Author's response to reviews

Title: Down-regulation of GRP78 is associated with the sensitivity of chemotherapy to VP-16 in small cell lung cancer NCI-H446 cells

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Author's response to reviews: see over
Dear editor:

I have carefully considered the reports from the reviewers on our manuscript “Down-regulation of GRP78 is associated with the sensitivity of chemotherapy to VP-16 in small cell lung cancer NCI-H446 cells (MS ID: 3112636332120043). Based on the review, we have revised the manuscript as the following.

**Reviewer:** Hae-Ryong Park

**Reviewer’s report:**

**Major Points**

1. *Previous studies revealed that* BAPTA-AM *exhibits the cytotoxic effects on the other cells. Thus, it must be described whether the concentrations of BAPTA-AM for its VP-16-chemosensitive activity are consistent with those for the cytotoxic antitumor activity in NCI-H446 cells.*

In our preliminary experiment, trypan blue was used to determine the effects of BAPTA-AM on cells in absence of VP16. The results showed that less than 1% of the cells pretreated with BAPTA-AM at concentrations from 10µM to 50µM for 24h died. When the concentration of BAPTA-AM was increased to 55, 60µM, the dead cells increased obviously. On the other hand, Jason Bakhshi reported that the percentage of cells viability was near 80% when melanoma cells were preincubated with BAPTA-AM at 50 µM for 3 h followed by treatment with 75 µM curcumin for 24 h. Then, the concentration of BAPTA-AM were decided as 10, 15, 25, 40µM in our experiment. Following is the reference:


2. *In Fig. 1, Could authors comment a little bit on the fact that basal levels of GRP78 are reduced apparently at treatment with 40µM of BAPTA-AM in A23187-induced stress condition? In similar manner, this is also related with Fig. 3(C). It seems to cell cytotoxicity induced before GRP78 was down-regulated.*

When the cells were exposed to BAPTA-AM alone at different concentrations (10, 15, 25, and 40 µM) for 2 h, the signals of GRP78 mRNA were too weak to be assayed easily with an approximately concentration-dependent manner (date not shown), whereas after the addition
of A23187 at the concentration of 2 μM for 2 h, the GRP78 mRNA signals of the BAPTA-AM pretreated cells could be assayed obviously.

3. In Fig. 3, although authors argued that BAPTA-AM was increased the sensitivity which target S-phase of VP-16-induced resistance, it is not seemed to associate with chemotherapy to VP-16. The drug resistance to VP-16 has the involvement of multiple pathways however, consideration for topoisomerase IIα which is a molecular target of VP-16 is necessary. Additionally, Why did not carry out the effect of BAPTA-AM on A23187-induced cells in absence of VP-16. These questions should be addressed and discussed in the paper.

Our results of apoptosis for the cells pretreated with BAPTA-AM or A23187, prior to the addition of VP-16 showed that, the apoptosis rate of cells with lower level of GRP78 in the BAPTA-AM→A23187-treated group increase greatly compared with A23187-treated group and control group with high or normal level of GRP78, indicating that the down-regulation of GRP78 by BAPTA-AM may increase the sensitivity to VP-16 in NCI-H446 cell. Our results on cycle distributions showed that there was a great decrease in G1 phase and a dramatic increase in S phase for BAPTA-AM→A23187-treated group cells, suggesting that BAPTA-AM may rendered more sensitivity to VP-16 through the change of distribution of cell cycle NCI-H446 cell. Till now, only few reports are involved on BAPTA-AM associated chemotherapy resistance. The mechanism for the chemotherapy resistance to VP-16 is complex, multiple pathways are involved. Topoisomerase IIα is an ATP-dependent nuclear enzyme that plays important roles in DNA replication and chromosome segregation by its ability to change the topological structure of DNA. VP-16 is the topoisomerase inhibitors, it can interact with the enzyme to stabilize topoisomerase-DNA complex, blocking strand-passing activity, thereby resulting in DNA breakage [27]. Furthermore, it had been proposed that the chaperone function of GRP78 could affect growth factor processing, creating a cell proliferation block to escape drug killing that only occurs in cycling cells [28]. Since VP-16 targets S phase cells [29] and here we confirmed that the inhibition of BAPTA-AM dramatically increased the percentage of S phase cells, we proposed that GRP78 might render the cells sensitive to VP-16-induced apoptosis through altering the cell cycle distribution in NCI-H446 cell line.

4. How dose DNA fragmentation in the nucleus when treated with BAPTA-AM in
VP-16-resistance cells.
The question has mentioned as above.

Minor Points
1. In Introduction, if the common used inhibitor of GRP78 is thapsigargin, indicate the Reference for that.

   the Reference:

The following have been corrected in the manuscript.

