November 04, 2023

Dear Editor and Reviewers,

We would like to thank the editor and reviewers for a careful and thorough reading of this manuscript and for the complimentary comments and beneficial suggestions, which helped to improve the value of our manuscript. The comment has been carefully considered point-by-point and responded. We hope that you find our responses satisfactory and that the manuscript is now on par with the standard of your prestigious journal.

In addition, we have considered and revised technical and formatting related comments from editors and editorial office. The manuscript has been edited and formatted as par requirements of the journal.

The following are our point-by-point responses and the changes are highlighted in yellow in the revised version of the manuscript.

Reviewer #1:

Specific Comments to Authors:

Reviewer #1: In this paper, the authors investigated the effects of 5-MTP on the proliferation, migration, invasion, and apoptosis of colorectal cancer cells. The paper is interesting and suitable for the Journal. However, some minor concern are for your consideration.

Comment 1: Abbreviations used should be with its full name when it firstly appears.

Reply: In the revised version, we have added the full abbreviation as their first use as following.

Traditional Chinese medicine (TCM)

5-Methoxytryptophan (5-MTP)

6-Cell-Counting-Kit-8 (CCK-8)

7- reactive oxygen species (ROS)

8-Colorectal cancer (CRC)

Phosphate Buffered Saline (PBS)

propyl iodide (PI)

Ethylene Diamine Tetraacetic Acid (EDTA)

Fluorescein isothiocyanate (FITC)

Dichlorofluorescin diacetate (DCFH-DA)

Comment 2: The sources of materials (ex. DCFHDA, 5-MTP..) are recommended to be added.

Reply: Unfortunately, we missed DCFHDA and 5-MTP purchase information in our initial version of the manuscript. DCFHDA is the probe in the ROS kit, and we purchase the ROS kit from Sangon Biotech (China) as well as 5-MTP from MedChemExpress (USA). We have added this information in the revision.

Comment 3: Figures: figure labeling '5-MT'' should be corrected as "5-MTP".

Reply: We appreciate your correction and we have changed "5-MT" to "5-MTP" in our revised edition. We are glad to do another revision if there is anything unsuitable.

Reviewer #2:

Specific Comments to Authors:

Reviewer #2: Comments about the manuscript: "5-Methoxytryptophan induced apoptosis and PI3K/Akt/FoxO3a phosphorylation in colorectal cancer" The study presented here concerns the investigation of the effects of 5-methoxytryptophan (5-MTP), a metabolite of tryptophan on the proliferation, migration, invasion and apoptosis of colorectal cancer cells with the aim of using this in treatment of colorectal cancer. To do this, the authors studied the effects of this molecule on proliferation, apoptosis and reactive oxygen species (ROS) on cell lines. A study of the effects of 5-

MPT on the PI3K/Akt signaling pathway in colorectal cancer cells was also carried out. This work provides interesting elements that could lead to treatment of colorectal cancer. However, the manuscript needs to be revised and improved before considering its publication.

Reply. We appreciate the constructive feedback and encouragement from the reviewer, we have changed the manuscript as the reviewers' suggestions.

Comment 1: More particularly, the description of methods and techniques need to be better developed.

Reply: Thank you very much for your suggestion. As par your suggestion, we have revised the "Method" part of the manuscript. We hope that revised manuscript is more clear and meet the requirement of the journal and also easier to understand.

Comment 2: (Question#1) Page 3, line 92. "Traditional Chinese medicine (TCM) has a long history of treating malignant tumors": This point is interesting: explain the link between TCM and use of 5-MTP. Page 3, line 110. (Question#2) "All cells were derived from ATCC": Some specifications on cell lines would be helpful. Page 3, lines 110-111. (Question#3) "cultured according to conditions defined in their instructions": Explain the method of culture:

Reply: We appreciate that you raise this point and provided us opportunity to further explain this crucial point. For the question#1, we add two related studies as citations and further expatiation as following. "Also, some previous study demonstrate the significant change of tryptophan after TCM treatment in cancer patients[9,10]. However, the potential anti-cancer role of tryptophan-related metabolites is still yet to be elucidated." As for the question#2 and #3, the HCT-116 is a kind of human colon cancer cells, HCT15 is a kind of human colorectal cancer cells, and SW480 is a kind of human colorectal adenocarcinoma cells, which are widely used to study the pathogenesis of colorectal cancer. All cells were derived from ATCC The HCT-116, HCT-15 and SW-480 cells were purchased from American Type Culture Collection(https://www.atcc.org/) and cultured in McCoy's 5A, RPMI-1640 and

DMEM medium (Gibco, USA), respectively, supplemented with 10% Fetal Bovine Serum (Gibco, USA) and 1% penicillin/streptomycin (Sangon Biotech, China). Cells were incubated with 5% CO_2 at 37°C. These statements have been added in the method part of the revised manuscript.

Comment 3: "according to conditions defined in their instructions" is not sufficient for a scientific paper.

Reply: Thank you very much for pointing out the problem. We have modified the content and written out the specific steps in detail in the revision. Specifically, in method part:" Cell culture The three major kinds of human colon cancer cellines, including HCT-116, HCT15, and SW480, were purchased from American Type Culture Collection(https://www.atcc.org/) and cultured in McCoy's 5A (Gibco, USA), RPMI-1640 (Gibco, USA) and DMEM medium (Gibco, USA), respectively, supplemented with 10% Fetal Bovine Serum (Gibco, USA) and 1% penicillin/streptomycin (Sangon Biotech, China). The colon cancer cellines were incubated in incubator (Thermo Scientific, USA) with 5% CO2 at 37°C. We added 5-MTP (MedChemExpress, USA) in different concentrations during cell culture. "

Comment 4: Page 3, lines 116 an 119. "collected by centrifugation": give some explanations of how the cells were collected.

