Author's response to reviews

Title: Curcumin-induced HDAC inhibition and attenuation of medulloblastoma growth in vitro and in vivo

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Author's response to reviews: see over
Curcumin-induced HDAC inhibition and attenuation of medulloblastoma growth in vitro and in vivo, Lee et al.

We thank the reviewers for their thoughtful comments on our manuscript and we feel that addressing their comments has significantly improved the quality of the manuscript. All changes are underlined in the revised version.

Reviewer: Caroline Saucier

1. I concur with the authors that curcumin treatment reduces HDAC activity. However, to conclude that curcumin-mediated reduction in HDAC activity and anti-tumorigenic effects is mainly caused by decreasing specifically HDAC4 expression is premature and a bit misleading. In fact, HDAC2 and HDAC7 appears to be reduced, particularly at earlier timepoints (Fig. 5B). Likewise, HDAC2 protein level is also reduced in D283 and D341 medulloblastoma cell lines treated for 24 hrs with curcumin (Supplemental Fig. 2). While the levels of HDAC activity and HDAC4 protein were measured as early as 3hr after curcumin treatment, the protein levels of the others HDAC isoforms were assessed at much later timepoint (15 and 24 hr). Measuring the expression levels of the other HDAC isoforms at earlier time point (at 3 hrs) might be of value.

We agree with this reviewer that we do not present enough evidence to make this statement and have reworded it accordingly in the abstract and the discussion section. As requested we have determined the expression levels of other HDAC isoforms such as HDAC5, 6, and 7 after 3 hours of curcumin treatment which are now included in the revised Fig. 5B. While HDAC4 showed a substantial reduction at this early timepoint, we did not find such a reduction in other isoforms. We also included the quantification data for the immunoblots of D283 and D341 cells in Suppl. Fig. 2 further confirming that the changes in HDAC4 expression are more substantial than these of other isoforms.

2. Likewise, it is a bit misleading to conclude that curcumin treatment reduced phosphorylation of HDAC4 in medulloblastoma cell lines based on the data shown in Fig. 5, where multiple bands were detected. The phosphor-specific HDAC antibody used recognized not only phosphorylated HDAC4, but as well as the HDAC5 and 7 isoforms, which all have relatively similar MW (120-140 kDa). Hence, I suggest the authors to tone down the claim that curcumin effects in medulloblastoma cells are mediated by reduced HDAC4 expression and activity within the abstract and the discussion.

We agree and have reworded our statements in the abstract and discussion sections. The changes are underlined on pages 2 and 18 in this revised manuscript.

3. The authors propose that HDAC inhibition in curcumin-treated cells contributes to the induction of apoptosis rather than being a byproduct of apoptosis. The authors should attempt to validate this hypothesis by measuring the HDAC activity and HDAC4 (and others HDAC isoforms) protein levels in cells treated with z-VAD-fmk, an inhibitor of caspases, which seemingly is blocking curcumin-
induced apoptosis (data not shown). Demonstrating that HDAC activity and HDAC4 protein levels are still reduced in presence of caspases inhibition would provide support for this hypothesis.

We agree and have now included new data showing that inhibition of caspase-3 with z-VAD-FMK did not prevent the reduction in HDAC4 expression (new Suppl. Fig. 4A) or HDAC activity (new Suppl. Fig. 4B) supporting our hypothesis that loss of HDAC4 expression or reduced HDAC activity is not just a byproduct of apoptosis in these cells.

4. Fig 1C, please provide protein loading control.

We have now included an immunoblot of α-tubulin as loading control in Fig. 1C.

5. Fig 2B, for the quantitative analysis of cell cycle profiles, please provide statistics.

In the quantitative analysis of the cell cycle profiles we have now included the error bars for the mean +/- SD of three independent experiments.

6. To facilitate interpretation, please indicate the MW standards for each immunoblot analysis and the expected MW of HDAC protein isoforms analyzed.

We apologize for the omission of the MW standards. These are now included for all the immunoblots throughout the manuscript.

7. The authors should discuss the possible mechanisms (e.g.: mRNA and/protein stability) by which curcumin mediates loss in HDAC4 protein (and of other HDAC protein isoforms).

