Re: MS ID#: 1536
MS TITLE: Influence of H2-receptor antagonists on intestinal mucositis induced by 5-fluorouracil in rats.

To reviewer 1, who raised four comments.

Thank you very much for your useful comments. The following is our reply to your comments.

Comment 1: the introduction could be expanded to include more detail of current literature in the area and more specifics with regards to their own past studies. They have them referenced but if using them as the rational for the present studies, there should be more detail provided here.
Response: In accordance with your recommendation, we have additionally described the rationale for the present study in the “Introduction”. (Page 3, lines 21~26 in the revised version)

Comment 2: abstract is misleading......Results: "lafutidine,...inhibited the 5-FU induced side effects, including weight gain, mucosal damage, and mucin accumulation. Written this way makes is sound as though 5FU caused weigh gain and mucin accumulation. This is an example of why rewriting certain sections is necessary so that the real point isn't misconstrued. The author means that Lafutidine prevents 5FU induced weight loss etc.... The aim section of the abstract is poorly written.
Response: In accordance with your suggestion, the style of abstract has been improved. (Page 2, lines 16-17)

Comment 3: Why two groups of rats? Why not take the intestinal tissues from the Yoshida rats? If there was a reason for the two groups, it should be explained. Also, it isn't clear in the results which group of rats is being used for which analysis. Had to read between the lines here. Are the Wistar rats, tumor bearing? Or just receiving drugs? Also not clear.
Response: This study have two aims. The first aim of this study to investigate the effects of cimetidine, famotidine, and lafutidine on the antitumor activity of oral administration of 5-FU in Yoshida sarcoma-bearing rats. Next, using Wistar rats, we compared the efficacy of these H2-receptor antagonists against 5-FU-induced rat intestinal mucosal injury and evaluated their effects on mucin accumulation in different areas of the intestinal tract. For clear the experimental procedure, we described separately “Experiment I” and “Experiment II” in the methods section. (Page 4~5)
Comment 4: There is a lot of detail about specific experimental analysis, that I think could be shortened and references to other articles where the methods are explained in detail could be used. This would allow for more space to expand the introduction, and experimental design/set up areas that are presently lacking detail.

Response:
In accordance with your suggestion, immunohistochemical staining procedure has been deleted.

We should like to thank the reviewers for their helpful comments and hope that we have now produced a more balanced and better account of our work. We hope that the revised manuscript is now acceptable for publication in “Cancer Research Frontiers”.
To reviewer 2, who raised four comments.

**Comment 1:** In the figure 2 the quantitative assessment to estimate the length/width ratio of villous in jejunum should be shown.

Response:
We wish to thank the referees for their useful comments. We have measured the villus height in the epithelium of the jejunum and ileum in four rats per group. The villus height was measured at three sites of three high-power fields (totally nine sites) in each rat, and the mean value and standard deviation were calculated. We made an additional statement in methods section and inserted a newly prepared graph in figure 2. (Page 5, lines 25~28; new figure 2 lower)

**Comment 2:** The authors should evaluate the biosynthetic activity of mucin or the influence of H2-receptor antagonists on diamine oxidase (DAO) activity that is associated with the maturation and the integrity of small intestinal mucosa. Apoptosis is considered to play a crucial role in the occurrence of intestinal mucositis caused by 5-FU chemotherapy. The authors should investigate the role of H2-receptor antagonists on the apoptotic response to 5-FU.

Response:
We appreciate very much for your very important suggestion. As you said, the analysis of the structural integrity (DAO activity and apoptotic response) of the basal epithelial layer is very important to improve our study on the regeneration of the intestinal mucosa damaged by 5-FU. In the forthcoming article, we wish to apply this method to our work collaborated with a pathologist.

**Comment 3:** The results obtained by the authors indicated that the protective effects of lafutidine involved the function of capsaicin-sensitive sensory neurons. The observed effects could be the activation of capsaicin sensitive calcitonin gene related peptide (CGRP) which produces nitric oxide (NO) in endothelial cells. The authors should evaluate the effects of lafutidine on capsaicin-induced NO production.

