Author's response to reviews

Title: Enhanced cytotoxic effect of radiation and temozolomide in malignant glioma cells: Targeting PI3K-AKT-mTOR signaling, HSP90 and Histone deacetylases

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Author's response to reviews:

Dear Editor and Reviewers,

We are grateful to you for your helpful comments and for giving us the opportunity to respond. We have performed additional experiments, integrated the resultant data, and rewritten major sections - from the Title to the Discussion - to improve clarity. Please find attached a thoroughly revised manuscript, which addresses the concerns raised and incorporates new figures, which continue to support our original conclusions. These revisions together should contribute to enhancing the appeal and relevance of our paper to the readership of “BMC Cancer”.

In the following paragraphs, we address specific points in detail:

Dr. Stefanie Galban

While potentially important observations were made in this in depth analysis, it remains unclear what GBM subtype patient population may benefit from such combination treatment. The radio-sensitizing effects for all investigated treatments were clearly demonstrated by investigating effects on DNA damage, cell death, autophagy, senescence, invasion and migration. As indicated in the title the main focus of the study was to target EGFR-associated signaling due to the lack efficacious EGFR targeting therapies. The authors chose to investigate three GBM cell lines: U251, U87 and T98G... Beyond the discrepancy between title, choice of cell lines and lack of indication which GBM subtype might benefit from such therapy, the scientific presentation and experimental evidence was felt to be typical of papers published in BMC.

We thank the Reviewer for bringing this to our attention. We agreed your opinion and addressed this issue by changing the title of manuscript as “Improved cytotoxic effect of radiation and temozolomide in malignant glioma cells:
Targeting PI3K-AKT-mTOR signaling, HSP90 and Histone deacetylases.

Dr. Jann N Sarkaria

The authors present an interesting study evaluating 4 different novel therapeutic agents in combination with radiation without and with Temozolomide. Overall, the studies are carefully done and results are convincing. However, there are numerous minor essential revisions, predominantly editorial issues that need to be addressed:

1. The title is somewhat misleading, since 2 of the 4 targets being evaluated are not specifically in the EGFR pathway. Inhibition of HDAC and HSP90 have pleiotropic effects that extend well beyond the EGFR pathway. Cell lines used are not EGFR over expressing, so the discussion within the results and discussion should acknowledge this limitation.

   Thank you for this thoughtful comment and we addressed this issue by changing the title of manuscript as “Enhanced cytotoxic effect of radiation and temozolomide in malignant glioma cells: Targeting PI3K-AKT-mTOR signaling, HSP90 and Histone deacetylases”.

2. In the results and discussion section, the authors suggest a mechanistic link between the effects of HDAC or HSP90 inhibition on specific signaling molecules and phenotypic responses. For example, they attribute suppression of EphA2, VEGF and MMP2 by HDAC inhibition to vasculogenic mimicry, invasion and migration. HDAC inhibition has numerous effects on many proteins, and the authors do not demonstrate a mechanistic link to these specific targets. Thus, they should reword the results and discussion sections carefully when describing the results for both HDAC and HSP90 inhibitors.

   We agreed to the reviewer and addressed this by adding the evidence of mechanistic link at p16.

3. Figure 2 not clear if results represent triplicate samples from a single experiment or results from 3 independent experiments

   Figure 2 is the result of three independent experiments and each experiment was performed with triplicates. We made this clear at the legend of Figure 2 and Results section.

4. Figure 3b - how many independent experiments were performed? PI103 certainly attenuated P-DNAPK, but not necessarily the other compounds. This result suggests that PI103 is directly inhibiting DNAPK, which could explain the radiosensitizing effects seen with this drug. This should be made clear in the discussion.

   We performed three independent experiments and got the similar results and made this clear at the legend of Figure 2 and Results section.

   We added following paragraph at the discussion section (page 15):

   “Although rapamycin was a strong inducer of autophagy, it did not increased cytotoxicity of radiation therapy combined with temozolomide. In contrast, PI103
which is a dual inhibitor of class I PI3K and m-TOR prolonged gammH2AX foci formation with downregulation of p-DNA-PK, increased autophagy and increased cytotoxicity of radiation and temozolomide. We speculated that the impairment of DNA damage repair following radiation is potential mechanism of radiosensitization seen with this compound.”

5. Figure 1 and 2 - statistical analysis should be provided for comparisons of the clonogenic survival curves.
We did statistical analysis at each dose point and the statistically significant differences were described at the Results section.

