Author's response to reviews

Title: Oleanane triterpenoid CDDO-Me induces apoptosis in multidrug resistant osteosarcoma cells through inhibition of Stat3 pathway

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Author's response to reviews: see over
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Sabina Alam, PhD
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Dear Dr. Alam

We appreciate the constructive comments and suggestions from both you and the reviewers regarding our manuscript, “Oleanane triterpenoid CDDO-Me induces apoptosis in multidrug resistant osteosarcoma cells through inhibition of Stat3 pathway” (MS: 2018554733007574). We have addressed each of the reviewer’s concerns point by point while including additional experimental results and clarifications. We have also made changes in the manuscript as a whole. Specific responses to the reviewers’ criticisms are listed below.

Referee 1.
Reviewer: Cheryl A London

This manuscript by Ryu et al is interesting and follows on the findings of others that STAT3 activation appears to be common in osteosarcoma. In the present work, the authors investigate the potential activity of oleanane triterpenoid CDDO-Me against osteosarcoma cells. Comments are listed below.

Major Compulsory Revisions

1. The authors refer to multi-drug resistant osteosarcoma lines and the potential role of STAT3 in this process. However, data from their laboratory indicate that GADD45a expression may be responsible for this phenomenon. Is it known that STAT3 also contributes definitely to chemotherapy resistance in the MDR cell lines? How does PGP1 (expressed only in the two MDR osteo lines) affect the observed drug resistance?

**Response:** We thank the reviewer for careful review of the work. There are several mechanisms in the acquisition of MDR and GADD45a deficiency is only one of the mechanisms (Apoptosis. 2009;14:124-33). Stat3 is another protein which may play a major role in acquired multidrug resistance. Several studies have shown that Stat3 is highly activated in high-grade as well as recurrent drug resistant cancer cells (Clin Cancer Res 2006;12:5055–5063, Oncogene 2005;24:1053–1065, Cancer 2006;107:2730–2740, Cancer Res 2004;64:3550–3558). Pgp1 is a drug efflux pumps which also contributes to the acquisition of MDR (Onecogene 2003; 22:280-295, Curr Cancer Drug Targets 2003;3:1-19). In fact, the osteosarcoma multidrug resistant cell lines used in this study are associated with these changes.

2. In the methods it is stated that doxorubicin resistant cell lines were periodically
cultured in doxorubicin to confirm drug resistance; there is no indication as to how often this was done, concentrations used, etc.

**Response:** Resistant cell lines were continuously cultured in 0.1 µM doxorubicin response (Page 7, line 10).

3. In the methods (and throughout the manuscript), the authors have mischaracterized the MTT assay as a cytotoxicity assay. This test only measures cell viability, and cannot be used to directly assess cytotoxicity or actual cell numbers. This should be changed throughout the text to represent cell viability rather than cell death/growth. This is particularly relevant for the results when CDDO-Me is used in combination with doxorubicin; the investigators are not assessing whether these cells underwent apoptosis or died when using the MTT assay; they would need to use assays that directly assess apoptosis, such as flow cytometry with AnnexinV staining.

**Response:** Characterization of MTT assay has been changed in the manuscript. Additionally we have performed caspase 3/7 assay to assess apoptosis and this data has been updated in the result section (Page 12 line 7) and in figure 3.

4. The method used for assessing drug combination effects is not technically valid. To determine whether two drugs are antagonistic, additive or synergistic in their effects on cell viability, a true combination index (CI) would need to be performed.

**Response:** We thank the reviewer’s suggestions and have calculated CI. The data have been updated in the result section. (Page 14 line 20).

5. The authors should provide IC50 calculations for the CDDO-Me against the various cell lines (Figure 2).

**Response:** We appreciate the reviewer’s suggestions and have calculated the IC50. The data have been updated in the methods and results section. (Page 11 line 23), (Page 15 line 7).

6. For the responses to IL-6 in U2OS cells, the authors should demonstrate that treatment of cells with IL-6 alone results in enhanced STAT3 phosphorylation prior to the EGFP-STAT3 translocation experiments. Additionally, in Fig 3, there should be a treatment group with CDDO-Me alone. Lastly, it would be nice to see the effects of IL-6 treatment on downstream STAT3 targets (i.e., survivin, etc) and show that the CDDO-Me can prevent upregulation of these targets (i.e., is actually preventing STAT3 transcriptional activity).

