Author's response to reviews

Title: Carbonate apatite-facilitated intracellular delivery of c-ROS1 siRNA sensitizes MCF-7 breast cancer cells to cisplatin and paclitaxel

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Version: 3 Date: 4 January 2013

Author's response to reviews: see over
January 3, 2013

To
The Editor-in-Chief
BMC Cancer

Dear Sir,

We have now revised our manuscript entitled “Carbonate apatite-facilitated intracellular delivery of c-ROS1 siRNA sensitizes MCF-7 breast cancer cells to cisplatin and paclitaxel” for your necessary consideration to publish in BMC Cancer.

Below are our responses to the comments of each referee in point-by-point manner:

REVIEWER 1

Major Compulsory Revisions

• In Methods and Results, the authors asserted that the anti-ROS1 siRNA was validated by the supplier and that the MCF7 cells constitutively expressed ROS1. However, the authors did not show any evidences that the siRNA effectively down-regulate the c-ROS1 expression, neither at the mRNA level, nor at the protein level. An analysis of ROS-1 expression (qRealTime-PCR and Western Blotting) in MCF7 cells before and after siRNA exposure should be carry out by authors. In addition, all the subsequent experimental analysis of the effects of siRNA on chemosensitivity of MCF7 cells should be accomplished including a scramble-siRNA as a control.

Answer: The siRNA used in the study for silencing c-ROS1 gene expression was designed, synthesized and finally validated in Qiagen laboratory by RT-PCR following knockdown. Therefore we do not feel that an additional validation study for this siRNA is actually required to confirm its silencing activity. Since siRNA is highly specific to the target gene transcript especially at the concentrations we used, we think that no other genes will be silenced. In the meantime, we have also carried out cell viability assessment using ‘AllStars Negative Control siRNA’, a scramble-siRNA at different doses and found no effect irrespective of the doses investigated. The following text has been added in the revised manuscript (in page 12): ‘Assessment of cell viability using ‘AllStars Negative Control siRNA’, a scramble siRNA (designed and synthesized by Qiagen) resulted in no cytotoxic effect irrespective of the siRNA doses used (not shown here), indicating the importance of siRNA specificity in gene knockdown and eventual change in cell functionality.’

• The MTT assay is used to measure an antiproliferative effect and not the cell survival. It cannot be considered a cytotoxicity test, thus the effect of the compounds on tumor cells should be defined as “antiproliferative”. Although both short- and long-term drug exposure can be employed in principle with this assay, it is recommended for long-term exposure (72-96 h), as the method, which
requires washing of small cell numbers after drug treatment, can result in high variability. Thus, the selected time of exposure (48 h) needs a different approach to evaluate the antiproliferative effect of combined treatment with siRNA and drugs (cell counter). In addition, the data analysis provided by the authors appears confusing, primarily because a method (MTT) that measured an antiproliferative effect (in the sense of “proliferation inhibition”), could not be used to measure “the percentage of cell death” as authors asserted.

**Answer:** MTT assay is widely accepted as a tool to assess the percentage of cell viability because the dye can only stain the mitochondria in living cells. Since we usually seed 50,000 cells per well of a 24-well plate, long-term exposure (72-96 h) condition could cause untreated cell to grow to over-confluence in the well, leading to the high variability of the data which would be difficult to be interpreted.

- The drug concentrations selected by authors to investigate the effects of 48 h combined treatment with anti-ROS1 siRNA on cell viability and protein expression in MCF7 cells, were 8 and 40 nM, for doxorubicin, cisplatin and paclitaxel respectively. The authors should explain such a choice, since the drugs are structurally uncorrelated and have different mechanisms of action. From Figs. 3, 4 and 5 it could be gathered that the drug concentrations were selected because, as single agent, they appeared subtoxic (the authors defined the doses “low and high”), but exhaustive data on cell sensitivity to the three drugs alone have to be included in the manuscript.

**Answer:** Indeed, we investigated much higher concentrations of doxorubicin, cisplatin and paclitaxel and in accordance with previous reports, observed more cytotoxic effects in MCF-7 as well as 4T1 cells. However, we felt that it would not be meaningful to include those data in this manuscript. Moreover, we did not find a synergistic effect when c-ROS1 siRNA was co-delivered with the individual drug presumably due to the dominant effect of the drug at the high concentrations. Thus, we chose much lower concentrations of the drugs (8 and 40 nM) on the basis of the notion that the effect of c-ROS1 gene knockdown would not be masked by the effects of these relatively lower concentrations of the individual drug. In addition, all of the three drugs irrespective of their different molecular structures, demonstrated similar levels of cytotoxicity in both breast cancer cell lines.

- An interesting review which has been recently published regarding ROS1 as a “druggable” receptor tyrosine kinase (Ou S. et al., Expert Rev Anticancer Ther 2012, 12:4, 447-56), suggested that several ALK inhibitors have been show to inhibit also ROS1. Preclinical data in such a way have provided rationale to develop ongoing trials of crizotinib (ALK/MET inhibitor) in ROS1-rearranged solid tumors (NCT00585195). Authors are highly invited to discuss this point.

**Answer:** We would like to cordially inform that in the current manuscript we have mainly discussed on the involvement of c-ROS1 gene in human breast cancer, although this particular gene is widely expressed in various human cancers including non-small-cell lung cancer (NSCLC) and cholangiocarcinoma.

**Minor Compulsory Revisions**
- This study has an evident weakness related to the data presented in Western blot analysis reported in Figure 6. In particular, although in Methods the authors
asserted that membranes were also tested for equal loading, no data with regard to this point was presented.

