Author's response to reviews

Title: Synergistic growth inhibition by acyclic retinoid and phosphatidylinositol 3-kinase inhibitor in human hepatoma cells

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Author's response to reviews: see over
Dr. Amancio Carnero,
Associate Editor, *BMC Cancer*,

Re: Revision of *BMC Cancer* manuscript MS: 5409740151005030: “Synergistic growth inhibition by acyclic retinoid and phosphatidylinositol 3-kinase inhibitor in human hepatoma cells”

Dear Dr. Carnero,

Thank you for your letter of July 13, 2013, and for the reviewers' comments concerning our manuscript entitled “Synergistic growth inhibition by acyclic retinoid and phosphatidylinositol 3-kinase inhibitor in human hepatoma cells”. Enclosed please find a revised manuscript, including new data (new Figs. 2A, 2D, 3A, 3B, and Table 1), which we believe address all of the concerns raised by the two reviewers, and a point-by-point list of our responses to the criticisms.

We thank you and the reviewers for these very helpful and constructive criticisms and we would like to request that our revised paper may again be considered for publication in *BMC Cancer*.

With best personal regards,

Sincerely,

Masahito Shimizu, M.D., Ph.D.
Responses to Reviewer #1 (Dr. Carmen Blanco-Aparicio)

We should like to thank the Reviewer #1 because this reviewer's comments were most helpful and gave us a better perspective of our work. We responded to concerns raised by the Reviewer #1 as follows.

Minor compulsory Revision:
1. The authors show that ACR and LY294002 alone have a moderate antiproliferative activity in hepatocarcinoma cells lines, but do not have any effect in Hc normal hepatocytes. Later they show that the combination is synergistic in hepatocarcinoma cell lines, but what happen with Hc normal hepatocytes do they render insensitive to the combination?

Based on this suggestion, we examined the possible combined effects of ACR and LY294002 on the growth of Hc normal hepatocytes. We found that the growth of Hc hepatocytes was not affected by the combination of these agents; even a combination of high concentrations of ACR (5 µM) plus LY294002 (15 µM) did not inhibit the growth of Hc cells in the present study (Page 8, line 24 to Page 9, line 2, Page 20, lines 13 to 15, and new Fig. 2D).

2. In figure 2A which is the effect of LY294002 or ACR at the single dose tested alone, this should be represented in the graph to visualize that the combination has a higher effect than each agent alone.

Following this suggestion, we revised Fig. 2A neatly and showed new Table 1, which clearly demonstrates that the combination has a higher effect than each agent alone (Page 8, lines 13 to 21). We appreciate your important suggestion.

3. Authors said that data obtained in figure 2A was used to calculate the CI. From which graph 2A was done. The graph in which ACR is in dose response seems to be more appropriate to do so, as the effects of the different doses of LY294002 are much clear.

As shown in new Table 1, the CI indices for less than 1 µM ACR (0.5 or 1 µM) plus less than 10 µM LY294002 (5 or 10 µM) were 1+ (slight synergism), 2+ (moderate synergism), or 3+ (synergism), respectively, when HLF human HCC cells were treated with a range of concentrations of these agents (Page 8, lines 13 to 21). In particular, our
finding that the combination regimen using 1 μM ACR plus 5 μM LY294002 synergistically inhibits the growth of HCC cells seems to be clinically relevant because this concentration (1 μM) is approximately the same as the plasma concentration of ACR (which ranged from 1 to 5 μM) in a clinical trial that demonstrated the chemopreventive effects of this agent in the recurrence of secondary HCC. We emphasized this point in the revised text (Page 14, lines 17 to 22 and Refs #10 and 11). We greatly appreciate your valuable comments concerning improvements to this paper.

4. Have the authors tried to repeat the experiments with a more potent PI3K inhibitors available in the market, to see if stronger effects are obtained.

Following this suggestion, we performed additional experiment and found that the combination of ACR plus BKM120, another selective PI3K inhibitor, significantly inhibited the growth of HCC cells (Page 5, lines 1 to 2, Page 5, lines 20 to 23, Page 9, lines 4 to 11, Page 20, lines 18 to 24, new Fig. 3A, new Fig. 3B, new Table 1, and new Ref #33). These findings suggest that combination therapy using ACR plus PI3K inhibitors might be an effective regimen for inhibiting the growth of HCC cells (Page 9, lines 11 to 13). We thank your important suggestion because we believe the results obtained from this additional experiment strengthen the value of our manuscript.

5. Is the combination effect dependent on the status of the PTEN/PI3k/AKT pathway of the hepatocarcinoma cell lines. How Do the cells that have the pathway activated respond in comparison with the cells that do not have the pathway activated?

We performed additional experiments and found that the combination of ACR and LY294002 significantly inhibited the growth of HLF, Huh7, and Hep3B HCC cells, whereas the growth of HepG2 cells, the other HCC cell line, was not suppressed by this combination (Page 8, lines 21 to 24, Page 13, lines 19 to 21, Page 20, lines 11 to 13, and new Fig. 2C). As suggested by this reviewer, this might be associated with the phosphorylation status of ERK and Akt proteins because the expression levels of p-ERK and p-Akt proteins are high in HLF, Huh7, and Hep3B cells compared with HepG2 cells as demonstrated in our previous study (Page 13, lines 21 to 24 and Ref #29). Based on this new finding, we also consider that HCC cells that overexpress p-ERK and p-Akt proteins might be more sensitive targets for combination therapy using ACR and PI3K inhibitors (Page 13, line 24 to Page 14, line 1). We appreciate your valuable comments again.
6. The authors treat the cells for 12h to see an effect on the phosphorylation of different proteins. Why the do a so long time, usually inhibition of a phosphorylation is a very fast process. Do the authors have done a time course for the downregulation of the biomarkers?

We have done preliminary time course study and found that this treatment time (12 hours) was appropriate for evaluating the expression levels of p-ERK, p-Akt, and p-RXRα proteins. This treatment protocol is also based on our previous reports (Page 6, lines 13 to 17 and Refs #25, 29, and 30). We thank your important suggestion.

**Responses to Reviewer #2 (Dr. Jordi Muntané)**

We are pleased that in the overall comments this reviewer found our study is interesting and the manuscript is clear and well written.