We have included a statement that we do not know the exact molecular mechanisms in the discussion section. These are studies that are ongoing in our laboratory at present and will be submitted for publication upon completion.

8. In supplemental Fig. 3, curcumin seems to induce the appearance of an HDAC phosphorylated species in the nucleus. The nature of this HDAC phosphorylated protein should be discussed.

We understand the concerns of this reviewer. However, we believe that the appearance of a phosphorylated band in the nuclear fractions upon curcumin treatment is a non-specific signal. Indeed, the antibody data sheet for the phospho-specific antibody for HDAC4/5/7 from Cell Signaling reports a non-specific cross-reactive band of about 80 kDa which would be in agreement with the observed band in Supplemental Fig. 3. In addition, the MW weights for HDACs 4, 5, and 7 are ~119, 121, and 100 kDa respectively. Our immunoblots with HDAC-specific antibodies (Fig. 5B, C) clearly indicate that these isoforms migrate between the MW markers of 100 and 150 kDa. We did not detect any significant bands of this MW in the nuclear fractions.
9. Please, provide a brief description of the Smo/Smo mice models.
We have included more information about the Smo/Smo model on page 16.

10. Fig. 1D, please indicate in the legend the duration of curcumin treatment.
We included the duration of treatment in the figure legend (16 hours).

11. Fig. 4A, Fig. 5A & D, please indicate in the text and legend the time of curcumin treatment.
In Fig. 4A cells were treated for 3 hours and in Figs. 5A and D for 6 hours. This information is now included in the text and the legend.

12. In Fig. 6A and its legend, please indicate when curcumin treatment was initiated.
This information is now included in the Fig. 6A (arrow) and its legend.

13. Page 5, line 21, “results. For” instead of “results, For”
We have changed the punctuation.

14. Page 7, 2 paragraph, line 8, space before Alexa.
The space is now included.

We included the comma.

16. Page 21, line 11, add a point after [10].
The point was added.

17. Page 22, “Curcumin has been used.....clinical trials in adults.” And “No adverse.....reported so far.” Please add references.
We have included two new references as requested.
Reviewer: Anat Erdreich-Epstein

1. Results: Second paragraph, authors describe that curcumin-induced apoptosis was inhibited by z-VAD-fmk, but do not show the data: this needs to be shown.

We agree with this reviewer and have now included these data as Supplementary Fig. 4.

2. Results: A number of western blots are lacking a panel for a housekeeping gene to ensure equal sample loading (e.g., fig 1C, fig 5B, Suppl fig 3). Additionally, when examining phosphorylation of proteins it is necessary to show the total amount of the protein on the same blot (e.g., Fig 5C, HDAC4/5/7 phosphorylation requires panel of the same blot probed for these HDACs).

We apologize for the omission of these blots and have now included loading controls in Figs. 1C (α-tubulin) and 5B (GAPDH), and Suppl Fig. 3 (HDAC5). We also included immunoblots for the total amounts of HDAC4, 5, and 7 and a GAPDH blot as loading control in Fig. 5C.

3. Results: In several figures authors have shown measures of apoptosis (PARP and caspase 3 cleavage etc.). However, in Figure 2A the panels do not show any accumulation of cells in the sub G1/G0 phase, even at 24 hrs. How do the authors reconcile these data?

Since submission of the previous version of this manuscript we have acquired a new Accuri C6 flow cytometer. We have repeated our cell cycle analysis and included the sub G0/G1 gates (revised Fig. 2). We found accumulation of cells in sub G0/G1 after 24 hours of treatment with curcumin which is consistent with the PARP and cleaved caspase-3 immunoblots.

4. Results: bar graphs should represent means of at least three independent experiments, and should include error bars to demonstrate the variability between experiments.

We agree and have now included bars in the revised Fig. 2.

5. Similarly, experiments shown an effect in a microscopic field need to have an accompanying panel that quantifies the effect in a number of fields over at three separate experiments and provides the means and error bars. Preferentially, these quantifications should be done by at least two independent blinded observers.

We agree. We have now quantified the number of abnormal mitotic spindles in curcumin-treated cells. These data are now included in Fig. 3.