Response:
As you know, the function of capsaicin sensitive sensory neurons is related to CGRP and NO. In our previous study, another second-generation H2-receptor antagonist, roxatidine, activated the mucin biosynthesis in rat gastric mucosa mediated by NO (Br J Pharmacol 1997; 122:1230-1236). Lafutidine could have the same function. In the forthcoming article, we wish to apply this method to our work.

**Comment 4:** The manuscript should be English proofread.

Response:
The manuscript was edited by Enago ([www.enago.jp](http://www.enago.jp)) for the English language review.
To reviewer 3, who raised seventeen comments.

Thank you very much for your useful comments. The following is our reply to your comments.

**Comment 1:** The Abstract section, AIM: Remove “however” from the first sentence.

**Comment 2:** The Abstract section, AIM: Replace “Furthermore” e.g. by “Simultaneously” in the third sentence.

**Comment 4:** The Abstract section, RESULTS: Remove “Moreover” from the second sentence.

**Comment 5:** The Introduction section: Remove “Therefore” from the sentence: “Therefore, these findings…”

**Comment 6:** The Introduction section: Correct the sentence: “The clinical practice guidelines recommend using either a proton-pump inhibitor (omeprazole) or an H2-receptor antagonist (ranitidine) for the prophylaxis of epigastric pain after treatment with cyclophosphamide, methotrexate, and 5-FU alone or in combination with folinic acid. Instead of “5-FU alone or in combination with folinic acid” use „5-FU or with combination of 5-FU and folinic acid“.

**Comment 11:** The Material and Methods section: Remove the word “all” in the sentence “Regarding PGM34, it was recently demonstrated that the epitope of this monoclonal antibody was a specific sulfated oligosaccharide of the mucin molecule; the antibody stains all the goblet cells.”

**Comment 12:** The Material and Methods section: Correct the centrifugation speed for 8,000 x g (page 5).

**Comment 17:** The References section: reference 11 – remove the dot at the end of the reference; reference 22 - remove the “x” and dot at the end of the reference.

Response: In accordance with your suggestion, the style of the manuscript has been improved. (Page 2, lines 2, 5, and 15; Page 3, lines 7, and 14~15; Page 5, lines 24 and 33; Page 11, line 1 and 33)

**Comment 3:** The Abstract section, METHODS: It is not clear what concentration of 5-FU was used in experiments with combination of 5-FU with H2 receptor antagonists. This information is not clear even from the Method section.

Response: The Materials and methods section has been improved. (Page 4, lines 12~22; Page 5, lines 10~13)

**Comment 7:** The Introduction section: Include the information about capsaicin-sensitive sensory neurons, as it is not clear why the experiments with capsaicin were done.

Response: “As lafutidine was reported to reverse the 5-FU-induced gastric ulcer healing delays mediated by capsaicin-sensitive neurons, ~” was added in the Introduction section. (Page 3, lines 3~4 from the bottom)
Comment 8: The Material and Methods section: The clear information about the number of rats used and what rats were used for implantation of sarcoma cells is missing. The passage about rats and their treatment has to be rewritten accordingly.

Comment 9: The Material and Methods section: The information about the number of Wistar rats used in particular experiments is missing.

Comment 10: The Material and Methods section: Add the information about the supplier of the anti-mucin antibody.

Comment 13: The Material and Methods section: Part 2.7 Statistical analysis: only Tukey’s test is mentioned. However, in the description of the table 1 authors mentioned Dunnett’s test. Include description of this test and clearly state in which experiments which one of the tests was used.

Comment 14: The Result section, page 6: Bodyweight change: In the Figure 1 it is apparent, that authors weighted rats also on 2nd, 4th and 6th day. This information is missing in the Material and Methods section.

Response:
The Materials and methods section has been improved. (Page 4, lines 9~10, and 32~34; Page 5, line 10, 22~25)

Comment 15: The Result section: Figure 1. Famotidine and cimetidine is related to the control group and lafutidine to 5-FU-treated group. It should be changed and/or explained.