2. In all manuscript, consist with SI units.
3. In Results, correct the subheading of title.
4. In Discussion, is it Table 3 or Table 2? Please check.
5. In Fig. 1, correct the style of graph and consist with font.
6. In Fig. 2, there is no data as control of BAPTA-AM only. Please add.
7. Although written reasonably well, the manuscript would still benefit from careful editing for English syntax and particularly for dropped words.

Reviewer: Kim Janda

Reviewers report:
This paper describes GRP78 expression in lung cancer cells and its down-regulation in the context of sensitivity to chemotherapeutic drugs.

Major Compulsory Revisions:
Overall, the manuscript is poorly written and needs significant improvement in word order and grammar. Relevant citations about targeting GRP78 are missing. The authors use immunofluorescence to assess the expression of GRP78 on the protein level; however, it would be more appropriate to do this by western blotting. The designation of the treated cells is misleading; the group “BAPTA-AM-inhibited cells” should be called “BAPTA-AM-treated cells”. The “A23187-induced group” should be called “A23187-treated group”. The authors should also consider generating a bar graph for the toxicity data. Furthermore, they should not only treat the cells with VP-16
but also with a known drug such as taxol. It would also be interesting to compare BAPTA-AM to another inhibitor of GRP78 expression.

1. The manuscript has been significant improvement in word order and grammar. Relevant citations about targeting GRP78 have been complemented.


2. In our preliminary experiment, we also did Western blots performed with the same antibodies directed against GRP78 as immunofluorescence. Because the results are consistent with that of immunofluorescence and PCR, especially the pattern of the expression of GRP78 mRNA were very clear, we just presented the results of immunofluorescence for the first manuscript. Maybe it is unsuitable. Following the reviewers suggestion, we show the Western blots results as followings this time:

![Western Blot Results](image)

Figure 3
The expression of GRP78 at the protein levels by western blots in NCI-H446. (A): untreated cells; (B): cells treated with BAPTA-AM at 10 µM alone for 2 h; (C): cells treated with A23187 at 2µM alone for 24 h; (D-E ) : cells treated with BAPTA-AM at 25 µM(D), 40 µM (E) for 2 h respectively .prior to the addition of A23187 at 2µM for 24 h.I: Electrophoregram II : Bar graph.
3. The three groups cells have been named as BAPTA-AM→A23187-treated group, A23187-treated group and control-group.

4. The question about toxicity should be explained as the responds to Hae-Ryong Park:
In our preliminary experiment, trypan blue was used to determine the effects of BAPTA-AM on cells in absence of VP16. The results showed that less than 1% of the cells pretreated with BAPTA-AM at concentrations from 10µM to 50µM for 24h died. Then, the concentration of BAPTA-AM were decided as 10, 15, 25, 40µM in our experiment.

5. Yes, our group has done some work on the relationship between the expression of GRP78 and the resistance to another commonly used anticancer drug, platinum in lung cancer, and the results are completely different from VP-16. The possible mechanism may lie in the different characteristics of the two drugs (the results are being collected).

**Minor Essential Revisions:**

The following have been corrected in the manuscript.

The authors should decide on either “Grp78” or “GRP78” and be consistent throughout the manuscript.

Page 2, line 3: reads “present”, should read “presence”
Page 2, line 5: reads “trough”, should read “through”
Page 2, line 6: reads “which is”, should read “which are”
Page 2, line 8: reads “GRP78/BiP, one of well-”, should read “GRP78/BiP, a well-”
Page 2, line 12: reads “The common used”, should read “Commonly used”
Page 3, line 29: reads “the cells were washed with PBS for twice”, should read “the cells were washed twice with PBS”
Page 6, line 5: reads “once within the cell”, should read “once inside the cell”
Page 6, line 14: reads “inhibited”, should read “inhibit”
Page 6, line 29: reads “sensate”, should read “sensitive”

**Figure 1 needs major improvements, the background in II should be white and the bars should be black. Error bars are missing. The Y-axis has not title.**
In the discussion the authors’ state: “In this work, we found that BAPTA-AM attenuated the expression of GRP78 significantly. In line with our results, Juliann G, et al. [21] and W hei-mei C, et al. [18] also respectively reported that BAPTA-AM caused a down-regulation of GRP78 in human breast cancer cell line and 9L rat brain tumor cells. But treatment with BAPTA-AM alone did not inhibit the expression of Grp78 mRNA, whereas treatment with BAPTA-AM followed by heat-shock induced could inhibit the expression of GRP78 obviously.” It is not clear who carried out the heat-shock treatment, the authors in this study or the aforementioned works by different groups. This needs to be clarified. Level of interest: An article whose findings are important to those with closely related research interests Quality of written English: Not suitable for publication unless extensively edited.

In our work, BAPTA-AM caused a down-regulation of GRP78 when the NCI-H446 cells were exposed to BAPTA-AM before the addition of A23187. In line with our results, JULIANN G reported that the expression of GRP78 decreased compared with control group when T47-D cells were treated with BAPTA-AM prior to heat shock.

The following is the reference:

With regards
Wang yingyan