Author's response to reviews

**Title:** Curcumin analogue T83 exhibits potent antitumor activity and induces radiosensitivity through inactivation of Jab1 in nasopharyngeal carcinoma

**Authors:**

Yunbao Pan (panyunbao@outlook.com)
Mengyao Wang (wangmengyao2002@yahoo.com.cn)
Xianzhang Bu (buxianzhang2002@yahoo.com.cn)
Yinglin Zuo (zuoyingling2002@yahoo.com.cn)
Sumei Wang (wangsumei2002@yahoo.com.cn)
Dujuan Wang (wangdujuan2002@yahoo.com.cn)
Qing Liu (liqing2002@yahoo.com.cn)
Tao Xu (xutao2002@yahoo.com.cn)
Chunhua Wang (wanchunhua2002@yahoo.com.cn)
Bojin Su (subojin2002@yahoo.com.cn)
Francois X Claret (fxclaret@mdanderson.org)
Huiling Yang (hlyangsums@hotmail.com)

**Version:** 2  **Date:** 13 May 2013

**Author's response to reviews:** see over
Professor Dr. Charistna Chap,
Editor-in-Chief
BMC Cancer

RE: MS# 6311286279049150

Dear Dr. Charistna Chap,

Thank you for considering our manuscript, “Curcumin analogue T83 exhibits potent antitumor activity and induces radiosensitivity through inactivation of Jab1 in nasopharyngeal carcinoma” for publication in BMC Cancer. We appreciate the insightful comments and constructive suggestions from the reviewers, which significantly improved the quality of our study. We have revised our manuscript based on their comments. Our responses to all specific reviewers’ comments are summarized below. New data are shown in Figs. 1, 2, 3, 5.

– Point-by-Point Reply to The Reviewers’ Comments –

Reviewer’s report

Title: Curcumin analogue T83 exhibits potent antitumor activity and induces radiosensitivity through inactivation of Jab1 in nasopharyngeal carcinoma

Version: 1 Date: 9 April 2013

Reviewer: Ingeborg Tinhofer

Reviewer’s report:

The study of Pan and colleagues investigates the antitumor activity of the curcumin analogue T83 and its potential to inhibit Jab1 in nasopharyngeal carcinoma. The study is based on previous results from the same groups which revealed upregulation of Jab1 in NPC with a phenotype of radio- and chemoresistance.

Major compulsory revisions:

1) The rationale of the study is not given in the abstract and the main goals can only be delineated from the title. This information should be given.

Response: We thank the reviewer for the suggestion, this information has now been added in the abstract (p.3).
2) The authors postulate that the antiproliferative and proapoptotic activity of T83 is based on its inhibition of Jab1. However, at doses at which significant growth inhibition was observed in clonogenic assays no effect on protein expression is visible in Figure 3 A. The authors should at least discuss this discrepancy.

Response: We used only 200 cells per well in the colony formation assay that last 48 hrs (double time as immunoblot analysis experiment) and the end point is cell survival (not Jab1) in contrast to $2 \times 10^5$ cells per well for 24 hrs in the Western blot analysis to assess the inhibition of Jab1; therefore, the amount of T83 should be lower in the colony formation assay. This phenomenon has also been demonstrated in our previous studies for cisplatin, ionizing radiation, ultraviolet radiation and Stattic in NPC cells (Pan Y, et al., Cancer Res, 2012, 72(7):1890-900; Pan Y et al., Oncogene, 2012, 326(2):155-60; Pan Y et al., PLoS One, 2013, 2013;8(1):e54565).

3) It remains unclear to the reviewer why the authors used the cell line CNE2 for generating the radioresistant subclone CNE2R. In their previous study published in Oncogene, the defined CNE2 as radioresistant and concluded from a comparative expression analysis of three cell lines that high expression of Jab1 in CNE2 was responsible for their low sensitivity to irradiation. In contrast, the cell line CNE1 had lower levels of Jab1 and was more sensitive to cisplatin and irradiation. The authors should validate their recent data from CNE2 cells by using at least one other syngenic cell line model of radioresistance and should include CNE1 cells in these studies.

