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ADVANCES CLINICAL MEDICINE TOWARD THE GOAL OF IMPROVING PATIENTS' QUALITY OF LIFE
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Reviewer A

This is an interesting study analyzing the role of irradiation in combination with a novel Hsp90 Inhibitor in vitro and in a mouse model. We appreciate your comments.

Comments

1) The abstract is missing in the ms.

We are sorry. We have included Abstract in the manuscript file.

2) The carcinoma cell line SCCVII needs to be explained in the abstract and in the introduction. Is this an ATCC cell line. Which tumor entity? please indicate this in the Materials and Methods.

The SCCVII cell line has been explained briefly in Abstract (Methods, Line 1) and Introduction (Para 3, Line 3 from bottom). This is not an ATCC cell line. The origin and characteristics of SCCVII cells have been explained in more details in Methods, *Tumor cells, mice, and drugs*, Line 1-2.

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3) Change title to something like: Effects of a combined treatment regimen consisting of Hsp90 inhibitor and radiation in vitro and in a tumor mouse model

Thank you for your suggestion. We have changed the title as you suggested.

4) Materials and Methods Use Mouse model instead of in vivo study

We have changed the title of the section from "*In vivo study*" to "*Mouse model*".

5) How can the authors guarantee that all mice received the same oral dose (please explain the procedure in detail).

We have stated the administration method in more details (Methods, *Tumor cells, mice, and drugs*, Para 2, Line 3-6 from bottom). To guarantee the same oral dose, administration of DS-2248 was carried out by a single investigator (T. K.).

6) Figure 2 Results: colony formin assay should be mentioned in the results describing Figure 2.

We have stated that Figure 2 is a result of colony formation assay (Fig. 2 legend).

7) Can the authors provide IHC analysis of relevant organs upon a combined therapeutic approach especially after 15 mg/kg body weight.

IHC analysis was not included in the present study, and we think we should do it in a future study. This has been commented on in the limitation paragraph of Discussion (Para 4, last 3 lines).

8) Figure 3: why is DMSO increasing y H2AX foci after 1h. this needs explanation. It appears that the time point chosen for yH2AX staining (6h, 24h) are too late.

The increase in γH2Ax at 1 h is due to irradiation, since all cells received 2 Gy. At 1 h,

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initial DNA DSB caused by irradiation after early DSB repair is evaluated, and at 6 and 24 h, subsequent DSB repair is observed. So, we think the timing was reasonable.

9) Could the authors provide other cytotoxicity assays (ie apoptosis assay etc.) instead.

Other cytotoxic assays were not included in the present study, and we think we should do it in a future study. This has been commented on in the limitation paragraph of Discussion (Para 4, last 3 lines).
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10) What is the mechanism of killing by Hsp90 inhibitor and radiation?

The mechanism of killing has been stated in more detail in Introduction, Para 2.

11) Statistics are missing in all Figures.

We have added *P* values and statistical methods for Figs. 2-5.

12) Can the authors include the combination 15mg/kg and 24 Gy of Figure 4 into Figure 5 for comparative reasons.

We have included the curve for 15 mg/kg + 24 Gy in Fig. 5.

13) Please provide statistical significances also in Figure 4 and 5.

We have done this as stated above.

14) How do the authors define additive effects, needs to be explained more detailed.

Whether the combined effect was additive or supra-additive was examined by a two-way factorial analysis of variance followed by post hoc Tukey's HSD test. This program is implemented in the statistical software "R". The method has been stated in more details in Methods, *Statistical analysis*, last 8 lines.

15) A bit more information on the advantages of the new Hsp90 inhibitor compared to older versions should be given.

Daiichi-Sankyo Co. Ltd., which is a developer of DS-2248, has data on comparison of DS-2248 and 17-AAG. Since this is not our data, a summary of the data has been stated in Introduction, Para 3, Line 8-11.

Reviewer B

The data presented in the ms are exactly in line with previously published studies with Hsp90 inhibitors and radiotherapy. However, the effect of combination therapy is only marginal.