Reply: Thank you very much for your questions. We re-write this part with details as follows in Cell cycle assays method part: "On the second day, 70% ethanol was discarded by centrifugation at 1000×g for 5 min at 4°C, washed with 1mL of precooled PBS."

Comment 5: Page 3, lines 119-120. "PI staining solution was prepared according to the kit instructions": what is "PI staining" Explain the method : "prepared according to the kit instructions" is not sufficient for a scientific article.

Reply: PI (propium iodide) can bind to DNA and RNA in cells. After RNA is digested

by RNA enzyme, the fluorescence intensity of PI bound to DNA detected by flow cytometry directly reflects the DNA content in cells. We have edited the above information in the revised version of the manuscript as well.

Comment 6: Page 3, lines 125-126. “Cells in each group were digested with EDTA-free trypsin, washed with PBS, and collected”: explain the centrifugation collection method.

Reply: Our centrifugation condition is “1000×g for 5 min”, and we have added the centrifugation condition in the revised manuscript.

Comment 7: Page 4, lines 129-130. Write “400μL of binding buffer solution” instead of “400μL of Binding buffer solution”. (no capital first letter to “binding”).

Reply: Thank you for correction. As suggested, we have changed "B" to" b" in the revised manuscript.

Comment 8: Page 4, line 133. “According to the manufacturer's instructions”: is not sufficient: explain the method.

Reply: We are very sorry for our negligence. In the Hoechst Staining assays method parts, we rewrite the whole part and rename the name of this part is “Apoptosis assays” as following: “Colon cancer cells in the logarithmic growth phase were seeded in 6-well plates, and 5-MTP-treated cells were added after the cells attached. Cells in each group were digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin, washed with PBS, and collected at 1000×g for 5 min. Binding buffer 100μL was used to resuspend cells, and Fluorescein isothiocyanate (FITC) staining solution 5μL and PI staining solution 10μL were added. They were blown and mixed well by the pipette. The cells were allowed to stand at room temperature for 15 min. Before loading the machine, 400μL of binding buffer solution was added to each tube and mixed well so that the final system was 500μL. Apoptosis of cells in each group was detected by flow cytometry.”

Comment 9: Page 4, line 134. “diluted DCFH-DA”: specify how the dye was diluted: concentration? Solvent?

Reply: The DCFH-DA does not need to be diluted, and we have corrected this mistake in the revised version of the manuscript as well.

Comment 10: Page 4, line 136. “Hoechst 33342 Viable Cell Staining Solution”: explain the preparation of the dye.

Reply: “Hoechst 33342 Viable Cell Staining Solution” does not need to be prepared. We purchased it from “Sangon Biotech, China”, and added the purchasing manufacturer in the revised manuscript.

Comment 11: Page 4, line 141. “staining working solution”: explain. Page 4, lines 144-145. “added an appropriate amount of cell culture medium”: clarify what is an “appropriate amount”?

Reply: As you suggested, we have revised the manuscript as following. “Before incubation, 25uL of 200X JC-10 concentrate (Sangon Biotech, China) was added to a 5mL Assay Buffer to dilute JC-10. 500μL of JC-1 solution was added to each well and incubated at 37 °C for 20 min. After incubated, washed twice with staining buffer, added 500uL cell culture medium, and observed under an inverted fluorescence microscope.”

Comment 12: Page 5, line 183. “fixed with methanol”: what is the composition of the fixative?

Reply: We are very sorry that we have omitted the specific paraformaldehyde concentration. We have increased the specific methanol concentration, that is, 4% paraformaldehyde purchased from Sangon Biotech, China.

Comment 13: Page 5, line 183. “crystal violet”: Some specifications on crystal violet (solvent, concentration) would be useful.

Reply: Thank you very much for your advice. We have increased the solvent and

concentration of crystal violet, namely 500 uL, 1% crystal violet purchased from Sangon Biotech, China.

Comment 14: Page 5, line 202. Write “Hoechst” instead of “Hochest”.

Reply: Thanks for your correction.We have changed "Hochest" to "Hoechst".

Comment 15: Pages 11-12, figure1. Figures e, g, I, representing cell cultures, must be explained: specify the dye used, explain what each figure represents.

Reply: Figure1 E, g, and I show the Colony formation ability of three types of colorectal cancer cells in each group after being treated with different concentrations of 5-MTP (5, 25, and 100 μM). The specific operation methods are described in detail in the "Methods" section.

Comment 16: Page 12, figure 2, line 504. Write “The Hoechst staining” instead of “The hochest staining”. Figures a and d: specify the scale bar in the legend (too small on the picture). Explain each figure.

Reply: We have changed "Hochest" to "Hoechst". We also checked our manuscript and determined that "Hoechst” is correct. In addition, we reworked the images in our Figure2 and reinterpreted each figure in the modified version.

Comment 167 Pages 14-15, figure 4: Specify the scale bars (too small on the picture). Explain each picture.

Reply: Thank you very much for your valuable suggestions. We have reorganized our Figure and increased the scale bars on the picture. We have also reinterpreted each image.

Once again, we thank the editors as well as the reviewers for providing very important and constructive feedback; we believe this has enhanced the quality of our work. We hope that the reviewed and refined manuscript is at par with the standards of your prestigious journal and manuscript is acceptable for publications.

With best regards!

Yabin Pu