Peer Review File

Article information: http://dx.doi.org/10.21037/tcr-22-1857

Round 1

Reviewer A

Comment 1:

Overall, while the effect is potentially interesting, the data is shown for only 1 cancer cell line, grown in an ectopic site. Additional data with more relevant/additional cancer models would increase the significance of the work.

Reply 1:

Thank you for your valuable suggestion. Unfortunately, we have used up available AAT-008, and obtaining additional samples may take a long time. So we cannot implement additional experiments. We have addressed this issue in the discussion section. (Page 9, Line 258-260)

Comment 2:

Much of the data presented regarding "first experiment" add little to the paper. Figures 3,4 and 6 describe the essence of the effect. However, this is just 1 experiment with the flow cytometry performed on very few animals.

Reply 2:

Thank you for your valuable suggestion. Since we cannot implement additional experiments as stated above, we would like to keep this figure; we have addressed the issue in the discussion section. (Page 9, Line 258-260)

Comment 3:

In several places in the manuscript, the authors refer to the effect they see as "…the effect is considered addit"ve". Since the compound alone has no effect, it is unclear what "additive" means.

Reply 3:

Thank you for your advice. The growth-delay effect of AAT-008 alone seemed minimal, so we did not carry out statistical analysis in the non-RT group. Upon revision, we have evaluated the effect in the non-RT group and found a statistical significance between the AAT-008 alone and no-treatment groups. This has been stated. (Page 6, Line155-156, 179-180 and Figure 5)

Comment 4:
The chemical structure or at least its full chemical name should be included in the paper, not just referenced.
Reply 4:
We agree with your comment. We have added a figure of chemical structure. (Page 4, Line 87 and Figure 1)

Comment 5:
The "weakening effect" of oral gavage and exclusion of mice due to this effect raises suspicion that additional stress was added to the mice that were used. The authors should read Hoggatt et al JAALAS 49:329-334, 2010 that describes a new method of oral gavage that reduces stress.
Reply 5:
Thank you for your valuable suggestion. We have read the article and have addressed this issue in the discussion section, referring to the paper. (Page 9, Line 260-262; Ref. 29)

Comment 6:
While EP4 may be a proto oncogene and inhibiting its signaling may have potential anti-cancer effects, EP4 plays critical roles in hematopoiesis and blood cell trafficking. The authors need to discuss this effect in light of potential clinical use of an EP4 antagonist.
Reply 6:
Thank you for your valuable suggestion. The safety including the effect on hematopoiesis of AAT-008 has been confirmed in many animal experiments (data not shown). We have added this point in the discussion section. (Page 9, Line 245-249)

Comment 7:
It would have been useful to show data using antagonists of the EP1-3 receptors to show specificity. In addition, no rational is provided for the preferential use of AAT-008. How/why is it better than other EP4 antagonists? Use of another EP4 antagonist would also be useful to add to specificity.
Reply 7:
Thank you for your valuable suggestion. AAT-008 has a great binding potency for EP4 receptors and has more than 1000-fold higher selectivity than other prostaglandin receptors. So we assumed the effect was obtained via EP4 receptor. We assumed that AAT-008 was useful to bring out the effectiveness via suppression of EP4 receptors

compared to other EP4 antagonists. Moreover, AAT-008 is ready to be used in clinical situations. These are the benefits to use AAT-008 compared to other EP4 antagonists. We have added these sentences in the discussion section. (Page 7, Line197-198 and Page 8, Line 250-255)

Reviewer B

Comment 1:

The in vivo growth data appear robust, with a clear association between tumor growth and AAT-08 administration+irradiation, suggesting an additive effect of both therapy forms. Unfortunately, there is only little data on further analysis, especially the proof for a causative role of the immune system. The authors could have used transgenic mouse models to proof their hypothesis. They could have used a more comprehensive FACS analysis, especially with a FACSverse machine at hand (6-10 parameter flow cytometer). It is unclear wether the obeserved effect is associated with decreased PGE2 activity. (...)

I would highly encourage the authors to proceed with their analysis to establish a much more detailed manuscript.

Reply 1:

Thank you for your valuable suggestion. Unfortunately, we have used up avaiable AAT-008, so we cannot implement additional experiments. We have addressed this issue in the discussion section. (Page 9, Line 257-260)

Reviewer C

Comment 1:

Use at least one human cancer cell line to repeat the tumor growth delay study to support the conclusion;

Reply 1:

Thank you for your valuable suggestion. Unfortunately, we have used up AAT-008, so we cannot implement additional experiments. We have added this point in the discussion section. (Page 9, Line 258-260)

Comment 2:

Display the in vivo images of tumors under X-ray;

Reply 2:

Although we did not take a picture for the tumor-bearing mice used in this study, we have shown a picture of the device to fix mice for irradiation. (Page 5, Line 117 and

Supplementary Figure 1)

Comment 3:
Display the flow figures of Teff/Treg proportions.
Reply 3:
We have added the representative dot plots of Teff and Treg in Supplementary figure 2.

Comment 4:
For figures 1 & 3, please mark out the unit of the Y axis (relative tumor volume).
Reply 4:
We have added the unit of the Y axis. (Figure 2 and 4)

Comment 5:
For figure 5, please label the legend for the empty and solid circles.
Reply 5:
We have added the legend. (Figure 5)

Round 2
Reviewer comments

Comment 1:
Although, the manuscript includes some interesting data showing combinational effects, it lacks logical explanation and rational of experimental designs.
Reply 1:
Thank you for your valuable suggestion. We have added logical explanation and rationale for experimental designs, thereby adding three papers. (Page 4, Introduction, Line 76-105 and References 4-7)

Comment 2:
Results section is quite poorly written, appears to increase the number of figures purposefully while showing the similar results.
Reply 2:
Thank you for your advice. We have removed two figures and added sentences regarding the results of the removed figures. (Page 7, Line 184-187 and 193-197)

Comment 3:

Choosing a single dose of IR (9 Gy) for showing radio-sensitizing effect of AAT-008 is not enough to make a conclusion until compared with lower doses of IR.

Reply 3:

Thank you for your valuable suggestion. We agree with you, and experiments using lower radiation doses are desirable. Unfortunately, however, we have used up available AAT-008, and obtaining additional samples may take a long time. So, we cannot implement additional experiments. We have addressed this issue as a limitation in the discussion section. (Page 9, Line 278-279)