1) I am curious about the dose-selection criteria for DS-2248,

In the data of Daiichi-Sankyo Co. Ltd. (developer of DS-2248), concentrations of DS-2248 to inhibit 50% cell growth was 9-51 nM as stated in Discussion. *In vivo*, investigators of Daiichi-Sankyo tested efficacy of DS-2248 at the dose of 2.5-20 mg/kg. Looking at these data, we chose the concentrations and doses for our experiments. We have stated that the concentrations and doses of DS-2248 were determined according to the unpublished data of Daiichi-Sankyo in Methods, *Tumor cells, mice, and drugs* (Para 2, last sentence).

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2) What are the oral bioavailability, PK in plasma, and the tumors related to given dose levels.

Daiich-Sankyo also has data on PK of this compound. Since the data are not our own, a summary of the PK study has been shown in Introduction, Para 3, Line 7-11 from bottom.

3) While tumor growth was delayed no regression in tumor volume was observed. What is the relative efficacy of this agent when compared to a much more effective Hsp90 inhibitor (AT13387) that shows the synergistic anti-tumor response when combined with radiotherapy.

Unfortunately, we do not have data on AT13387, but Daiichi-Sankyo has data on comparison of DS-2248 and 17-AAG which is another famous Hsp90 inhibitor; a short summary of the result has been added in Introduction, Para 3, Line 8-11.

4) Also how general these results are as all in vivo data is from one mouse tumor model.

We only used one tumor line, and this is a limitation of this study. So, it has been stated in the limitation section of Discussion (Para 4, Line 1-5). In a previous study, the efficacy of a radiosensitizer was quite similar among SCCVII tumors and four human pancreatic cancers (Ref 33, newly added). So, the effect may not be specific to this tumor. We will plan to evaluate the effect in other mouse models *in vivo*. This has been stated in Discussion (Para 4, Line 5).

5) Also, the tumor growth data needs to be plotted as actual tumor volume, not as relative tumor volume.

We have plotted tumor growth data as actual tumor volume (Fig. 4 and 5).

Reviewer C

Kondo et al. present a manuscript investigating novel Hsp90 inhibitor DS-2248 in combination with radiation. The study shows that DS-2248 has an effect against SCCVII cells, increased by radiation. Furthermore, the authors show that DS-2248 cells have more gamma-H2AX puncta after radiation treatment compared to DMSO control. Lastly, they show a delayed on-set of SCCVII tumors in mice when treated with DS-2248 alone and in combination with radiation. Here there was no observed synergistic effect.

Also other newer Hsp90 inhibitors with seemingly improved toxicity profiles in clinic compared to the first generation exists. However, the authors refer to unpublished data from clinical trials that DS-2248 also has a favorable toxicity profile, anti-tumor activity and orally bioavailable which makes it interesting.
However, while the authors demonstrate an effect in combination with radiation, they only do so in one (1) murine cell line, which makes any translational value of findings doubtful.

The limitation that we used only one tumor line has been mentioned as stated above in Discussion, Para 4.

Please see below for additional remarks:

1. Considering the novelty of the inhibitor, there is yet no results presented that Hsp90 is the target of DS-2248. The authors should preferably demonstrate any effect on any gene expression or protein regulation associated with Hsp90 inhibition, such as Hsp70/Hsp27 upregulation.

Daiichi-Sankyo (developer of DS-2248) has data on Western blotting, which show that DS-2248 treatment upregulated Hsp70. So, the results have been presented as a new figure (Fig. 6) and the method has been added in Methods (*Mouse model*, Para 4). The result has been stated in Results (last section, *Western blotting*) and commented on in Discussion, Para 3, Line 1-2. To do so, please allow us to add a new coauthor Koichi Nakamura from Daiichi-Sankyo.

2. The study only uses one cell line, SCCVII – a squamous cell carcinoma cell line. Has the identity of the cell line been verified in the lab by STR analysis and has regular mycoplasma testing been performed? If so, please add the method section. If not, this should be done. Also please also state from where you obtained the cell line.

The SCCVII cell line was a gift from Kyoto University Department of Radiation Oncology; STR analysis and regular mycoplasma testing had been done there, and before the start of this experiment, mycoplasma testing was again performed, showing a negative result. These have been stated in Methods, *Tumor cells, mice, and drugs*, Line 1-4.

3. The SCCVII is a murine squamous cell carcinoma cell line, please clarify this in the material and methods section.

We have clarified it and added an explanation about the cell line in more detail in Methods, *Tumor cells, mice, and drugs*, Line 1.

