

Dear Editor,

Please find enclosed the edited manuscript in word format (file name 61978 revised manuscript .doc).

Title: Tetramethylpyrazine inhibits proliferation of colon cancer cells in vitro

Manuscript No: 61978

We have revised the manuscript carefully according to the comments and suggestions of reviewers and editor, and responded, point by point to, the comments as listed below.

I would like to re-submit this revised manuscript to your journal, and hope it is acceptable for publication in the journal.

Looking forward to hearing from you soon.

Yours sincerely

Deng-Xiang Liu

Replies to Reviewers and Editor

First of all, we thank both reviewers and editor for their positive and constructive comments and suggestions.

Reviewer

1. In the introduction, the text starting from “Although” line 82 till line 92 lacks references.

Response: Thank you for raising this issue. We have noticed that, and added a suitable reference.

2. In the experiments addressed in the first part of the results, did the author study the colon cancer cells at different time points (24 hours or 72 hours for example)? If not,

why was the 48 hours' time point chosen to assess the cell viability and IC50?

Response: Thank you for raising this issue. We have done some pre-experiments at different time points (24 hours 48 hours and 72 hours) and the results are partially shown in Fig.3. The results showed that TMP suppressed the cell viability in a dose- and time-dependent manner at all time points. We believe that the effect is already obvious at the 48 hours' time point, so we choose it.

3. Concerning the concentrations of TMP used in the experiments, did the author base the choice of concentration on any previous study? For example, Zheng et al. (2011) have used concentrations of TMP similar to those used in this study. Also, in the second part of the results, the concentrations of TMP used to examine cell viability and morphology were stated differently in the materials and methods section; it would be flawless if this is better clarified and corrected.

Response: Thank you for raising this issue. We accept your suggestion. Actually, we learn from some other studies and have done some pre-experiments to chose the concentrations of TMP. In the second part of the results, we found the mistake and want to correct concentrations to 0 $\mu\text{g/ml}$, 300 $\mu\text{g/ml}$, 600 $\mu\text{g/ml}$, 900 $\mu\text{g/ml}$, 1200 $\mu\text{g/ml}$ or 1500 $\mu\text{g/ml}$.

4. In figure 3C, the authors should present their data in a different way to support their statement of time and dose dependence for TMP . With the current presentation, the dose dependence is clearly visible, whereas the time dependence remains difficult to interpret as there is also not statistics. The authors may consider splitting the data in two different graphs.

Response: Thank you for raising this issue. According to our experiment data, we can observe obvious time dependence at low concentrations of TMP.

5. The authors mention that TMP at a concentration of 600 $\mu\text{g/ml}$ most efficiently induces cell cycle arrest and apoptosis. Is that based on any further experiments? From the figures, TMP of concentration 1200 $\mu\text{g/ml}$ showed the highest effect. Song et al. (2013) have shown that TMP could have some side effects in vivo upon increasing TMP concentration further above 100 μM . Is it similar in this case so that the author relied on 600 $\mu\text{g/ml}$ being the most efficient without side effects? This

should be made clearer in the text. Also, it is recommended to specify exactly the statistical significance of these 2 results and show it on the graph. Please adapt the text and figures accordingly.

Response: Thank you for raising this issue. We observed that when the TMP concentration reaches 600 µg/ml, it showed a relatively good effect. The effect is more obvious as the concentration increases. Therefore, 600 µg/ml is the lowest concentration. For the TMP concentration of 1500 µg/ml in our experiment is far below 100 µM, we don't need to pay attention to the side effects.

6. In the discussion, it would be more convincing to point out the *in vivo* effect of TMP in other cancer types in which it induced similar alterations to those uncovered by the present study. For example, Zhou et al. (2017) have shown that upon treating mice with TMP, they detected a decrease in tumor growth due to the inhibition of cell proliferation. For the discussion it would be interesting if the authors could speculate based on their current and past data if similar could be the case in colon cancer? Also, it might help to write some lines about the advantage of TMP compared to other natural products that have been shown to act on cancer cells.

Response: Thank you for raising this issue. We accept your suggestion. We have noticed that, and we will detect the *in vivo* effect of TMP on colon cancer in our further experiment.

Minor

1. The authors state in the beginning that they aimed to assess the mechanisms by which TMP act. Did the author assess for any cell cycle regulators (CDK4, p16...) or apoptotic markers (caspases 3 for example). If so, then it would be a nice addition to extend on that.

Response: Thank you for raising this issue. We accept your suggestion. We have noticed that, and we will detect the mechanisms of TMP on colon cancer in our further experiment.

2. In Figs 4+5 concerning the cell cycle and apoptosis analysis, did the authors check for different time points? If not, why did they rely only on the 24 hours assessment instead of 48h?

Response: Thank you for raising this issue. According to our experiment data, we can observe obvious effect at all time points. We believe that the effect is already obvious at the 24 hours' time point, so we choose it.

3. The graphs throughout the paper would profit from unified design and colors.

Response: Thank you for raising this issue. We accept your suggestion. We have noticed that, and made some changes in our further experiment.

4. Line 159: please fix the font and change case status of “statistical analysis” to be similar to the previous titles.

Response: Thank you for raising this issue. We accept your suggestion. We have noticed that, and we will correct it.

5. Line 164: Replace Graphgraph by Graph pad.

Response: Thank you for raising this issue. We accept your suggestion. We have noticed that, and we will correct it.

Science Editor

1. The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

Response: Thank you for raising this issue. We accept your suggestion. We have noticed that, and will provide the original figure documents.

2. The “Article Highlights” section is missing. Please add the “Article Highlights” section at the end of the main text.

Response: Thank you for raising this issue. We have added the “Article Highlights” section at the end of the main text.

Major: Figure/Images raw data needs to be provided to the journal as well as the Figure still need to be improved in terms of resolution and quality.

We submit the editable figures.