7. For the drug combination experiments (Fig 6), studies should be performed with both the drug sensitive and drug resistant lines to demonstrate a true difference in the resistant vs sensitive lines.

Response: We appreciate the reviewer’s suggestions. In figure 7, we have added the MTT data of drug sensitive cell lines.

Minor Essential Revisions
1. There are several grammatical errors throughout the entire text. The manuscript should be thoroughly reviewed for spelling and grammar and all errors should be addressed. Also, the authors need to insure that the references are correct. For example, the references on page 4 for the U2OS line are incorrect.

Response: We thank the reviewer for the careful review. We have edited the manuscript accordingly.

Discretionary Revisions
1. With respect to the tumor samples used, it is assumed that these were all collected prior to neoadjuvant chemotherapy administration?

Response: These samples were all after the chemotherapy.

Referee 2.
Reviewer: Konstantin Leskov

Reviewer’s report:
The article by Keinosuke Ryu et al., titled “Oleanane triterpenoid CDDO-Me induces apoptosis in multidrug resistant osteosarcoma cells through inhibition of Stat3 pathway” is the first to date to explore the effect of CDDO-Me on osteosarcoma cell lines. The paper essentially re-iterates the results found in ovarian, breast and lung cancer treated with CDDO-Me. The changes in Stat3 phosphorylation after treatment with CDDO-Me had been previously reported in other types of cancer. The authors of this paper are the first to report such changes in osteosarcoma.
The methods presented are adequate to demonstrate the alterations of Stat3 status in osteosarcoma.
The paper in general is a bit light on data.

Only the MTT assay was used to estimate compound toxicity. Colony forming assay would be a good compliment to MTT to assess reproductive cell death in addition to metabolic cell death and apoptosis.

**Response:** We have utilized caspase 3/7 assay to compliment MTT in assessing cell death and apoptosis and this data has been updated in the result section (Page12 line7) and in figure 3.

No animal data was presented. The sensitivity of osteosarcoma xenografts to CDDO-Me would strengthen author’s point of usefulness of this compound in clinic.

**Response:** We agree with the reviewer’s suggestions. We are currently undergoing animal study, and it is our decision to submit this article without in vivo results on this occasion.

The measurement of IC50 of CDDO-Me was mentioned in methods, but I did not find the actual values of IC50 for MDR and non-MDR osteosarcoma cells anywhere else in the paper. Since no IC50 was shown, the reason for using specific doses of the compound is unclear. In particular, using 1 μM of CDDO-Me in the nuclear translocation experiment is unclear. 1 μM seems a bit high to be clinically relevant. I wonder if 0.1-0.2 μM would have the same effect.

The writing in general is acceptable; the conclusions drawn are adequate for the experimental results.

**Response:** We agree with the reviewer and have calculated IC50 and added the data in the methods and result section (Page11 line23), (Page15 line7). From the results of IC50, we believe that the concentration of CDDO-Me used in each experiment is adequate.

Referee 3.
Reviewer: Jiayuh Lin

**Reviewer's report:**
In this manuscript, authors examined the inhibition of Stat3 pathway Oleanane triterpenoid CDDO-Me in multidrug resistant human osteosarcoma cells.

Major Compulsory Revisions:

1. In figure 5, it will be necessary to include data on cleaved PARP as in figure 2B or cleaved caspase-3.

**Response:** We agree with the reviewer and have added additional data of PARP in figure 6 (Time-dependent inhibition) as in figure 2B.
Minor Essential Revisions:

1. In Figures 2-6, what is (are) the serum concentration being used?

**Response:** The serum concentrations used in these figures are all 10%.

2. In Figure 3, it will be helpful to include a DAPI staining.

**Response:** We have included the result of hoechst staining. (Figure 3).

Discretionary Revisions:

1. In Figure 3, it will be helpful to include a Western blot showing P-STAT3 is induced by IL-6 and inhibited by 1 microM of CDDO-Me.


2. Are other targets of CDDO-Me (besides STAT3) may be associated with the induction of apoptosis in these osteosarcoma cells?

**Response:** CDDO-Me inhibit not only Stat3 pathway, but also other targets as well (Cancer Res 2008; 15: 2927-33, J Neurooncol 2007; 84: 147-57. J Exp Ther Onco; 2008; 7: 31-9). The exact mechanism downstream of Stat3 has not been completely elucidated to date.

We hope these changes will now make this manuscript acceptable for publication in BMC Cancer.

Respectfully,

Zhenfeng Duan