4. Your experiments show that DS-2248 (without radiation) on its own causes a marked H2AX foci increase. A. What is the cause of this? B. With this in mind, can the higher amount of H2AX foci at all time points in DS-2248-treated cells be due to this rather than DSB repair inhibition?

DS-2248 as well as other Hsp90 inhibitors are anti-cancer agents, and they cause DNA DSB. A reference (No. 9) for this has been added and the mechanism of action has been stated in Introduction, Para 2. Therefore, the higherγH2AX index produced by the combination may be in part due to the cytotoxic effect of DS-2248. This possibility has been stated in Discussion, Para 1, Line 12-16.

5. Figure 2: Was N=1 for each combination for each experiment? Please clarify.

We have stated that N was 3 for each point in Fig. 2 legend.

6. For in vitro experiments you state the effect if 50 nM was "mild". Please specify with numbers. Also, why do you think the compound only caused a mild effect at 50 nM for this cell line when you refer in the discussion to Honma et al. who found the IC50 of 4 other cell lines to be 9-51 nM.

We have shown the surviving fraction of cells treated by 50 nM DS-2248 without irradiation (i.e., 0.72 ± 0.03; Results, *In vitro* activity, Line 3). As you point out, 50 nM is not a low concentration, but SCCVII is an established murine cell line that is relatively resistant to anti-cancer therapy compared to human tumor cell lines. This has been stated in Discussion (Para 2, Line 6-8).

7. Throughout the manuscript you write that the combined effect is additive or supra-additive without stating method or giving P-values for this. Please amend.

Whether the combined effect was additive or supra-additive was examined by two-way factorial analysis of variance followed by post hoc Tukey's HSD test implemented in the statistical software "R". This method has been stated in more

detail in Methods, *Statistical analysis*, Line 3 to end.

Minor remarks:
Line 20: Please provide reference or rephrase/remove.

We have removed this sentence (Introduction, top).

Line 21-23: This is not very on point, I'd rather see you relate the role of radiation in the clinical treatment of squamous cell carcinoma.

We have modified this part to address the importance of radiation and radiosensitization in squamous cell carcinomas (Introduction, Para 1, Line 1-2).

Line 44: This sentence refers to two references I have not successfully been able to use to verify the statement. Ref #15 is a patent application and #16 a conference paper (?) I cannot find.

Yes, Ref #15 is a patent application document, and Ref #16 is a conference abstract which is not easy to get. Therefore, Ref #16 has been deleted from the reference list and the contents have been explained in the text (Introduction, Para 3, Line 8-11).

Line 69: Please clarify "appropriate numbers".

We have stated the actual numbers in Methods, *Treatment and colony formation assay,* Line 1-4.

Line 75: Please write out the procedure instead of writing "standard".

We have stated the actual procedure in Methods, *Treatment and colony formation assay,* Line 2-4 from bottom.

Line 99-100: Please clarify "with differing tumor volumes evenly distributed among the groups".

We have explained it in Methods, *Mouse model*, Para 1, Line 4-6.

Line 134: Please state statistical method and P-values.

We have stated them (Results, *In vitro* study, last sentence).

Line 136: For results section and figure 3 legend, state that experiments were performed on SCCVII cells.

We have stated it in Results, *In vitro activity*, Line 1 and the Fig. 3 legend.

Line 139-140: DS-2248 produced more foci compared to the DMSO control. Please amend.
Line 140: Control cells => DMSO-treated cells

Thank you for your suggestion. We have corrected them (Results, *Changes in γH2AX foci per nucleus*, Line 4-5).

Line 153: Please state full P-value.

We have stated full P-values (Results, *Tumor growth delay assay*, Para 1, Line 7-8; Para 2, Line 5-6).

Line 199-200: Ref 22 does not support the claim that this is a general phenomena.

We apologize for the mistake. The correct reference was No. 21 and we have corrected it (Discussion, Para 3, Line 7).

Line 203-205: You cannot state this based on body weight.

The statement has been modified (Discussion, Para 3, Line 10-11).

Line 220: An extra K.

Thank you. We have deleted K (Acknowledgements, Line 5).