cells were similar to non-transfected cells in response to PEITC treatment. It was not possible to repeat all the experiments
with vector transfected cells. Since the stable transfection and selection process was performed in other researcher’s lab, we do not have the colonies of paired controls and manipulated cell lines, so apologize for that.

3. *Because of the lack of the appropriate control groups, most conclusions are not supported by the results. For example, the authors concluded that HER2-overexpressing cells were more sensitive to PEITC, but the results (cell proliferation data) could be simply due to cell manipulation effect.*

**Response:** We have now performed the experiments with the vector transfected control cells and observed that HER2 overexpressing cells showed more cleavage of PARP as compared to the vector control cells when treated with PEITC. In order to clearly demonstrate increased sensitivity of cells expressing high HER2 towards PEITC, we also evaluated histone associated DNA fragments (cell death assay) in the parent cells, vector control cells and high HER2 cells in response to PEITC treatment. Our results show more cell death in the high HER2 cells treated with PEITC as compared to parent or vector control cells, indicating enhanced sensitivity of high HER2 cells to PEITC (Fig 5B). The new data with appropriate control clearly suggests that HER2 overexpressing cells are sensitive to PEITC and the effect of PEITC is not due to cell manipulation. The new data has been included in the revised manuscript.

4. *Since the stable HER2-overexpressing cell lines are available, it is unclear and seems unreasonable to use transient overexpression to determine the effect of HER2 overexpression on apoptosis-inducing activity of PEITC.*

**Response:** Initially we looked at the effects of PEITC in MCF-7 and MDA-MB-231 cells which had constitutive levels of HER2 and observed that PEITC was causing downregulation of HER2. To identify the target of PEITC, we transiently overexpressed HER2 in these cells expecting that HER2 overexpression would abolish or reduce the effect of PEITC. But surprisingly we observed that HER2 overexpression enhanced the effects of PEITC. To validate this observation, we decided to use cells with stable HER2 overexpression and were lucky to obtain HER2 overexpressing MCF-7 and MB231 cells from two different labs.

5. *Most results of biomarker analysis in figures 2 and 3 are based on qualitative western blot images, but not quantitative results. In addition, the comparison between figure 2 (the parent MDA-MB231 and MCF-7 cell lines) and figure 3 (the Her2-overexpressing cell lines) does not show dramatic effect of HER2 overexpression on modulation of these biomarkers, thus does not seem to support the conclusions made by the authors in the related parts in results and discussion sections.*

**Response:** We apologize for not providing the quantitation of western blots. We have now quantitated the blots and the results do show more cleavage of caspase-3 and/or PARP by PEITC.
treatment in cells overexpressing HER2 as compared to cell shaving constitutive level. The quantitation of western blots has been included in the revised manuscript.

**6. Similarly, the parallel animal study using the vector control MDA-MB231 cell line should have been performed.**

**Response:** The aim of the current study was to show that PEITC maintain its efficacy *in vivo* in the cells overexpressing HER2. The *in vivo* experiment showed that PEITC was effective in reducing the growth of HER2 overexpressing tumor xenografts. We apologize for our inability to provide *in vivo* data in vector control cells also as it will take at least two more months to perform the experiment. Moreover, it may not add much to the overall conclusion of the study.

**7. Many places in the manuscript are overstated. Most times, the results only “suggest”, but not “demonstrate”, “support” or “indicate” … Also, “significant” should not be used if the results are not statistically analyzed or the p value is >0.05. Particularly, the last paragraph in the discussion and the conclusion are the most overstated ones.**

**Response:** We have revised the manuscript and the changes have been made in the terminology, as suggested.

Minor essential revisions

1. **All abbreviations need to be spelled at the first time.**

**Response:** A list of abbreviations has been included in the manuscript as suggested.

2. **Annexin-FITC assay: the concentrations of annexin V and propidium iodide should be provided, and the composition of binding buffer should be given.**

**Response:** This assay was performed based on the manufacturer’s protocol (BD Biosciences, catalog #556547). According to the protocol, we used 5µl of each Annexin V FITC and propidium iodide respectively for every sample. The composition of binding buffer was not provided in the data sheet. However, the general composition of Annexin V binding buffer is: 0.1 M HEPES/NaOH, pH 7.4, 1.4 M NaCl, 25 mM CaCl2.

