Author's response to reviews

Title: Curcumin potentiates antitumor activity of 5-fluorouracil in a 3D alginate tumor microenvironment of colorectal cancer

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Revision/Rebuttal Notes of BMC Cancer
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Title: Curcumin potentiates antitumor activity of 5-fluorouracil in a 3D alginate tumor microenvironment of colorectal cancer

Authors: Mehdi Shakibaie, Patricia Kraehe, Bastian Popper, Parviz Shayan, Ajay Goel and Constanze Buhrmann

Response to comments of the reviewers:

Reviewer #1: It is an excellent paper and deserves to be published in BMC Cancer, as it demonstrates that alginate can provide an ideal tumor environment and curcumin potentiates and chemosensitizes the HCT116 cells to 5-FU based chemotherapy and thus useful in overcoming the drug resistance problem in CRC treatment. However, the authors may address certain minor points and it may be pointed out that they need not always have to agree with the reviewer’s view point.

We are grateful to the reviewer #1 for the positive and highly constructive remarks on our manuscript. This reviewer clearly understands the field and the specific aims and scope of our research. In the following pages we provide detailed responses to the minor comments made by the reviewer.

Specific comments:

1. Cell lines and culture: Page 6: How 5-FU resistant cell lines generated and characterised in terms of malignancy. Give references of previous publications.
   Response: Thank you. We followed the suggestion of the reviewer and this has been corrected in the revised version of the manuscript.

2. How are the combination doses arrived of 5-FU + curcumin. Is there any
preliminary observation.
Response: Thank you. We followed the suggestion of the reviewers and this has been corrected in the revised version of the manuscript.

3. Invasion assay: Page 7: Invasion through matrigel matrix chamber could have been performed (Naksgawa et al. 2005, Carcinogenesis. 26, 1044) for metastasis.
Response: Thank you. Actually, the aim of the paper was to employ and represent a well-established in vitro model (alginate tumor microenvironment as a 3D-scafold) that mimics in vivo processes of invasion environment (like ECM in vivo) during metastasis in well controlled and standardized conditions.

4. Western Blot: Page 8: What is meant by semiquantitative evaluation. Are the values arrived with Image J software and also the values do not seem to be normalised with #-actin.
Response: Thank you. We followed the suggestion of the reviewers and this has been corrected in the revised version of the manuscript.

5. Apoptotic cell death: Page 9: FACS analysis could have been done. For eg. With Annexin V/PI or with apoptotic bleb assay.
Response: The reviewer is correct in pointing out that it would be better instead of TEM, we could use FACS scan for analysing Annexin V/PI Expression. However, we do not have these assays established in our laboratories at present. However, we appreciate the reviewer’s suggestion, and will plan to establish these assays in our laboratories for future studies. Nevertheless, we have established already this method to quantify the apoptotic cells under the electron microscope and have published this often in the past. Taken together, Our group has many years of experience with these methods, so we can be confident in saying that our results are indeed reproducible.

6. MTT assay: Page 9: The last sentence “This experiment was repeated 3 times........ etc.” can be deleted. It is already mentioned in the legend to the figure.
Response: Thank you. We agree with the reviewer and this issue has been dealt with in the revised version.

7. Results: Page 10: A suitable picture of the colonosphere may be added or if published earlier, the reference may be given.
Response: We believe that our current manuscript already contains quite comprehensive suitable picture of the colonosphere (light microscopy: Fig. 1; electron microscopy: Fig. 3).

8. Page 13: Along with CXCR4, MMP-9 and NF-#B, the angiogenic factors could have been studied such as VEGF/VEGFR2, since angiogenesis is an integral part of invasion and metastasis.
Response: The reviewer is quite correct. We plan to include the areas that you
suggested in your comments above in a new article for another journal and we hope to be able to nominate you as a reviewer.

9. Page 14: Gelatin zymography of MMP-9 could have been done. Also, beside MMP-9, MMP-2 could have been added.
Response: A: The reviewer is quite correct in pointing out that Gelatin zymography of MMP-9 could have been done. However, we do not have this assay established in our laboratories at present. We need to invest a considerable amount of effort to establish this assay in our laboratories for future studies.
Response: B: We plan to include the expression of MMP-2 that you suggested in your comments above in a new article, which is in preparation.

10. Page 15: Similar cytotoxic profile and apoptosis induced by 5-FU and curcumin in a dose dependent manner (not shown), such data can be put as supplementary.
Response: Actually, we have not shown the electron microscopic pictures from HCT116R cells for space reason. But we have already included in Fig. 7C statistical results of ultrastructural and morphological features of apoptotic cell death of HCT16 and HCT116R. For that reason, we did not see the necessity to show the similar pictures.

11. Page 17: Assuming that same number of cells was taken for MTT assay, the OD value given on 14 days is about 4-5 at 550 nm (Fig 2), while in Fig 8 the data is shown as % viable cells and also in parenthesis the absorbance at 550 nm. A note could be given of how the % viable cells arrived or what is the OD value at 100% viable cells.
Response: A: Yes, the same number of cells (1x106/ml) was taken for the whole MTT assays.
Response: B: We agree with reviewer on this point. We corrected and mentioned the OD value at 100% viable cells in the legend to the figure 8. It was for HCT116 (4.4) and for HCT116R (6.7).

12. Page 19: Considering the effects of 5-FU and curcumin are not additive but synergistic, what are the mechanisms, alternative or parallel signal transduction pathways. A couple of lines could be written indicating the pathway or molecular crosstalk.
Response: We are extremely grateful to the reviewer for this insightful comment. We have cited new references and discussed this important point in the Discussion part of the revised MS, as suggested by the reviewer.

13. Page 21: Certain integral proteins of NF-κB such as IKK and IκB could have been done for their expression as claimed on Page 23, for the phosphorylation of NF-κB.
Response: We have previously shown that curcumin down-regulated NF-κB pathway through inhibition of IκB kinase activation and IκB phosphorylation in
CRC cells. We followed the suggestion of the reviewer and this is now mentioned clearly in the Discussion of the revised version of the manuscript.

14. Page 23: Curcumin is shown as chemopreventive agent. But isn’t it more appropriate to describe it as an anti-inflammatory agent.

Response: We agree with the reviewer and this issue has been dealt with in the revised version.

Once again, the authors thank the reviewer for the incisive comments and suggestions.

Reviewer #2: An article of outstanding merit and interest in its field

We are grateful to reviewer #2 for the positive and highly constructive remarks on our manuscript. Below we provide detailed responses to the major comments made by the reviewer.

Comments to the Author

Several concerns have been addressed. The experiments have been improved. It may be accepted for publication. The hypothesis of using curcumin as potential chemotherapeutic agent for treating colorectal cancer is good but not new and has been reported earlier by several authors. References after 2010 have not been cited. Thus several relevant studies have been missed. Please add.

Response: The reviewer is correct to point out that references after 2010 should be cited. We have now additionally cited new references after 2010 in the Discussion of the revised version of the manuscript.

We feel that these revisions address all the points raised by the reviewers and significantly improve the flow of the paper. Thank you again.