Author's response to reviews

Title: Effect of troglitazone on tumor growth and pulmonary metastasis development of the mouse osteosarcoma cell line LM8

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Author's response to reviews: see over
Dear Dr. Graham,

Thank you and the reviewers for your careful consideration of the manuscript entitled "Effect of troglitazone on tumor growth and pulmonary metastasis development of the mouse osteosarcoma cell line LM8 (MS # 5681320133008800)” by J. Aizawa et al. Following the helpful comments of you and the reviewers, we have modified our manuscript and Figures.

I have added the sentence “Troglitazone is generously donated by Daiichi-Sankyo Co., Ltd., Tokyo, Japan.” in the Competing Interests Section.

We hope that you will consider this revised version suitable for publication in BMC Cancer.

Sincerely yours,

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Answers to Dr. Olivia Fromigue

1. **Abstract:** As suggested, we have mentioned the method of the determination of the microvessel density (MVD) within the tumor (p. 2, lines 19-20) and the result of the MVD in the control and TGZ group (p. 3, lines 3-4).

2. **p. 6, lines 10-11 and line 21:** As suggested, we have expressed the cell number as cells/cm² instead of cells/plate or /well.

3. **p. 6, line 23; p. 7, lines 5-6; p. 7, lines 16-17:** We have indicated that subconfluent cells were treated with or without 50 µM TGZ for 3 days.

4. **p. 13, lines 20-21:** We have changed “GW9662” to “the PPARγ antagonist GW9662”.

5. **LM8 cells in both the untreated and TGZ-treated cultures secreted the proform and active form of MMP-2. We have shown the proform of MMP-2 by arrowhead in the upper panel of Figure 3C. We have explained arrowhead in the legend of Figure 3 (p. 30, lines 17-18). Similarly, the tumors of the control and TGZ groups contained both forms of MMP-2. We have shown the proform of MMP-2 by arrowhead in the upper panel of Figure 6C. We have explained arrowhead in the legend of Figure 6 (p. 32, line 3).

6. **p. 19, 2nd paragraph and p. 20, 1st paragraph:** We have discussed about the relationship among Akt signaling, MMP-2 activity, and cell invasion and motility, previously described. We have added the literature of Yang et al. that TGZ inhibits cell motility and decreases the level of p-Akt in human ovarian carcinoma cell line, ES-2 (Ref #32) (p. 20, lines 4-5).

7. **p. 8, lines 4-19:** We performed the cell invasion and motility assays using LM8 cells, which had been previously treated with or without 50 µM TGZ for 3 days. These untreated and TGZ-treated cells were harvested by trypsinization, and suspended in DMEM containing 0.1% BSA. In the cell invasion assay, we added the same number of cells (5 × 10⁵ cells) to individual invasion chambers containing a matrigel-coated filter (lines 4-8). In the cell motility assay, we added the same number of cells (2.5 × 10⁵ cells) to individual motility chambers (lines 17-19).

8. **We used the analysis software, Diversity Database™ (v. 1.1, Toyobo Co., Ltd., Osaka, Japan) to determine relative densitometric units of bands. It is hard to determine p-Akt/Akt ratio using this software. We have mentioned the name and version of this software (p. 9, line 10).**
Answers to Dr. Avudai Maran

1. We have provided better pictures of Figures 3C and 6B. LM8 cells in both the untreated and TGZ-treated cultures secreted the proform and active form of MMP-2. We have shown the proform of MMP-2 by arrowhead in the upper panel of Figure 3C. We have explained arrowhead in the legend of Figure 3 (p. 30, lines 17-18). Similarly, the tumors of the control and TGZ groups contained both forms of MMP-2. We have shown the proform of MMP-2 by arrowhead in the upper panel of Figure 6C. We have explained arrowhead in the legend of Figure 6 (p. 32, line 3).

2. p. 19, 2nd paragraph and p. 20, 1st paragraph: We have discussed about the relationship among Akt signaling, MMP-2 activity, and cell invasion and motility in detail. We have added the literature of Yang et al. that TGZ inhibits cell motility and decreases the level of p-Akt in human ovarian carcinoma cell line, ES-2 (Ref #32) (p. 20, lines 4-5).

3. p. 22, the last paragraph: We have indicated that the PI3K-Akt signaling pathway may be a chemotherapeutic target in osteosarcoma treatment. We have added this paragraph in the Discussion section; therefore, we could not shorten this section.

4. p. 13, lines 20-21: We have changed “GW9662” to “the PPARγ antagonist GW9662”.