Response:
The Results section has been improved. (Page 6, lines 25~27)

Comment 16: The Result section: From the graph in Figure 1 it is not apparent that cimetidine caused virtually no changes in the weights of rats as compared to 5-FU treated group. After 8-days the BW change was around 15% comparing to 5-FU treated group. Does it mean non-significant change?

Response:
Yes, it does.

We should like to thank the reviewers for their helpful comments and hope that we have now produced a more balanced and better account of our work. We hope that the revised manuscript is now acceptable for publication in “Cancer Research Frontiers”.
To reviewer 4, who raised seven comments.

Thank you very much for your useful comments. The following is our reply to your comments.

**Comment 1:** There is no explanation in the manuscript about the characteristics of the Yoshida sarcoma and why it was used in the present work.

Response:
The Yoshida sarcoma is one transplantable allograft tumor model utilized in the study of antitumor activity. As suggested by reviewer 4, several words have been added to the text on page 3, paragraph 3 to explain.

**Comment 2:** For the immunohistochemistry, there is no information about the anti-mucin monoclonal antibody PGM34: from which company/researcher it was obtained, which dilution was used.

Response:
We have established several monoclonal antibodies that react with mucin synthesized and secreted from specific mucus-producing cells of the rat gastrointestinal mucosa (Ishihara et al., Glycoconju J 1996; 13:857-864; Biochem J 1996; 318:409-416). Regarding PGM34 established by us, it was recently demonstrated that the epitope of this monoclonal antibody was a specific sulfated oligosaccharide of the mucin molecule; the antibody stains all the goblet cells of the small intestine of the rat (Tsubokawa et al., FEBS J 2007; 274:1833-1848). Several words have been added to the text on page 5, paragraph 3 to explain.

**Comment 3:** Fig 2. Scale bars should be included in the images, as “magnification 25X” is not a precise way to describe the final magnification of an image.

Response:
In accordance with your suggestion, the scale bars are included in Figure 2.

**Comment 4:** Fig. 2 - images are very poor in contrast and resolution and therefore they need to be changed/improved.

Response:
In accordance with your suggestion, Figure 2 has been improved. (Please see figure 2 in the Table & Figure-RK4.pdf, separately submitted.)

**Comment 5:** Fig 2. Legend says “Note that goblet cells in the jejunum and ileum show positive staining with PGM34” but since the images are very poor in resolution it is not evident where is the PGM34 positive staining. Arrows are needed.

Response:
Statement in the figure legends, “Note that goblet cells in the jejunum and ileum show positive staining with PGM34”, has been deleted because it is unclear. We have measured the villus height in the epithelium of the jejunum and ileum in four rats per group. The villus height was measured at three sites of three high-power fields (totally nine sites) in each rat, and the mean value and standard deviation were calculated. We made an additional statement in figure legends and inserted a newly prepared graph in figure 2. (Page 5, lines 25~28; new figure 2 lower)

Comment 6: Image H in Figure 2 (5-FU + cimetidine) is the same as image Figure 3B (5-FU in capsaicin pretreatment). How the same image is shown for two different treatments?
Response:
Image in Figure 3B (5-FU in capsaicin pretreatment) was mistaken. The correct photograph has been inserted in Figure 3B.

Comment 7: Since 5-FU is known to cause the degeneration of the basal epithelial cells in the intestinal mucosa, one important missing experiment is the analysis by immunohistochemistry of the structural integrity of the basal epithelial layer using antibodies against: specific keratin molecules, tight junction proteins (occludins, claudins and ZOs), and apoptosis markers (activated caspase 3). Valuable information could come from these analysis in all the experimental conditions: control, 5-FU, and 5-FU + cimetidine, famotidine, and lafutidine.
Response:
We appreciate very much for your very important suggestion. As you said, the analysis by immunohistochemistry of the structural integrity of the basal epithelial layer is very important to improve our study on the regeneration of the intestinal mucosa damaged by 5-FU. In the forthcoming article, we wish to apply this method to our work collaborated with a pathologist.

We should like to thank the reviewers for their helpful comments and hope that we have now produced a more balanced and better account of our work. We hope that the revised manuscript is now acceptable for publication in “Cancer Research Frontiers”.

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