Author’s response to reviews

Title: Oridonin Stabilizes Retinoic Acid Receptor Alpha Through ROS-activated NF-kappa B Signaling

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Author’s response to reviews: see over
Dear Ms. Cherry Battad,

Thank you very much for your email of January 5, 2015, regarding my manuscript entitled *Oridonin Stabilizes Retinoic Acid Receptor Alpha Through ROS-activated NF-κB Signaling* (ID: 1942176560136099). Your comments and those of the reviewers were highly insightful and enabled us to greatly improve the quality of our manuscript. In the following pages are our point-by-point responses to each of the comments of the reviewers as well as to your own comments. We hope that the revisions in the manuscript and our accompanying responses will be sufficient to make our manuscript suitable for publication in *BMC Cancer*. We are willing to modify the manuscript a second time if more changes are recommended by the reviewers and/or the editor.

We look forward to hearing from you at your earliest convenience.

Yours sincerely,

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Responses to the comments of Reviewer 1:

Comment 1: Major Compulsory Revisions:

(1) INTRODUCTION. More information about oridonin and its potential clinical utility are needed;

Response: As suggested by the reviewer, we have added more information about oridonin and its potential clinical utility (please see page 5, lines 96-104).

(2) RESULTS (paragraph “suppression of oridonin-induced RARα stability by chemical inhibition of NF-kB signaling”). To definitely rule out the involvement of activation of JNK, p38, and ERK in the oridonin-induced RAR alpha stabilization, the activity and specificity of the kinase inhibitors SP600125, SB203580, and PD98059 need to be tested and showed in Fig. 4. In addition, the description of the results depicted in Fig. 4 B (pg 13 lines 272-279) is not adequately illustrated and needs to be re-written more clearly.

Response: Thank you for the suggestions. We have added the experiment assessing the activity and specificity of the three kinase inhibitors suggested. The results are shown in Figure 4 as the reviewer suggested. In addition, the description of the results depicted in Fig. 4B have been re-written to be more clear (please see page 13-14, lines 300-305).

(3) DISCUSSION. In this section the results obtained in the study are nicely summarized but poorly discussed. The clinical relevance of RARα regulation in relationship with the ability of oridonin to modulate its stability involving NF-κB/TNFα signaling would need to be addressed more in detail.

Response: We thank the reviewer for this critical suggestion. The discussion part has been re-written (please see page 15-18). We hope the reviewer is satisfied with our improved and additional discussion. As suggested by the reviewer, we have addressed
in more detail the clinical relevance of RARα regulation with the ability of oridonin to modulate its stability via NF-κB/TNFα signaling (please see lines 349-372).

Comment 2: Minor Essential Revisions:

(1) ABSTRACT. It would be helpful to read in the background that oridonin is a natural diterpenoid isolated from Rabdosia rubescens and that the compound regulates RARα expression by increasing its protein level. In addition, there is no mention in the Methods of the primary leukemia cells used in the study;

Response: As suggested by the reviewer, we have added a brief description of oridonin and its ability to increase RARα protein levels to the Background section (please see page 2, line 30-32). In addition, we have also added the use of primary leukemia cells to the Methods section (please see page 2, line 40-41).

(2) METHODS. In the cell culture details there is no mention of COS-7 cells, NB4 GFP and NB4/GFP MAD cells, 293T cells, and primary leukemic cells. In “Reagents and antibodies” paragraph the sentence at lines 109-110 is incomplete. In “Plasmid construction and transfection” paragraph I could not find the description of RARα expressing plasmid construction (or related reference) as well as the description the plasmid used to generate NB4/GFP MAD cells. In the paragraph entitled “Patient samples” the last sentence is incomplete.

Response: Thank you very much for your comments. We apologize for the error. As suggested by the reviewer, we have added details of COS-7 cells, NB4 GFP and NB4/GFP MAD cells, 293T cells, and primary leukemic cells in the cell culture section of the Methods (please see page 5-6, line 110-117). We have also completed the two sentences in “Reagents and antibodies” (please see page 6, line 123-124) and “Patient samples” paragraphs (please see page 10, line 212-214). Finally, we have described the construction of the RARα expressing plasmid (please see page 9, line 194-198) and added relevant information regarding generation of NB4/GFP and
NB4/GFP-MAD cells (please see page 5-6, line 110-112).

(3) RESULTS. In the text describing FIG. 3B (page 12) there is no mention of pERK1/ERK2 decrease at 12h after oridonin treatment.

Response: This is a great point that we overlooked. As rightfully suggested by the reviewer, we have added a description of the change of pERK1/ERK2 levels, which rapidly increased 6 h after oridonin treatment, and then declined after 12 h (please see page 13, lines 280-282).