Response: To avoid the genetic interference from different cell lines, we established radioresistant subclone CNE2R cell to study the mechanism of radioresistance in NPC. Based on the reviewer's comment, we have repeated experiments in CNE1 cells and have added the new data in Figs.1, 2, 3, 5.

4) In line with point 2 a causal relationship between downregulation of Jab1 and radiosensitization by T83 remains questionable. The experiments presented in Figure 4 rather suggest that the inhibition of Jab1 by T83 is insufficient and/or not relevant for the observed effects on clonogenic growth. If the authors want to establish T83 as specific inhibitor of Jab1, they should demonstrate that ectopic overexpression of Jab1 reduces the sensitivity of cells to T83 treatment.

Response: To test the effect of T83 on different cytological phenomenon, we used different cell number in different experiments (colony formation assay, apoptosis, protein expression), therefore, the amount of T83 should be optimized/adjusted according to the experiments. Several studies have demonstrated that suppression of Jab1 inhibits cell viability and induces apoptosis. In our study, we focus on the antitumor effect of T83 in NPC cells. T83 works synergistically with Jab1-siRNA in reducing cell growth and inducing apoptosis. This could be explained by suppression of more Jab1 enhancing the antitumor effect. Consistent with our studies, two other studies in Stat3 support our findings. First, a study by P. Joana et al. demonstrated that JAK/Stat3 inhibitor, AZD1480, was effective in blocking the growth of Stat3-deficient TPC-1 xenografts (Joana P, et al., PLoS One. 2012; 7(10): e46869). Second, a study by Xin-Sheng et al. also identified metformin as a Stat3 inhibitor; metformin significantly induced growth inhibition and apoptosis in Stat3-knockdown cells compared with control cells (Xin-Sheng, et al., Cell Cycle. 2012;11(2):367-76). These researchers concluded that tumor growth inhibition observed on treatment with AZD1480 or metformin are dependent on inhibition of Stat3.
5) The reason for using such high doses for showing the differences in PARP and caspase-3 cleavage in figure 5E should be given. It would be more interesting to see whether there are any differences at those doses which have been used in the clonogenic survival assays.

**Response:** Based on our previous studies, it is hard to detect cleaved PARP and cleaved caspase-3 when the cells are exposed to less than 10 Gy ionizing radiation (IR). The difference for the IR doses between clonogenic survival assays and apoptosis could be also attributed to the different cell number and exposed time and the end point we used in different experiments. It is important to note that in certain tumor cells, apoptosis may be promoted in response to DNA damage, but cells may survive or experience G1 or G2/M cell cycle arrest upon DNA damage because of their inability to undergo apoptosis. In our study, clonogenic assay was used to test the cell proliferation, however, sub-G1 content and cleaved PARP and caspase-3 were examined in apoptosis experiments. Thus, we optimized the IR doses in different experiments. These optimized IR doses was also demonstrated in our previous studies (Oncogene, 2012; Plos One, 2012)

Minor essential revision:

1) In figure 5 A-D, the same scale on the y-axis should be used, otherwise the graphs are misleading.

**Response:** We agreed that the same scale on the y-axis should be used in the same experiments and situation. However, in our studies, the highest IR does in Fig.5A is 4 Gy, which is different from Fig.5B and 5C, therefore, the different response to IR between CNE2 and CNE2R would be concealed if we take the same scale as Fig.5B. For Fig.5D, we take another scale to show another experiment (sub-G1), which may show the aesthetic illustration for the data.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests.
Reviewer's report

**Title:** Curcumin analogue T83 exhibits potent antitumor activity and induces radiosensitivity through inactivation of Jab1 in nasopharyngeal carcinoma

**Version:** 1  **Date:** 10 April 2013

**Reviewer:** Peter Daniel

**Reviewer's report:**

The authors study the anti-tumor effect of a curcumin analogue, T83, in a nasopharynx carcinoma cell line, CNE2. They show that T83 induces cell cycle arrest in a tetraploid state and induces apoptosis with processing of caspase-3. Moreover, they show that the radio-resistant sublime CNE2R shows a high sensitivity to T83 and a more pronounced down-regulation of the c-Jun activation domain-binding protein-1 (Jab-1). To establish a functional role of Jab-1 in regulating sensitivity to T83 they perform siRNA experiments and show that Jab-1 siRNA reduces clonogeneic survival and increases apoptosis. As it stands, the data are performed only in a single cell line system with a limited set of analyses. More-in depth signaling analyses and mechanistic insights would be desirable. The following points need to be addressed:

**Major points:**

1. The authors should state whether the CNE2 cells are of human origin and how they confirmed the cell line identity.

**Response:** We have add the NPC cell line information in the materials (p.6). Both human NPC CNE1 and CNE2 cell lines have been widely used in NPC studies.