5. Why decreasing effect of TGZ on cell invasiveness was small is unclear. We used the BD BioCoat Matrigel™ invasion chambers with polyethylene terephthalate-filters coated with matrigel basement membrane matrix (6 wells, 8 µm pore size; BD Biosciences, Franklin Lake, NJ) and performed the invasion assay according to the manufacturer’s instructions (p. 7, lines 24-25 and p. 8, lines 1-2).

6. We have changed the presentation of the results of Western blot for Akt and p-Akt from “TGZ decreased the level of p-Akt without affecting Akt expression” to “TGZ decreased the level of p-Akt (p. 20, line 7)”. 
Answers to Dr. Tong-Chuan He

1. **p. 12, 1st paragraph:** Asai et al. reported that fillopodial and lamellipodial structures surrounding the cell surface of LM8 play a pivotal role in cell motility (Ref #16). This is the reason why we examined the morphological changes of LM8 cells. We have mentioned this (lines 3-4). TGZ induced morphological changes of LM8 cells, suggesting that TGZ-treated cells may display lower motile activity than untreated cells (lines 12-13). This is confirmed by the finding of cell motility assay.

2. **p. 14, 3rd paragraph:** We have modified the presentation of the results of cell motility assay. We have added the sentence “This low motile activity of TGZ-treated cells may result from the above-mentioned TGZ-induced cell morphological changes (lines 21-22).”

3. As suggested, we measured alkaline phosphatase (ALP) activity. Subconfluent cells were treated for 3 days with or without 50 µM TGZ, harvested in 0.6 ml solution A, sonicated briefly, and centrifuged. The ALP activity in the supernatants was measured using a kit for ALP (Wako Pure Chemical Industries Ltd., Osaka, Japan). No difference in the ALP activity between the untreated and TGZ-treated cultures was observed [the untreated cultures (n=4), 55.8 ± 1.0 nmol/min/µg DNA; the TGZ-treated cultures (n=4), 53.3 ± 1.7 nmol/min/µg DNA; p<0.01]. Based on this finding, it is hard to say that the suppression of cell growth results from the TGZ-induced differentiation of LM8 cells into osteoblasts. So, we have not added this data in the revised manuscript.

4. **p. 19, 1st paragraph:** We have mentioned the effect of GW9662 on PPARγ ligand-induced cell growth. We have changed the presentation from “These findings raise the question of whether the TGZ-induced decrease in cell proliferation was mediated by PPARγ.” to “These findings raise the question of whether the TGZ-induced decrease in cell proliferation was mediated by PPARγ, because the PPARγ ligands have antitumor activity against a wide variety of tumors in vitro [7] (lines 3-6).” In addition, we have mentioned the literature of Seargent et al. (Br J Pharmacol 2004, 143: 933-937). They reported that RGZ inhibits the growth of MDA-MB-231 cells and that GW9662 enhances rather than reverses RGZ-induced growth inhibition (lines 15-17).

5. **p. 19, 2nd paragraph and p. 20, 1st paragraph:** Whether Akt silencing renders the cells more sensitive or resistant to TGZ treatment is unclear. We have discussed about the relationship among Akt signaling, MMP-2 activity, and cell invasion and motility in detail. We have added the literature of Yang et al. that TGZ inhibits cell motility and decreases the level of p-Akt in human ovarian carcinoma cell line, ES-2 (Ref #32) (p. 20, lines 4-5).

6. **p.22, the last paragraph:** We have indicated that the PI3K-Akt signaling pathway may be a chemotherapeutic target in osteosarcoma treatment.

7. **p. 16, lines 24-25 and p.17, line 1; p. 17, lines 12-14 and 21-22:** We have mentioned the reason why we chose three proteins (MMP-2, VEGF, CD34). We have added two literatures (Ref. #26 and 27).

8. We administered TGZ according to the method of Magenta et al. (Ref. #9). They dissolved RGZ in ethanol and administered drinking water containing 100 µM RGZ to mice inoculated with LMM3 mammary tumor cells. In their case, the final ethanol concentration was 0.01%. In our case, the final ethanol concentration was 0.5% (p.10, line 12).

9. We asked Daiichi-Sankyo Co. the stability of TGZ. They have performed animal experiments using powdered diet containing TGZ. They said that the decrease in the activity of TGZ during the 7-day storage period (at room temperature) is very small (~4%). We changed drinking water every 2-3 day (p. 10, line 14).

10. We will consult about animal treatment with the local Animal Ethics Committees at the Ehime University Graduate School of Medicine and at the Ehime Prefectural University of Health Sciences, Ehime, Japan.