Title: Effects of Salubrinal on Development of Osteoclasts and Osteoblasts from Bone Marrow-Derived Cells

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Author's response to reviews: see over
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Dr. Takanobu Nakase
Editor
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Dear Dr. Nakase,

We appreciate your editorial assistance and the valuable comments and suggestions from the reviewers to our manuscript (MS: 1786360012979546, Effects of Salubrinal on Development of Osteoclasts and Osteoblasts from Bone Marrow-Derived Cells) by H. Yokota, K. Hamamura, A. Chen, T.R. Dodge, N. Tanjung, A. Abedinpoor, and P. Zhang. The point-to-point responses are described in this letter, and the revised parts are highlighted in red in the revised text.

Responses to the First Reviewer

Minor Essential Revisions:

1) In Figure 2, 3, 5 and 6, authors should describe each conditions of cell culture on photographs.

   As suggested, we added description of each of the cell culture conditions in these figure legends.

2) The method of isolation of TRAP-positive cells is not clear in the sections of migration and adhesion assay. After fixation and staining, how can TRAP positive cells be isolated?

   Sorry for unclear description. Prior to the migration and adhesion assays, we evaluated the number of TRACP-positive cells that were isolated from the same sources but cultured in separate wells. Based on the results in TRACP staining, we then employed the equivalent numbers of TRACP positive cells in each of the experimental groups and conducted the migration and adhesion assays.

3) Does alfa-v-beta-3 mean integrin? Authors should mention that.

   Thank you for your comments. We changed to “αvβ3 integrin.”

4) Authors assessed osteoblastic differentiation only by ALP staining. How are osteoblastic mRNA expressions, such as osteocalcin, bone sialoprotein, Runx2?

   This manuscript describes the effects of salubrinal on RANKL-administered mice, and the development of osteoclasts and osteoblasts from bone marrow derived cells. We provided several lines of evidence that support salubrinal’s dual roles – the stimulation of osteoblastic differentiation and the inhibition of osteoclastic development. We agree that the examination of mRNA expression levels for
ostecalcin, bone sialoprotein, Runx2, etc. is a next logical step to understand the mechanism of salubrinal’s action in the development of osteoblasts. Thank you for your suggestion.

Responses to the Second Reviewer

Major issues

1. More details about methods are needed.

   a) Give some references or explanation to the concentration of salubrinal used, and also the incubation time chosen in the study.

      As suggested, we added description of the concentration of salubrinal, and the incubation time in the method section.

   b) Method for establishing RAW264.7 cells need to be given in details.

      RAW264.7 cells are an established cell line. In the revised manuscript, we added more information on this line.

   c) Give more information about lysis of RAW264.7 cells.

      Bone marrow-derived cells or RAW264.7 cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer, containing protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, Billerica, MA, USA).

   d) How do you quantify signal intensities protein?

      The bands were scanned with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA) and their intensities were quantified using Image J.

   e) Explain how do you calculate the values of mRNA?

      The relative mRNA abundance for the selected genes with respect to the level of GAPDH mRNA was expressed as a ratio of $S_{\text{treated}}/S_{\text{control}}$, where $S_{\text{treated}}$ is the mRNA level for the cells treated with salubrinal, and $S_{\text{treated}}$ is the mRNA level for control cells.

2. Results about BMD are confused.

   a) What is the meaning of analyzing the BMD in OVX-mice if only use cells from RANKL-injected mice?
Besides RANKL-injected mice, we employed OVX mice. OVX mice are more frequent used for inducing osteoporosis than RANKL administration. In order to show efficacy of salubrinal, we first employed the OVX mouse model and then moved to the RANKL injection model. We believe that the results with OVX mice strengthen the observed effects of salubrinal.

b) What values of total BMD had the RANKL-injected mice? Why you show values of ulna and humerus of BMD and not of femur or tibia?

We used the tibia and femur as a source of bone marrow derived cells, while the ulna and humerus for radiographic imaging for determination of BMD and BMC. The primary purpose of this arrangement was to reduce the number of animals involved in the study.

c) Is there any effect of the salubrinal on the growth and proliferation of the cells (HSCs, MSCs and RAW264.7) in vitro?

In response to the concentrations of salubrinal employed in this study, we did not observe significant changes in the growth and proliferation of MSCs and RAW264.7 cells.

3. Discussion is a little poor.

a) Discussion explains the role of eIF2a but does not show any results for this gene, however not explained and not provided bibliography of NFATc1, which is the gene study. I suggest rewrite the discussion of NFATc1 gene.

As suggested, we added more description of NFATc1 gene in Discussion.

b) A general lack of references and data from other authors. You may compare your work with relevant studies of others.

As suggested, we added more references and compared with other studies in the discussion section.

Minor issues

1. Background p.3: I think the sentences about bisphosphonates “… though long-term usage may cause osteonecrosis of the jawbone and fracture of the femur” is a little hard, perhaps is better to say that some bisphosphonates may be associated with an increased risk of osteonecrosis of the jawbone and atypical femur fracture.

As suggested, we changed “… though long-term usage may cause osteonecrosis of the jawbone and fracture of the femur” to “bisphosphonates may be associated with an increased risk of osteonecrosis of the jawbone and atypical femur fracture.”
2. Sometimes write TRACP and other times write TRAP.

   We changed “TRAP” to “TRACP.”

3. Results: p.12 use comma or no in the numeric results (e.g., 53,213 ± 3545).

   We corrected. Thank you.

4. Salubrinal at 2 μM reduced CFU-GM with a p=0.01 no 0.001. The authors of the manuscript should also include both limitations and strengths of the study.

   We revised the p value. Furthermore, we added description on the limitations and strengths of the current study in Discussion.

In summary, we greatly appreciated the comments and suggestions. We hope that the responses in the revision are satisfactory.

Yours sincerely,

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