Dr. Peter Sminia

The manuscript concerns an important issue in the therapy of high grade glioma patients: their resistance to radiotherapy (and chemotherapy), and how to improve glioma therapy by targeting therapy. The laboratory observations on cell lines are interesting.

We thank the Reviewer for the supportive comments for the implication of our work and thoughtful comments for our work.

1. Title: The title of the manuscript is not representative for the presented work.
“Enhancement of the Radiosensitizing Effect of Temozolomide” is not or only partially covered by the presented data. Also, HDAC inhibition is not only EGFR-associated. Please change the title.

We agreed your opinion and addressed this issue by changing the title of manuscript as “Improved cytotoxic effect of radiation and temozolomide in malignant glioma cells: Targeting PI3K-AKT-mTOR signaling, HSP90 and Histone deacetylases”.

2. Abstract: The results section should be revised (see comments below).
We revised the result section as you requested.

3. Background:
1) The paper of Stupp et al. (2005) is not a recent paper. In the meanwhile, long follow-up data are published (Stupp et al., Lancet Oncology 10: 459-466, 2009), which should be mentioned. With regard to the genetics of glioma, the next reference could be incorporated into the text: Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature. 23;455(7216):1061-1078, 2008.

Thank you for this thoughtful comment and we revised reference section as you suggested.

2) The rationale for using HDAC inhibition as well as ligand-independent modulation as enhancement strategy should be explained in more detail, or by referring to the literature.
We described the rationale for using HSP90 and HDAC by referring the references at Introduction section as you requested.
3) The final para (page 4) of the background chapter contains a conclusion as mentioned in the abstract, and should be deleted here. We revised this part as you suggested.

4. Materials and Methods:
1) Cell culture: details about treatment of cells with TMZ should be moved to the para Clonogenic assays.
We moved this paragraph to “clonogenic assay” section at page 5.

2) Clonogenic Assays:
The concentration of 25 microM TMZ has no effect at all on the MGMT unmethylated T98 glioma cell line (literature data, and data from this reviewers lab), while it is a cytotoxic dose on U251 and U87 cells. Why was the TMZ not adapted to the sensitivity of the three cell lines to the drug, by taking an isoeffective drug dose? See also my later remarks.

3) Please add irradiation details. X-rays or gamma rays? Dose rate?
We added “Cells were irradiated with 6 MV X-ray from a linear accelerator (Clinac 6/100, Varian Medical Systems, Palo Alto, CA) at a dose rate of 2.46 Gy/min” at page 6.

4) Wound healing assay-How was exactly the cell migration distance scored? How many wells were counted. Please add details.
We added the details as you requested at page 8: “The distances between the edges of the wound were measured by using Image J software. The sixty measurements were taken for each experimental condition. The degree of mobility is expressed as percent of wound closure as compared with the zero time point. Migration rates were calculated using the following equation: (initial distance-final distance /initial distance) × 100”.

5) Statistical Analysis
Were the experiments performed in triplicate, i.e. three independent experiments and were controls included in each separate experiment? Were three independent experiments performed for all the assays?
We performed minimum three independent experiments for all clonogenic assays and each experiment was performed with triplicates. We made it clear this point at p6 and the legend of Figure 1 and 2.

5. Results / Figures
1) Please move text and belonging references (e.g. second para, lines 1-5 and several other places) to the appropriate chapter of the manuscript, either the introduction or discussion.
We moved those paragraphs and belonging references to the Introduction section at page 4.
2) Figure 2 and Supplementary table: the SERs of TMZ alone groups, for all three cell lines, are the same. Does this mean that the cell survival assay with TMZ was performed only one time, and not repeated for each separate experiment with addition of the test drug, either RPM, PI103, 17DMAG or LBH? Each individual experiment should have its own TMZ + irradiation control curve. Since the IR alone and IR + TMZ cell survival curves presented in Figures 2ABCD are exactly the same for U251, as well as for the T98 cell line, this was probably not done. Because the additional effect of the three drugs were compared with the TMZ + IR curve alone – and enhancement ratios as presented in the supplementary table 1 calculated on basis of those curves, the obtained SER values and conclusions, if not performed with controls in each separate experiment, might be wrong.

As you know, clonogenic assay is labor-intensive work especially it involves combined treatment of two different compounds and radiation. In order to reduce the absolute number of dishes, we performed clonogenic assays for all four inhibitors as the one set for each cell line last one or two round of experiments; we already knew the optimal condition and just wanted to confirm the reproducibility of data from previous experiments. That is the reason why the control graph is the same.