6. Results: Demonstration of an anti-tumor effect of curcumin in two in vivo models is the major strength of this manuscript. However, the p-values in Fig 6A (subcutaneous tumor model) are all >0.05. The authors used t-tests to compare
tumor measurements at three time points to tumor size in the corresponding control time points, which may not be the optimal method for assessing these differences. Consultation with a statistician will likely permit use of a more efficient (hence more powerful) statistical analysis for this experiment, and thus better evaluate the differences in tumor growth.

We tried different statistical analyses including a two-way ANOVA test but the p-value did not change significantly. We observed some obvious outliers inside each group, but decided to include them because 1) we were not sure the judgment is unbiased and 2) the number per group was becoming too small. While preparing the revised version of this manuscript, we undertook an additional xenograft study with a similar scheme. The results are now included as a new Fig. 6A and showed strong p-values (P<0.0001 with both t-test and two-way ANOVA). We have now included Fig. 6A of the previous submission as a new Supplemental Figure 5.

7. Discussion: Authors state in the first paragraph that they have demonstrated that curcumin induces apoptosis in medulloblastoma cells “BY” reducing HDAC4 etc. However, although they showed that both apoptosis and reduced HDAC4 do occur following curcumin treatment, they have not demonstrated that the HDAC4 decrease mediates the apoptosis. In the third paragraph of Discussion they further elaborate on this potential causal relationship but do not show data to support it. Addition of mechanistic experiments to examine the causal relationship between curcumin, HDAC4 and apoptosis will significantly enhance this manuscript. Alternatively, they should amend the language in the relevant sections.

We agree with this reviewer that we do not present enough evidence to make this statement and have reworded it accordingly in the abstract and the discussion section. We have also included new data that inhibition of caspase-3 with z-VAQD-FMK did not prevent the reduction in HDAC4 (new Suppl. Fig. 4) supporting our hypothesis that loss of HDAC4 expression is not just a byproduct of apoptosis. In addition, while we are in the process of performing more mechanistic experiments to examine causal relationship between curcumin, HDAC4 and apoptosis are required we feel that this is beyond the scope of this manuscript. The results obtained from these experiments will be submitted for publication in another manuscript.

8. Background: Last sentence in second paragraph of Background is not clear.

We have changed this sentence.

9. Background: Please add references to support that curcumin crosses the BBB (first sentence in third paragraph in Background). Also, last sentence in that paragraph implies that the BBB is “one of the major obstacles for chemotherapy in pediatric brain tumors”, ignoring the fact that although some drugs can not cross the BBB, many chemotherapy agents sufficiently enter the CNS and are in fact used in therapy of medulloblastoma (e.g., CCNU, cisplatin, etoposide, vincristine, cyclophosphamide, thiotepa, carboplatin). Please adjust the language.
We now included references that curcumin can cross the blood brain barrier and removed the statement of “one of the major obstacles...”.

10. Background: In the first paragraph of the Background section, the authors should choose references that directly address the statements they provide when introducing medulloblastoma incidence, prognosis and treatment outcome. In the present version the two first references address the points they seek to support only indirectly or in quoting other references.

We agree and included an appropriate reference.

11. In Methods, when describing preparation of lysates for the various methods (in vivo, HDAC, immunoblotting), authors indicate both 1% and 0.1% Triton X-100: please verify that this variation is not due to a typographical error.

We are sorry for the confusion. It was a typographical error, 0.1% Triton X-100 is the correct concentration.

12. Results: in the first paragraph authors state that the morphological changes (shrinking, rounding, detachment) ‘indicate’ that curcumin induces cell death. Although such morphological changes can accompany cell death, they do not definitively indicate its presence.

We changed that statement to “suggesting that curcumin might induce”.

13. Results: third paragraph first/second line: there is no need to explain to readers in the cancer field that carcinoma is a cancer derived from epithelial cells.

We agree and removed the explanation.

14. Discussion: Parts of the discussion are repetitious and should be significantly trimmed, especially in sections that have only background, with little direct connection to the work presented. Additionally, all but the last two sentences in “Conclusions” consist of only background that has already been presented and should be omitted.

We agree with this reviewer and have significantly shortened the discussion and conclusion section.