2. Evidence should be provided that the CNE2R cells are in fact a sublime of CNE2 and not an unrelated cell line contaminant.

**Response:** We thank the reviewer for this suggestion, we have identified cell lines by analysis of DNA microsatellite short tandem repeats (STR) and confirmed that the CNE2R cells is a subclone of CNE2 cells. The new data are showed in Additional file 1.

3. The authors should show the therapy effect of T83 in vitro in relation to the activity of curcumin.

**Response:** Based on the reviewer’s suggestion, we have tested the effect of curcumin on NPC cells. Our data showed T83 is much more potent in inhibiting NPC cells than curcumin. New data are showed in Fig.1B and 1E.

4. At least one additional cell line should be studied with regard to T83 activity and the role of Jab-1 to show the broader relevance of these findings.

**Response:** Based on the reviewer's suggestion, we have tested the effect of T83 on another NPC CNE1 cells and got the similar data. New data are showed in Figs. 1, 2, 3 and 5.
5. A second, independent apoptosis assay should be performed, e.g. Annexin-V-FITC/PI double staining.

**Response**: Based on the reviewer’s suggestion, we have tested the effect of T83 on anther NPC CNE1 cells using Annexin-V/PI staining. New data are showed in Fig.2.

6. The functional role of Jab-1 should be put on a broader experimental basis. The authors should show that T83/Curcumin/Jab-1 siRNA affect aspects of Jab-1 signaling such as, e.g. regulation of p27KIP1 and stabilization of complexes of c-Jun or JunD with AP-1 sites.

**Response**: We thank the reviewer for this suggestion, we have detected the effect of T38 on p53 and p27 in CNE1 and CNE2 cells. The new data are showed in Fig.3C.

7. The authors show that CNE2 cells arrest in a tetraploid "G2/M" stage. Do they have information on the p53 status of CNE2? Failure to activate p53 and p21CDKN1 would facilitate arrest in G2/M.


8. In the same vein: JAB1 is also known as COP9 signalosome subunit 5(CSN5), which is a component of the COP9 signalosome regulatory complex(CSN). The COP9 signalosome has been shown to regulate p53 stability. Would T83/Curcumin/Jab-1 siRNA affect expression of p53 protein and induction of p21CDKN1? Such analyses would provide more mechanistic insights.

**Response**: Based on the reviewer’s suggestion, we have detected the effect of T38 on p53 and p27 in CNE1 and CNE2 cells. The new data are showed in Fig.3C.

Minor points:
1. typo y-axis label figure 2B: apo(p)totic

**Response**: We thank the reviewer for the carefully reviewing our manuscript, we have corrected the label.

2. The shading of the bars in the figures is a bit unfortunate. It is recommended to use black versus white bars as the current shading is not easy to differentiate in all figures.

**Response**: As suggested, we have modified the figures.

**Level of interest**: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.
Editorial request(s): In addition to addressing the referees’s comments we require the following editorial points be addressed:

1.) Kindly provide more details about the synthesis of T83;
   
   **Response:** As suggested, we have provided the details of T83 in the methods section (p.6).

2.) please move Competing Interest from title page to after conclusions,
   
   **Response:** We have moved Competing Interest from title page to after conclusions.

3.) Kindly include Authors’ contributions:
   
   **Response:** We thank the editor for the suggestion, we have added Authors’ contributions.

4.) and Acknowledgements:
   
   **Response:** We have added Acknowledgements.

5.) Lastly, kindly move funding statement (grant support) to Acknowledgement section.
   
   **Response:** As suggested, we have moved funding statement to Acknowledgement section.

Again, we thank the reviewers and editors for their suggestions, which have strengthened the presentation of our work in this revised manuscript.

Sincerely,

Yunbao Pan