**Author’s response to reviews**

**Title:** Smac mimetics LCL161 and GDC-0152 inhibit osteosarcoma growth and metastasis in mice

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**Author’s response to reviews:**

Dear Dr Chiu,

Thank you for organizing the review of our manuscript, which we have re-titled “Smac mimetics LCL161 and GDC-0152 inhibit osteosarcoma growth and metastasis in mice”. We greatly appreciate the thorough and constructive feedback the reviewers provided. We are pleased to now submit a revised manuscript that we have modified in response to their suggestions.

Below, we have itemized the changes we made to the manuscript to address each of the points raised by the reviewers.

Yours sincerely,

Christine Hawkins
Reviewer 1’s comments and our responses

1) “The authors can add a picture to describe the core ideas and key conclusions of this manuscript. For example: Smac mimetics can cooperate with TNFα secreted by tumor-Associated myeloid cells to kill osteosarcoma cells in vivo.”

Figure 7 of the revised manuscript presents a model of the mechanism by which we propose Smac mimetics exert anti-osteosarcoma activity in vivo.

2) “The author also mentioned: co-treatments were also more toxic. How to avoid this problem and further improve the safety of biological treatment?”

We have expanded our discussion of the issues regarding combined toxicities, and risks versus benefits of combination treatment, adding these sentences into the discussion (lines 471-484):

“Subsequent studies will also be required to accurately model the potential benefit of co-treatment of osteosarcoma patients with Smac mimetics plus doxorubicin, versus single agent treatment, and to consider the balance between efficacy versus toxicities conferred by co-treatment, relative to Smac mimetics alone or coupled with other chemotherapy drugs”…. “It will be important to determine whether cooperative toxicities would be avoided by sequential exposure, in which case subsequent Smac mimetic treatment may be considered for patients whose cancers persist or recur after administration of the maximal cumulative dose of doxorubicin recommended to avoid dose-limiting cardiotoxicity.”

3) “I want to ask whether the authors can consider to prove that the addition of TNFα on the basis of the combination of the two drugs can effectively inhibit the development of the tumors in vivo.”

The revised discussion includes extra text (lines 444-450) addressing this point (new text is underlined): “Although we did not formally test the requirement for TNFα in order for Smac mimetics to exert anti-osteosarcoma effects in our model, this conclusion is consistent with our data showing that (a) Smac mimetic sensitivity of osteosarcoma cells in vitro depends on exogenous TNFα, (b) these drugs retard growth of tumors derived from these cells in vivo, and (c) implanted osteosarcomas contain TNFα produced by intratumoral immune cells. We would predict that Smac mimetic treatments would be ineffective in osteosarcoma-bearing animals animals treated with TNFα-blocking agents, or TNFα-deficient mice. Indeed, the presumed deficiency in TNFα-producing tumor-associated myeloid cells within SCID mice probably explains the relatively poor anti-osteosarcoma efficacy of LCL161 in treating SCID mice bearing patient-derived xenografts [21].”

Reviewer 2’s comments and our responses
1) “The authors use two way ANOVA in several instances, and this is not correct. Most of their data should be analyzed by one way ANOVA since they are not testing two independent variables.

   a. Figure 1: All data should be analyzed by one way ANOVA with multiple comparison testing. Especially, since all data is compared to saline, there is only one variable.

   b. Although some may consider a four arm experiment of no drug, drug 1, drug 2 vs drug 1 & 2 as two variables, it is really one variable: treatment. This should also be tested with one way ANOVA (either repeated measures or testing time points of interest on their own).

   c. Check all instances of two way ANOVA usage: I don't think any are appropriate.”

We had originally used two-way ANOVAs for experiments comparing the effect of treatments over time, specifying (1) time and (2) treatment as the two factors. However, we defer to the reviewer’s statistical expertise. Therefore, in accordance with the reviewer’s request, we reanalyzed the data presented in Figures 1, 2, 4 and 6 using one-way ANOVAs with Sidak’s corrections for multiple comparisons, to compare responses as measured by bioluminescence at week 5.

2) “The authors' conclusions for results presented in figure 2 are probably not substantiated by the data. To be clear, I think transparent presentation and conclusion drawn from the data is the important component here and it will not change the overall conclusion of the manuscript, but it may for this figure. Again, the two way ANOVA is not appropriate. And, more importantly, the authors claim that the smac mimetics "cooperate with doxorubicin", yet the statistics are compared to saline. Figure 1 basically demonstrates that the smac mimetics have anti-tumor effect, but the doxorubicin effect is minimal. It would follow that they would be testing whether doxorubicin + smac mimetic have better anti-tumor activity than smac mimetic alone. Thus, treatments should not be compared to saline. I suspect that the double treatment may not be better than smac mimetic alone, in the 1029H model, and this is what should be stated and discussed. This lends credence to new drugs, like smac mimetics, over standard of care.”

