Author's response to reviews

Title: Small Interfering RNA Targeting Mcl-1 Enhances Proteasome Inhibitor-Induced Apoptosis in Solid Malignant Tumors

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Author's response to reviews: see over
Dear Dr Marinette Lacson,

Thank you so much for your letter of Sep 8.2011, regarding our manuscript (Small Interfering RNA Targeting Mcl-1 Enhances Proteasome Inhibitor-Induced Apoptosis in Solid Malignant Tumors). We also want to thank the reviewers for reviewing our manuscript and providing helpful comments again. In this letter we provided our responds to the comments from the reviewers (see below). We will welcome any rebuttal or feedback from the reviewers about our following responses. It is certainly understandable that peoples may have different opinions about the facts presented below.

For Comments from Dr Gian Luigi Russo:

1. In my view, the ms contains an evident contradiction which must be explained or, at least, discussed. PIs are considered anticancer molecules able to induce apoptosis. However, as reported here and by others, they also increase stability and, consequently, expression of Mcl-1 which is a pro-survival factor and it is expected to counteract the pro-apoptotic effect of the inhibitors. How these two events can coexist in the cell? Is it possible that Mcl-1 increase following PI treatment is an epiphenomenon without a functional meaning?

Response: The ubiquitin-proteasome pathway is critical for maintaining the homeostasis of most intracellular proteins in eukaryotic cells. Cell cycle progression, transcription factor activation, apoptosis, and other cellular events may be directly or indirectly controlled by the ubiquitin-proteasome pathway (Palombella et al., 1994; King et al., 1996). Previously, we had reported that proteasome inhibitors could induce Bik accumulation in various cancer cells (Zhu H et al, 2005). Here we reported that proteasome inhibitors could also induce Mcl-1 accumulation in these cells. Although both Bik and Mcl-1 protein were accumulated in these cells, they should play distinct role for cell survival. We had demonstrated that Bik accumulation induced by proteasome inhibitors might play a pro-apoptotic role in these cells (Zhu H et al, 2005). However, in this study, we showed that Mcl-1 siRNA could enhance the cell killing effect induced by proteasome inhibitor. These data suggested that Mcl-1 could partial prevent cells from death. Base on these, we don’t think that Mcl-1 increase following proteasome inhibitors treatment is an epiphenomenon without a functional meaning. We had added the discussion in the first paragraph of page 14.

2. One of the key experiment is presented in Fig. 5B where the relative cell viability in the combined treatment (MG132+Mcl-1 siRNA) is slightly better than MG132 + control siRNA. However in considering potential clinical application, issues regarding costs and toxicity of a
combined treatment must be taken into account. The relatively limited benefit resulting from the analysis of Fig. 5B does not justify the positive message transmitted by the authors throughout the text on the efficacy of the combined treatment.

**Response:** Although the absolute value of difference between control siRNA+MG132 and Mcl-1 siRNA+MG132 is not so large, it reaches to a statistical significance. However, what reported here is just a preliminary result from the basic research and only suggests that it could be a potential direction for next anti-cancer therapeutic study. Anyway it is still far away from its clinical application. We modified that statement in the second paragraph of page13.

3. The Authors stated in the abstract and introduction that “The current study was designed to analyze the levels of several anti-apoptotic members of Bcl-2 family in different human cancer cell lines after they were treated with proteasome inhibitors...”. However, they didn’t mention any data on other Bcl-2 family members. Why? No effect following PI treatment? Please clarify this point.

**Response:** For this issue, we had added the data of Bcl-XL and Bcl-2 into Figure 1A. They had no change following PI treatment. We had made changes in the legend of Figure 1A and in the first paragraph of page 8 which were marked by red font.

4. About sensitivity to bortezomib, the Authors stated that “These results demonstrate that the cells’ susceptibility to bortezomib was not obviously associated with the amount of Mcl-1 accumulation”. To reach this conclusion a correlation index must be calculated combining data from Tab. 1 and Fig. 4.