**Answer:** We think that equal loading was justified by the similar levels of phosphor AKT as observed for various samples in Fig. 6.

- In the manuscript the authors argue of a possible therapy with siRNA technique, also to be used as potential sensitizer for chemotherapy in the treatment of breast cancer. Such an assumption seems to be technically and conceptually pretentious, because the biggest obstacle to developing siRNA-based therapies remains the delivery of the siRNA molecule to the target tissue, in addition to the difficulties, handled by the authors, correlated to the delivery across the cell membrane. Thus the authors should emphasize that major prerequisites for the in vivo applicability of siRNA include efficient transfer across the cell membrane, favourable blood half-life and adequate tissue bioavailability, as well as stability of the siRNA. Additionally, for clinical applicability, these requirements need to be achieved in the context of minimally invasive administration (see also Medarova Z. et al., Nat Med 2007, 13:3, 372-77).

**Answer:** Although delivery of siRNA to the target tissue (breast) remains a strong barrier, only siRNA delivery seems to be inadequate for effective killing of cancer cells. An effective combination of a particular drug and a target siRNA would be far better than either drugs or siRNA alone for proper treatment of breast cancer.

3. Discretionary Revisions
- Regarding the Fig. 3, 4 and 5, the relative captions reported that “**” indicates significant difference from the control value with ANOVA test and p< 0.05 compared with untreated cells”. Authors are invited to better clarify this feature, since no asterisk is reported in the above mentioned figures.
- Table 1, as a consequence of the major issues raised in point 1, has to be modified to improve the clearness of the results.

**Answer:** We have checked the figures as well as the Table 1 based on your sincere concerns and would like to cordially mention that we are still satisfied with our statistical analysis and the contents of Table 1.

**REVIEWER 2 EVALUATION**

1. Some of the authors of this work have already published results using carbonate-apatite as a delivery system for siRNA (ref 30-32). These results are referred to different siRNA and experimental cellular models other than those used in this study. The authors of this study should show the siRNA loading efficiency on the nanoparticles and an example of efficient cellular uptake, determined by using a labelled siRNA.

**Answer:** We have already published the data on siRNA loading efficiency and cellular uptake using labeled siRNA (Ref. 30).

2. For overall accuracy, the authors should show the targeting efficiency of the
RNAi c-ROS1 by demonstrating downmodulation of RNA by RT-qQPCR or end point PCR (24-72h after transfection) and confirm the degree of inhibition of ROS1 protein induced by RNAi targeting.

**Answer:** We have answered this in the beginning in response to the concern raised by the first Reviewer.

3. Have the authors used a negative control siRNA for transfection? Specific results on c-ROS1 RNA and protein expression are required.

**Answer:** We have also answered this in the beginning in response to the comments of the first Reviewer.

4. Why the authors analyzed the effect of drugs and siRNA combination 48h after treatment?

**Answer:** We have already answered this while responding to the comments of the first Reviewer. An incubation period shorter than 48h was not examined on the basis that siRNA-mediated silencing usually takes more than 24h.

5. Authors should calculate if the antiproliferative effect of drugs (cisplatin and paclitaxel) combined with RNAi is additive or synergistic since they refer to a synergistic effect of the combination with paclitaxel.

**Answer:** In our previously published papers (Ref. 30-32), we clearly demonstrated the patterns of additive and synergistic effects. Indeed, in the case of siRNA-facilitated gene knockdown, the additive effects are usually reflected by a minimal or no change in the percentage of cell viability.

6. In order to validate the potential therapeutic implications of the proposed combination regimen of cisplatin and paclitaxel plus ROS1 suppression, more prolonged experiments of inhibition of cell proliferation are required. This can be easily done by assessing the clonogenic potential of MCF7 cells transfected with control RNAi or c-ROS1 RNAi in the presence or absence of drugs using a conventional soft agar colony formation assay.

**Answer:** Having been motivated with our original and exciting findings as presented in the current manuscript, we are currently progressing to carry out the experiments in a breast cancer model using c-ROS1 siRNA and the cancer drugs. We cordially like to mention that we do not feel it necessary at this stage for assessing the clonogenic potential of MCF7.

7. (pag 14) Authors proposed that downmodulation of c-ROS1 mRNA might deactivate the Raf/MEK/ERK pathway that is involved also in resistance to paclitaxel thereby synergistically enhancing the cell apoptosis induced by drug. Have the authors measured apoptotic cells after treatment?

**Answer:** Since we are using the conventional and popular anti-cancer drugs that are well-known to kill the cancer cells through apoptosis, we have not presented the data in this manuscript.
8. Representative immunoblot in Fig 6: authors should add AKT expression and Useful reference for protein expression. Moreover an adequate quantitative analysis is required.

**Answer:** In this study, we have only detected the activated forms of MAPK and AKT in order to demonstrate the activation levels of these two pathways (which are mainly responsible for cell proliferation and survival) in response to the c-ROS1 gene knock-down and the conventional anti-cancer drugs.

Minor point
1. Pag 8. I suggest to shorten the description of “Cell culture and seeding”
   **Answer:** The contents have been shortened.
2. Asterisk in Fig 4 and Fig 5 should be added.
   **Answer:** We have already answered this while responding to the comments of the first Reviewer.
3. Authors should check the SD in Fig 5.
   **Answer:** We have checked the SD in Fig 5. The error bars are too short to be seen for several samples.

We would like to thank the reviewers for their valuable suggestions. We would welcome any more suggestion to improve our manuscript.

Sincerely Yours,
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