3. **Western blot analysis: the CHAPS buffer composition should be provided; the antibodies used should be given detailed information, such as the vendor, type, and dilution.**

**Response:** The CHAPS buffer composition and the antibody details used for the study is now included in the revised manuscript along with respective sources and dilutions for the antibodies.

4. **Was MCF-7-HER2 cell line provided by Fei (as described in cell culture) or Fei and Reeves (in acknowledgements)?**
Response: The MCF-7-HER2 cells were provided by Dr. Reeves with permission from Dr. Fei.

5. Fig 2D: the images of nucleus and overlay in the treatment group may be switched.

Response: We apologize for the misplacement of the figures and have now switched the panels as required.

6. The statistical analysis results should be presented in a consistent way (either the letter or *).

Response: We have made the changes to make the statistical analysis consistent throughout the manuscript.

7. The literature review is not up to date. There are several PEITC and breast cancer related publications in the past years.

Response: The manuscript has been updated with more recent publications on PEITC and breast cancer.

8. HER2 silencing using siRNA: the sequencing of the siRNA and the dose used should be provided, and the control siRNA should be used.

Response: The HER2 siRNA was purchased from Cell Signaling Technologies. siRNA is a proprietary product of the company, so sequencing was not provided by the manufacturer. We purchased scrambled (control) siRNA and performed the experiments again as suggested. The dose of siRNA and the data with control siRNA has been included in the manuscript. Overall, the results were not affected.

9. For the combination studies, it is essential to determine the dose-dependent combination effect, and the nature of combination.

Response: We agree that the studies on PEITC and doxorubicin combination are preliminary. Our purpose in this study was to give some indication that PEITC treatment can enhance the effects of doxorubicin through the same mechanism. However a complete detailed study on the combination of PEITC with doxorubicin in vitro and in vivo will be done separately later where the suggested studies will be included.

Discretionary revisions:

1. The data of PEITC and doxorubicin are very weak and very preliminary.

Response: We agree with the reviewer; however the purpose of this study was to indicate the future potential of PEITC against breast cancer along with conventional chemotherapy agent.

Quality of written English: Needs some language corrections before being published.
Response: As suggested, the manuscript was corrected for language.

Reviewer #3

1. The authors show several lines of evidence that HER2 overexpression sensitzes MCF-7 (and MDA-MB-231) to PEITC. This is very interesting. Is this because the cells become addicted to the growth induction by HER-2 signaling? Is the growth rate of HER2 overexpressing cells higher than the vector transfectants? Please include the source of PEITC and its molecular weight. Similarly for the animal study, state the dose of PEITC in mg/kg.

Response: We are thankful to the reviewer for a critical review of our manuscript. HER2 is a growth factor receptor and its expression is usually required for enhanced cell growth and survival and for the same reason it is known to play detrimental role in cancer. It is very well possible that the effects are due to the dependency of cells on HER2 signaling. The growth rate of HER2 overexpressing cells is higher than the parent cells. There is a report where caspase-3 mediated cleavage of HER2 lead to cell apoptosis through mitochondrial pathway, a process elicited by HER2 fragments. In our study, although we show cyt c release, we did not consistently observed Her2 fragmentation [Strohecker AM, Yehiely F, Chen F, Cryns VL: Caspase cleavage of HER-2 releases a Bad-like cell death effector. The Journal of Biological Chemistry 2008, 283(26):18269-18282].

The source, molecular weight and dose of PEITC (mg/kg) have been included in the revised manuscript.

2. Please list all the abbreviations.

Response: The list of abbreviations has been included in the manuscript before the abstract as suggested.

3. Given the translational potential of the study, the authors may want to change the title of their article to more translational. One suggestion – Antitumor activity of PEITC in HER2 positive breast cancer models.

Response: We have modified the title of the study as suggested.

We again thank the reviewers for their constructive suggestions and hope that the revised manuscript would now be accepted for publication in BMC-Medicine.

Thank you,

Sincerely,

Sanjay K. Srivastava, Ph.D.