**Response:** The pearson correlation index for the data from Tab. 1 and Fig. 4. is 0.781 (P=0.066), suggesting the cells’ susceptibility to bortezomib was not obviously associated with the amount of Mcl-1 accumulation. We had listed the value of r and p in the second line of page 10.

5. Regarding Fig. 5A, the Authors stated “Nevertheless, treatment with MG132 resulted in obvious Mcl-1 accumulation in cells pretreated with Mcl-1 siRNA, although the level of this accumulation was dramatically lower than in cells pretreated by control siRNA” To say the true, analysis by eyes suggest that Mcl-1 level in the data point MG132+Mcl-1 siRNA is not so “dramatically lower” as described. How many immunoblottings have been performed regarding data in Fig. 5A? Probably more than one. It is mandatory to add a graph showing densitometric analysis plus/minus SD.
Response: Three times immunoblottings had been performed regarding data in Fig. 5A. We had added a graph showing densitometric analysis plus/minus SD as Fig 5B. We also made changes in the second paragraph of page 10 as well as in the legend of figure 5.

6. Fig. 6 present several problems. Probably a copy/paste mistake happened regarding Fig. 6B which is identical to Fig. 5B. This is impossible since in Fig. 5 and 6 cell viability has been calculated using two different assays. Lack of the real Fig. 6B makes difficult to judge data reported in Fig. 6A. Percentages in the different squares of Fig. 6A indicating positivity to propidium and annexin-V are missing. Control data point in Fig. 6A doesn’t help Authors’ conclusions since it shows a clear signal (top-left) probably attributable to propidium staining.

Response: We are sorry for making a mistake for Fig6B in the previous edition. Now we had added the correct data in the Fig6B. Because the values in the Fig.6B represent means ± SD of a triplicate assay, we didn’t add the percentages in the different squares of Fig.6A.

7. The rationale behind the selection of the cell lines used in the present study must be clarified.

Response: We chose these cell lines (Human colon cancer cell lines DLD1, LOVO, SW620, and HCT116; human lung cancer cell lines H1299; human ovarian cancer cell line SKOV3) because they were owned by our lab. That’s all. No special reason behind the selection. We had made change in the first paragraph of page 5.

8. In Fig. 3B and in Table 1 SD is not indicated

Response: We had added SD data in Fig. 3B and in Table 1. We had re-calculated the IC50 of bortezomib in different cell lines by CurveExperter Professional Version 1.2. Because the IC50 of LOVO is much lower than the minimal concentration used in our experiments, we can not calculate the precise value for it.

9. Several controls are missing. As an example, MG132 mono-treatment in Fig. 5B and Fig. 6.

Response: We had added the data of MG132 in Fig. 5C (previous Fig.5B) and Fig. 6.

10. How do the Authors explain that Mcl-1 siRNA alone in Fig. 5B does not decrease cell viability considering the anti-apoptotic nature of Mcl-1?

Response: We think that there are two potential factors in charge of this result. One is that the treatment time of Mcl- siRNA is not enough long to induce obvious apoptosis. The other is that Mcl-1 siRNA used here is only partial blocking the expression of Mcl-1 protein which
was showed in the Fig.5A. Both of these causes to slight cell death in Mcl-1 siRNA alone group in Fig. 5C (previous Fig. 5B).

For Comments from Dr martine amiot:
1. The authors claim that Mcl-1 accumulation was caused by stabilization of the protein against degradation. However, the mechanism of stabilization has not been investigated while the E3 ligase (Mule) involved in Mcl-1 degradation is now well described. Thus, the authors should address the regulation of Mcl-1 ubiquitination and the regulation of the Mcl-1/Mule complexes.

Response: As the reviewer mentioned, the E3 ligase (Mule) involved in Mcl-1 degradation is now well described. The ubiquitination of Mcl-1 is mediated by Mule-a BH3-only E3 ubiquitin ligase [Zhong Q et al, Cell. 2005]. This process requires the association of Mcl-1 with Mule and is controlled by Noxa through the regulation of the Mcl/USP9X interaction [Warr MR et al, FEBS Lett. 2005; Gomez-Bougie P et al, Biochem Biophys Res Commun. 2011]. The level of Mcl-1/Mule complex would determine the sensitivity of cancer cells to apoptosis [Pervin S et al, Br J Cancer. 2011]. Base on the articles, we set the main aim of our manuscript is try to clarify the effect of Mcl-1 accumulation on the apoptosis induced by proteasome inhibitors. The underlying mechanism of Mcl-1 stabilization would be another story for us. We had added the content in the discussion [the third paragraph of page 12].

