Peer Review File

Article information: https://dx.doi.org/10.21037/tlcr-22-856

Reviewer A

The manuscript by Kaytor et al. examines the effects of an oral nanopreparation of genistein for tissue protection in a murine model of NSCLC.

Comments for Revision:
1) How were tumor volumes determined? Were these determined by a researcher blinded to the treatment groups?
Reply 1A: We thank the reviewer for their comment. Tumor volumes were determined by external measurements of the tumor dimensions using calipers. Tumor volumes were then calculated using the formula for an ellipse [(4/3)pR1•R2•R3] where R3=(R2+R1)/2 and R1, R2, and R3 are the orthogonal radii of the tumor. This calculation was based on a modification and extension of the method used by Gallo, et al (PMID 18567761). The method is similar to published ellipsoid formulas used to calculate tumor volumes (Rodallec, et al PMID: 36178898). The researchers making the tumor measures were blinded to treatment groups.

Changes in the text: We revised the text within the **Methods** section (**lines 122-130**) to include the above details on how tumor volumes were determined.

2) Please provide Kaplan-Meier curves with the tumor volume curves. Although this information is provided in the table, it would be more informative to see next to the tumor volume.
Reply 2A: We thank the reviewer for their comment and agree that Kaplan-Meier curves would be more informative than just the number at risk tables.

Changes in the text: We have updated **Figures 1 and 2** to include new panels C-D that feature Kaplan-Meier curves for the two xenograft studies detailed in the manuscript. Revisions have been made throughout the **Methods** and **Results** sections to incorporate these revised figures.

3) Please provide histology of the tumors in each treatment group if this tissue is available after the experiment. This is important information for this study, to view the effects of genistein on the tumor structures.
Reply 3A: We thank the reviewer for the comment, but unfortunately, we did not have the histopathology lab provide sample images of the tumors from the first experiment with thoracic irradiation, and we can no longer obtain these images. This study was focused on the effect of genistein on normal tissue rather than the tumor. While we agree it is important to also view the effects of genistein on the tumor, as noted in the text, findings in tumor tissue were not related to any particular treatment group. However, in relation to your comment, we have now included a supplementary table that provides the detailed findings from the H&E stained sections of the tumor implants from 5-6 animals from each treatment group. We did not submit tumor samples for histopathology evaluation for the second study with abdominal irradiation.

Changes in the text: We have revised the **Results** section (**line 267**) to include reference to new **Supplementary Table 1**, which details the histopathology analysis of tumors from the thoracic irradiation xenograft study.

4) Please provide histology of the terminal ilium that was analyzed during these experiments. This information is currently provided in table form.
Reply 4A: We thank the reviewer for their comment. We only have terminal ileum images from vehicle and nano-genistein treated animals without irradiation. We have included these as a supplementary figure. We also have revised the manuscript to include a new supplementary table that lists the specific findings for the terminal ileum and skin from animals in experiment 2.

Changes in the text: We have revised the **Results** section (**line 274**) to include reference to new **Supplementary Table 2**, which details the histopathology analysis of the terminal ileum and the skin adjacent to the tumors from the abdominal irradiation xenograft study. We also included (**line 274**) reference to new **Supplementary Figure 1**, which has representative terminal ileum images.

5) Please provide vivarium conditions in the Methods: temp, humidity, light/dark cycle.

Reply 5A: We thank the reviewer for bringing to our attention that we omitted these important details in the methods.

Changes in the text: We have revised the **Methods** section (**lines 100-101**) to include more details on vivarium conditions.

Reviewer B

The authors found that the combination is associated with reduced tumor growth and improved normal lung histopathology compared to those receiving vehicle/placebo. The findings may support the translation to Phase-I trial due to safety and efficacy in the mouse model.