We reanalyzed the data using one-way ANOVAs (adjusting P values for multiple comparisons), comparing responses of saline, doxorubicin or Smac mimetics to the combination treatments at the final bioluminescence timepoint. As the reviewer suspected, combined treatment never provoked a significantly different response from GDC-0152 or LCL161, despite suppressing average tumor growth more profoundly than sole agent treatment in Figures 2B, 2C, 2D and 6A. Our gut feeling is that combined Smac mimetic plus doxorubicin treatment is more effective than Smac mimetics alone, but we recognize that these statistical analyses of these data sets do not warrant this conclusion. Therefore we have reworded text pertaining to combination treatments in the results (lines 305-309) and discussion (lines 462-463, 471-474), to acknowledge this. We have also emphasized in the discussion that additional studies will be needed to ascertain whether cooperative efficacy does occur, and whether any improved anti-tumor effect would outweigh additional toxicities (lines 479-484). We have also deleted the reference to cooperation
with doxorubicin from the title and the conclusions section of the abstract, and have amended the results portion of the abstract (lines 20-22).

3) “Readers would benefit from an explanation of what they are looking at in the PET and MRI images in figure 1C. Is this a whole mouse with part of the image representing the tumor? Is this a whole tumor? What do the colors or greyscale represent? Arrows pointing to described areas, a key and/or heat map details or cartoon would be helpful.”

The PET/MRI data are transverse images on a plane through mouse at the location of the tumor. The tumor is on the upper left, with the spine at the top and the legs at the bottom. We have added a cartoon illustrating this imaging plane (Figure 1C), and included descriptions of anatomical landmarks in the legend to Figure 1. Scale bars for PET and MRI are included in the panel, and described in the legend.

4) “As discusses above, present statistics on smac mimetic alone vs smac mimetic + doxorubicin in figure 2.”

See response to point 2

5) “The described gating in figure 3 legend does not necessarily make sense for the described cell types. F4/80 as the sole marker of macrophages is probably ok may be a stretch. Neutrophils should be positive for Ly6G. Either describe more clearly, or describe the cells by their markers. I think the overall conclusion that myeloid cells are present and contain TNFalpha will be maintained. In discussion, the authors are careful to not dwell on a particular TNFalpha-expressing cell and it may be best to present the data that way if they can re-gate to show myeloid cells as expressing TNFalpha. Or, double check that their gating is truly representing macrophages, NK cells and neutrophils.”

We have provided a more detailed account of the markers and gating strategy we used, listing the phenotypic features we attribute to each cell type within Figure 3A and B and listing markers not detected in the legend. We reanalyzed the flow cytometry data relating to the cell types containing TNFα, just comparing in revised Figure 3B the proportion of osteosarcoma/other (ie lacking the immune markers) versus immune (NK, macrophage, neutrophil) cells that do and do not express TNFα.

6) “The authors discuss statistical results in Figure 4a legend, but no statistics (*) are presented in the figure panel.”

In that case, no statistically significant differences were observed. We had previously included a statement to this effect in the legend, but the revised manuscript makes this clearer. In all figures that feature statistical tests we have now included a label defining the comparator (eg “P vs
saline”, or “P vs combo”) and have included “ns” labels for groups that were not significantly different from the comparator.

7) “Clarify how a luminescence-based viability/proliferation assay is affected by luciferase positive cells.”

We have added extra text about this into the relevant part of the results section (lines 369-372, 383-389). The CellTiter-Glo kit is designed to provide a linear relationship between cell number and ATP content over a huge range of cell densities. To accomplish this, the luciferase concentration in the reagent is very high, to prevent it becoming the rate-limiting factor in the reaction when ATP is abundant. We therefore suspect that some extra luciferase in our cells, encoded by the reporter transgene, will not affect the reaction rate and luminescence intensity. We have acknowledged that this is formally possible however, and have pointed out that in that case some of the loss of signal induced by drug toxicity may be due to a decrease in transgene-encoded luciferase, in addition to the signal decrease due to a reduction in ATP within the well. Either way, a decrease in signal would reflect cell death.

8) “It is unclear what statistical comparison is being made in figure 5c. Is it pairwise comparison of human vs mouse TNFalpha? If so, one way ANOVA cannot be used. Mann-Whitney or T test would be appropriate.”

That’s correct, we compared the effects of human versus murine TNFα, at various concentrations on the cells, either alone or with Smac mimetics. We have reworded the legend, and added a label into the figure “P human versus murine TNFα” to help readers understand this. We have reanalyzed the data using T tests with adjustments for multiple comparisons.

9) “Figure 6c is unclear as presented. I think it would be easier to interpret if the data were separated into two panels: C) KRIB-Luc cells per lung in each treatment group; D) Bioluminescence in each treatment group. Again, if doxorubicin + LCL is not better than LCL alone (or dox alone in this case), then discuss this. This would not alter the overall conclusion about smac mimetics, but may alter the conclusion about combination and risk of combination. Comparing to saline in Fig 6a is not terribly helpful..”

The bioluminescence data is provided in panel A. We added the suggested figure showing the qPCR data (new Figure 6C). We decided to also retain the panel comparing primary and metastases responses for each tumor (now panel D), as we feel readers may be interested to see this relationship. We thought it was important to test whether the drugs provoked statistically significant responses in this model, but we agree that assessing whether combined therapy had significant additional benefit was also important. Therefore we performed six statistical comparisons (using one-way ANOVA with Sidak corrections): saline versus each of the four treatments, and combination treatment versus weekly LCL161 and weekly doxorubicin.