(4) FIGURES. Fig 2H (right panel) should firstly show the expression levels of catalase and then RAR alpha, RXR alpha and beta-actin.

Response: As suggested by the reviewer, we have re-arranged Fig. 2H (right panel).

Comment 3: Discretionary Revisions:
(1) For consequentiality in result description I suggest to build FIG.1 following an alternative panel order: 1) B (western of primary leukemic cells), 2) C (clinical information), 3) A (western blot in NB4 cells), 4) (mRNA analysis of the same samples showed in A), 5) E and 6) F.

Response: We agree and in accordance with your comments, we have re-arranged Fig. 1.

Comment 4:
Quality of written English: Needs some language corrections before being published.

Response: As suggested by the reviewer, we have carefully made modifications throughout the original manuscript. Furthermore, to thoroughly improve the presentation of our work, the revised manuscript has been edited and proofread by a native-English-speaking science editor from Edanz Group Ltd. Because of the number of changes made as part of this editorial process, these changes are not marked in the
text.

Comment 5:
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Response: Regarding the statistical analysis, we chose the Student’s $t$-test to analyze the results, which were derived from at least three independent experiments and expressed as the mean ± standard deviation.

Responses to the comments of Reviewer 2:
Comment 1: While the topic about biological mechanism of oridonin is interesting, the manuscript is poorly written. The presentation is tough to read and understand with numerous language issues.
Response: We apologize that our manuscript was poorly written and we are grateful to the editors and reviewers for their consideration in assessing our study. As suggested by the reviewer, we have made careful revisions throughout the original manuscript. Furthermore, to thoroughly improve the presentation of our work, the revised manuscript has been edited and proofread by a native-English-speaking science editor from Edanz Group Ltd. Because of the number of changes made as part of this editorial process, these changes are not marked in the text. We hope the reviewer is satisfied with the presentation of the revised manuscript; however, we are willing to pursue further modifications if the reviewer so requests.

Comment 2: In the section of background, the authors failed to provide a brief description of oridonin and its reported mechanisms in the research background. Many important publications in the field could be cited. (Cell Res, 2007, 17, 274; Autophagy, 2009, 5, 430; Blood, 2007, 109, 3441; J Surg Res. 2014, 190, 55-63; J Med Chem. 2013, 56, 8814-25; Cancer Res, 2014, 74, 4409; J Med Chem. 2013, 56, 5048-58.)
Response: We thank the reviewer for this valuable comment. We have added a brief description of oridonin and its reported mechanisms of action as well as potential clinical utility (please see page 5, lines 96-106). Several important publications have been added to the reference list and compiled according to the journal format (please see page 20-21, references 16-22).

Comment 3: The authors previously reported that oridonin induced the apoptosis of human acute promyelocytic leukemia cell lines via ROS pathway (Int. Jnl. Lab. Hem., 2010, 32, e114). In this manuscript, the authors put too much attention to present and discuss the same/similar findings again, and it is thus redundant.

Response: As mentioned in this manuscript, our research group previously reported that oridonin could induce ROS-initiated apoptosis and enhance ATRA-induced differentiation in APL cells. More interestingly, we noticed that the differentiation-enhancing effect of oridonin was accompanied by increased levels of RARα protein. However, the underlying mechanism of RARα accumulation was not fully illustrated at that time. Besides, in that study, our research group focused on the effect of ROS on oridonin-induced apoptosis, not on the RARα protein accumulation or differentiation. In this work, we further investigated the mechanisms underlying oridonin-stabilized RARα protein. Fortunately, after 2 years of study, we found that oridonin also increased RARα abundance in primary leukemia cells and increased ectopically expressed RARα protein. The stabilized RARα showed transcriptional activity in the presence of its ligand, ATRA. Most importantly, we demonstrated that the moderate production of ROS induced by oridonin was able to activate the NF-kB signaling pathway and cause nuclear translocation of p65, which is responsible for oridonin-induced stabilization of RARα. From our point of view, the results presented in this manuscript are a progression from our previous study. To clearly present our novel findings, we have re-written a large section of the Discussion (please see pages 15-18). We hope the reviewer is satisfied with our improved and additional discussion.
If the reviewer feels there are still improvements needed, we would appreciate their suggestions and would try our best to accommodate them.

**Responses to the comments of Reviewer 3:**

**Comment:** Minor Essential Revisions

The authors should add in the manuscript the data about only the DMSO, that was used as vehicle for oridonin. Should be clear that DMSO did not induces effects.

**Response:** Thank you very much for your comments. Actually, when cells were treated by any reagent, the matching concentration of vehicle was used in the control and the final concentration of DMSO was kept at or below 0.1% in all experiments. As suggested by the reviewer, we have added this information to the revised manuscript (please see page 6, lines 127-129).