3) Figure 3A: the photographs are not convincing. Please add quantitative data. We added the bar graph displaying quantitative data.

4) Figure 4B: the quantitative data presented in this figure do not show a radioenhancing effect of TMZ alone. The compound PI103 seems to protect for the effect of IR, if correct: is the effect significant? The effect of PI103 on the caspases3/7 which indicates apoptosis was insignificant although this compound significantly increased autophagy as a mode of cell death.

5) Figure 4C: The presented fluorescence photographs require better explanation in the text. It is difficult to see that addition of TMZ to each of the combination treatments of drug with irradiation enhances the lysosomal localization of LysoTracker. We added the bar graph showing quantitative data for punctate fluorescence.

6) Figures 5A&B: How exactly was cell migration scored? Because the test took 24 hours, the effect of (drug induced inhibition of ) cell proliferation might result in a higher reduction of cell migration in the test groups where drugs were present. How about the effects of the drugs alone, without TMZ and or IR? “The distances between the edges of the wound were measured by using Image J software. The sixty measurements were taken for each experimental condition. The degree of mobility is expressed as percent of wound closure as compared with the zero time point. Migration rates were calculated using the following equation: (initial distance-final distance /initial distance) × 100”. We added this paragraph at p8-9. PI103 or 17-DMAG or LBH589 alone also increased migration
and invasion of U251 cells.

7) Figure 5C: Addition of TMZ has probably no extra effect on all combinations, and also no radiosensitizing effect. Quantitative data are required to draw a definite conclusion.

We added the bar graph showing quantitative data to clarify this point.

8) Results, Page 14: move literature data to the introduction or discussion.

We moved those literature data to the Introduction section at page 4.

6. Discussion

This discussion in its present form is rather an overview of the literature than a discussion of the own findings, and placing those into perspective of current knowledge in this field of research. The discussion should start with a short summary of own findings, the main message from the – in the results section - presented experimental data. Then, own data and data from the literature should be criticized and discussed. The main message from own data, future steps and clinical relevance should be part of the discussion. Taken together, the discussion has to be rewritten.

We appreciate this thoughtful comment and addressed by rewriting significant part of this section.

Dr. Chann Lagadec

1. Most of the biological effects have been presented as pictures, without any quantification... please quantify them.

We added quantitative data at Figure 3, 4 and 5, respectively.

2. Authors never explained why they picked up 6Gy to analyze biological effects of combined therapies... is it the dose for best SER?

Yes

3. Authors claimed that RPM increases expression of LC3-II, as well as 17DMAG and LBH589... unfortunately, it’s not obvious at all on presented western blot... please provide a quantification bar graph of the 3 repeats.

We added quantitative data at Figure 4C and 4D.

4. To test potential side effects of combined therapies, authors used normal astrocytes irradiated with 2 Gy...

We chose 2Gy as it is the conventional fractionated dose used in daily treatment of GBM and SF2 (Survival fraction at 2 Gy) is widely accepted as an important parameter of intrinsic radiosensitivity.

5. Authors never specified how they chose the dose concentrations of each drugs? Since RPM does not have effect, is the dose sufficient? RPM seems to induce autophagy but does not have effect on radiosensitivity... authors should use this opportunity to discuss about potential mechanisms differences or main
pathways involved which should be explored to bypass GBM radioresistance...
We thank the Reviewer for bringing this to our attention. We agreed your opinion and added the in depth discussion at page 15.

6. Authors used siRNA... this has never been described in material and methods. How long after siRNA transfection did you perform the western blot and the clonogenic assay? Since you don’t observe a decrease of p-Akt, that might be due to phosphorylation induced by transfection-stress... please use an additional control: untrasfected cells.
We added the paragraph for RNAi at page 5 and 6. We actually used untrasfected cell and nonspecific control both as the control at the initial phase of study and could not find any significant differences in terms of clonogenic survival and expression of proteins we looked at.

7. Authors claimed that rapamycin-treated cells exhibit a staining for EphA2 as strong as the control or TMZ-treated cells... nevertheless according to the picture (and the western blot), RPM reduces expression of EphA2... Please quantify the staining.
We added quantitative data at Figure5E.

8. “low level of MGMT expression, which indicated a high level of methylated MGMT”... has to be change by: “low level of MGMT, as previously described (ref) which might highlight a high level of MGMT promotor methylation”.
We made the changes at p10 as you suggested.

We deeply appreciate all your helpful comments and excellent suggestions to improve our work.

Respectfully,

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