2. The authors should also address in their manuscript whether Bortezomib induces Mcl-1 up-regulation in primary tumor cells.

Response: We had spent more than two years for primary tumor cells culture from patient's samples according to the request of a program from our province (#2005C23002). We tired various different ways to get primary cells. However, fibroblast contamination is always an obstacle for us and it is so difficult to passage that we cannot get enough cancer cells for the next experiments. Now we are trying to establish primary tumor derived from patients with cancer in nude mice. If succeeded, we would detect the effect of proteasome inhibitors on the primary tumors in future.

3. The Mcl-1 siRNA has been only investigated in one colon cell line, therefore, it is highly recommended that the authors add data from other cell lines.

Response: For this issue, we had detected the effect of Mcl-1 siRNA on the other colon cancer SW620 cell line and a similar result was detected. We had added the results in the
figure 5C. Also, we made changes in the second paragraph of page 10 as well as in the legend of figure 5.

For Comments from Dr Shehla SP Pervin:

1. The authors have extensively used human colon cancer cell lines, one lung and an ovarian cancer cell line. It will be good to use one or two breast cancer cell lines also, because most of them are resistant to proteosome inhibitors and knowing the status of Mcl-1 in these cells will advance the field.

Response: For this issue, we detected the effect of PS-341 in the human breast cancer MCF7 cell line. However, we did not find the change of Mcl-1 protein level after treatment with PS-341. The data was reported in the Fig.1D. We had added the changes in the text (in the first paragraph of page 8).

2. A reference for the concentrations of CHX used should be included in the text.

Response: For this issue, we previously had the reference [20] in the last paragraph of page 6. Now we also added this reference in the text in the first paragraph of page 9.

3. Difference between relative cell viability between control siRNA+MG132 and Mcl-1 siRNA+MG132 is small. Cell viability with only MG132 should be added. The authors should comment on this in the discussion.

Response: For this issue, we had added the data of MG132 alone in Fig.5C (previous Fig.5B), Fig.6A and Fig.6B. Although the absolute value of difference between control siRNA+MG132 and Mcl-1 siRNA+MG132 is not so large, it reaches to a statistical significance. Also, we had added comment in the second paragraph of page 13.

4. In fig 6A, showing increased Annexin V levels, which is an early indicator of early membrane flipping, does not suggest the cells undergo apoptosis. The western blots in fig 6C show small amount of cleaved caspase-9 and 3. To convincingly show that apoptosis is induced in MG132+ Mcl-1 siRNA cells, caspase-3 assay using fluorometric substrate should be done.

Response: In Fig.6A, the distribution of DLD1 cells in apoptosis measured by annexin-V and propidium iodide staining using flow cytometry. Cells with both annexin-V and propidium iodide staining positive were judge as undergoing apoptosis. We had presented the apoptotic data in Fig.6B. Fig.6C was just another evidence for apoptosis. We don’t think it is necessary
to quantitate apoptosis again by caspase-3 assay using fluorometric substrate. So we had
corrected the Fig.6B, but not performed the caspase-3 assay using fluorometric substrate.

5. In fig 3B, where relative levels of Mcl-1 fall with DMSO, CHX should be added.
Response: We had added the “CHX” symbol in Fig 3B.

6. A few blots reprobed with other anti apoptotic proteins should also be included.
Response: For this issue, we made changes in Figure 1A and its legends 1. We also made
changes in he first paragraph of page 8 which were marked by red font.

Based on above discussion and evidences, I would fully appreciate that you will allow us to
revise our manuscript and resubmit it to you for further consideration.
I look forward to hearing from you!

Sincerely,

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