Several issues need to be considered:

The authors only used one cell line (A549 cells) in two separate studies. Please comment. Why not Lews Lung Carcinoma (LLC1) or another lung cancer cell line?
Reply 1B: We thank the reviewer for their comment and acknowledge that the use of a single cell line is a limitation to these studies. We chose to only evaluate a single cell line because this was an exploratory study with the goal of obtaining enough preliminary to support opening an early phase trial to evaluate the drug in patients with non-small cell lung cancer (NSCLC). Accordingly, we chose A549 cells because they are a human cell line that has been classically used as a model of NSCLC. As our pilot clinical trial has already completed accrual, the benefits from performing experiments in a second cell line at this time are likely more limited as we wait for the clinical data to mature. We do, however, acknowledge that the use of LLC1 cells in an immunocompetent mouse model would be informative given the anti-inflammatory aspects of the drug's mechanism of action.

Changes in the text: We have revised the **Discussion** section (**lines 327-330**) to mention the limitation of using a single cell line and the importance of using a syngeneic mouse model in future studies.

Why did you use a single dose of 12.5 Gy rather than fractionated radiotherapy with 2,00Gy daily or radiation dosage above 40Gy?
Reply 2B: We thank the reviewer for their comment and acknowledge that using fractionated radiotherapy is more clinically relevant. Our goal was to deliver a radiation dose that would elicit

measurable growth delay in the tumor while also causing detectable damage to normal lung tissue. Given that these were proof-of-concept studies, we chose to deliver a single 12.5 Gy dose that in previous studies caused detectable lung tissue damage (Jackson et al. PMID: 28963717). Additional published data with A549 cells in nude mice supported the use of 12.5 Gy to cause tumor growth delay without completely ablating the tumors (Storozhuk, et al. PMID: 22607554).

Changes in the text: We have revised the **Methods** section (**lines 143-145**) to specify the rationale behind the use of a single 12.5 Gy dose.

Given the latest evidence that lung fibrosis could be seen 6 months after the end of radiotherapy with radiation dosage above 40Gy per fraction (Ref: 10.3389/fmed.2021.794324)
Please comment on the used fractionation regimen.
Reply 3B: We thank the reviewer for their comment and for bringing to our attention the publication on the use of arc delivery in mouse models. We agree with the reviewer that exposure to higher doses, particularly with the highly precise arc therapy radiation technique, can cause lung fibrosis to develop. The present study used a lower dose based on the radiation technique available and a previous publication supporting that a 12.5 Gy was sufficient to elicit lung tissue damage (Jackson et al. PMID: 28963717). We note that our study was not carried out long enough for significant lung fibrosis to develop, and any tissue damage detected was likely due to pneumonitis, which often precedes, and can lead to, late fibrosis. A higher dose and/or longer in-life phase, and fractionated radiation treatment should be considered for future studies in order to evaluate lung fibrosis in a more clinically relevant setting.

Changes in the text: We have revised the **Discussion** section (**lines 313-315**) to mention that future studies will need to be carried out for at least 6 months in order to detect lung fibrosis in mice.

Why did the authors only use hematoxylin and eosin (H&E) staining. It would be highly recommended to add Masson's staining to detect collage or another staining e.g., immune subsets?
Reply 4B: We thank the reviewer for their comment, and we agree that additional staining, including Masson's trichrome would have been useful for the detecting of collagen as a surrogate marker of fibrosis. We initially chose to only use H&E staining because it would be sufficient for the pathologist to assess tissue damage and immune cell infiltration.

Changes in the text: We have revised the **Discussion** section (**lines 326-329**) to mention the use of a single tissue stain as a limitation.

Discussion is rather short. Please add important references for the development of nano- genistein, mechanism, potential synergistic mechanism with radiotherapy.
Reply 5B: We thank the reviewer for their comment and agree that the discussion was short.

Changes in the text: We have revised the **Discussion** section (**lines 281-306**) to include more detailed discussion on the development of nano-genistein and its mechanism, as well as the corresponding references.

I can not recommend to accept the manuscript in the latest form. Substantial improvements are warranted.
Reply 6B: We thank the reviewer for all their comments, which in our opinion have helped to significantly strengthen the manuscript.