Author's response to reviews

Title: Evaluation of a curcumin analog as an anti-cancer agent inducing ER stress-mediated apoptosis in non-small cell lung cancer cells

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Author’s response to reviews: see over
Dear Dr Francois Vallette and Ms Cherry Battad
Journal Editorial Office
BioMed Central

Thank you so much for giving us the opportunity to revise our manuscript with the title “Evaluation of a curcumin analog as an anti-cancer agent inducing ER stress-mediated apoptosis in non-small cell lung cancer cells (6082800539531192 in BMC Cancer)”. We also really appreciate two reviewers for their insightful comments and constructive suggestions. Accordingly, we have substantially revised our manuscript. The point-to-point responses to each reviewer comments are provided as a separate file “Response to Reviewers”.

Especially, in the revised manuscript,

1) a human lung (bronchial) epithelial cell line, BEAS-2B, was used as a comparison, replacing the fibroblast MRC-5.

2) we supplemented a real time cell function analysis in H460 cells with or without B82 treatment to determine the time-course effects of B82.

3) we found that B82 could not induced CHOP expression in BEAS-2B cells.

4) we added the test of several else pro-apoptotic markers in B82-treated H460 cells.

5) we highlighted the change with blue font.

We are looking forward to the positive response. Thank you for your further consideration.

Best regards

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Response to Reviewers

“Discovery and identification of new non-ATP competitive FGFR1 inhibitors with therapeutic potential on non-small-cell lung cancer (CAN-D-13-01070)”

Reviewer #1-1:
There are some grammatical and spelling errors, please correct.

Response: We have smoothed our English and refined the grammar carefully.

Reviewer #1-2: On page 10, last line. authors mention that "siRNA reversed the effect of B82...). This is not correct, if at all it reduces the effect of B82

Response: Revised.

Reviewer #1-3: Do normal non-cancerous cells show any sign of ER stress? I would like this to be discussed as how cancer cells are vulnerable to ER stress

Response: Thanks. In revision, we used a human lung (bronchial) epithelial cell line, BEAS-2B, as a comparison control. The new Figure 2D showed a significantly higher IC_{50} value of B82 against BEAS-2B cell growth than H460 cells. The new Figure 3C showed B82 at the same concentrations could not induce the expression of CHOP in BEAS-2B cells, suggesting a ER stress-activating selectivity of B82 against cancer cells.

Reviewer #2-1: The siRNA CHOP only attenuated the apoptosis in the Fig. 3 but not completely abolished. What is the possible explanation on these results? Is there any possible of others apoptosis-mediating pathways?
**Response:** Firstly, the siRNA CHOP could not completely wipe out the ER stress activation. Secondly, the leading curcumin has been reported to exert anticancer effects by multitargeting mechanisms. Indeed, the siRNA CHOP only partly attenuated the apoptosis in H460 cells. We agree the reviewer’s comment that there may be other apoptosis-mediating pathways in B82-treated cells. Caspase-3 activation and p53 phosphorylation also play important roles in mitochondria-mediated apoptotic pathway. Therefore, we declaimed that B82-induced cell apoptosis is, at least partly, mediated by CHOP. Although this work only focuses on the ER stress-mediated apoptosis, further studies in our lab are necessary to establish such notions. In the revised manuscript, we have discussed this in Page 11.

**Reviewer #2-2:** The effect of curcumin and its analog on cancer cells apoptosis either by ER-stress or others have been intensively investigated. The authors should do more work on reviewing and added more informative information in the introduction as well as discussion parts. Also, the authors are asked to provide the explanation regarding the novelty.

**Response:** Thanks for this suggestion. We have added more information on reviewing the anti-cancer effects of curcumin and its analogs in the Background as well as the Discussion part. Also, we provided more explanation regarding the novelty of our work in discussion through the comparison with previously reported curcumin analogs.

**Reviewer #2-3:** The results from Fig. 2 indicated that only late apoptosis increased in a dose-dependent manner. Please discuss why such compound did not alter the early apoptosis? Is it possible to decrease the time of the assay to assess only the early apoptosis?

**Response:** Firstly, although the Annexin V-positive early apoptosis in Figure 2B and 2C is not as evident as the late apoptosis, the comparisons of early apoptosis in B82-treated groups with that in DMSO-control group are statistically significant. Cells treated with B82 at three concentrations appeared early apoptosis in a dose-dependent manner, except for a slight deviation in B82 (5µM)-treated group. Secondly, both this comment and the following one raised the question that the time points for apoptotic detections including MTT and flow cytometer are different. Therefore, in revision, we supplemented a dynamic monitoring of H460 cell proliferation with or without B82 using the real time cell electric sensor assay system (RT-CES™ System). Inhibitory effects of B82 at 2.5 and 10µM on the proliferation of H460 cells on laminin-coated plates were tested using the RT-CES system. H460 cells were continuously monitored up to 96h. At the time point of 40h, B82 at 2.5 or 10µM and vehicle control DMSO were added into the corresponding wells. As shown in Figure 2A, B82 treatment strongly suppressed the proliferation of H460 cells in time-course manner. About 10-16h after B82 addition, H460 cells were undergoing death or apoptosis.
Reviewer #2-4: The IC50 evaluation was carried out by incubating the cells with compounds for 72 h; however, the Annexin V/PI assay used 12 h for such treatments. It would be more precise if the authors could provide MTT results by the same treated condition. Also, at 12 h late apoptosis could be detected as shown in Fig. 2; what will happen if the cells were further exposed to the compound for 48 h more.

Response: Please see the response to the comment of Reviewer #2-3. I think that a 48-h MTT assay, plus the present 12-h MTT assay, may not elucidate the whole time course. However, it is really difficult to carry out a series of MTT assays in different time points. In revision, we provided a real time cell assay during 0h-96h. The RT-CES is a convenient way to continuously determine cell number and cell activity and may offer a full-range detection of B82-induced effects. Figure 2A showed a continuously change of H460 cells during 0h-56h after B82 treatment.

Reviewer #2-5: What is the final concentration of DMSO used in the experiment? Fig. 2 clearly showed that vehicle alone can cause apoptosis?

Response: In in vitro experiments, the final concentration of DMSO in cultural medium is 0.1%. This concentration of DMSO has been demonstrated to show no effect on cell growth. Figure 2A and Figure 3 also showed DMSO did not affect cell function and apoptotic markers. Figure 2 may showed a relatively high background in DMSO vehicle group caused by the flow cytometer operation. Importantly, the pro-apoptotic results in other groups were all normalized with DMSO vehicle group.

Reviewer #2-6: Again the time for MTT assay in Fig 3 is not along with other experiments

Response: According to the process of ERAD and our previous data, we detected the different markers in ER stress-apoptosis pathway at different time. GRP78, ATF-4, and XBP-1 in initiation phase of ERAD were detected at 3h-6h after treatment; CHOP in commitment phase was detected at 6-12h after treatment; while caspase in execution phase was detected at 24h. Then, the MTT assay was carried out at 48h after treatment.
Reviewer #2-7: Other marker for apoptosis should be addressed.

Response: Thanks for this advice. We supplemented the test of other apoptotic markers in revision and showed the results in Supporting Information. As shown in Figure S1, B82 at 10µM could increase the expression of cleaved-PARP (a downstream protein of caspase-3), P53, and Bax. These data confirmed that B82 treatment for 24h induced apoptosis in H460 cells. The results were added in the revised manuscript.

![Figure S1](image-url)