Dear Prof. Ladiges,

Thank you very much for considering our manuscript entitled: “**Specific** **regulation of senescence-associated secretory phenotype by a senescence-targeted activatable senomorphic**”. We would also like to thank the reviewers for their time and thoughtful comments. Herein please find our point-by-point responses to the critiques from the reviewers, as well as our revised manuscript with highlighted changes.

**Reviewer #1:**

*In this study, Xie and colleagues produced a senescent cell targetable aptamer and investigated the effect of this aptamer on gene expression relating to senescence associated secretory phenotype (SASP) and cell viability in H2O2-induced senescent cells. This aptamer can be activated by the action of senescence associated beta-galactosidase (SA-β-gal) and carbonic anhydrase and produces H2S. The phenotype for the senescent cells might be caused via the production of H2S. This may provide a new strategy to build senescent cell targetable senomorphics via SA-β-gal. This study is potentially interesting and useful for the researchers in the fields of aging and age associated diseases. However, there are a couple of concerns in this manuscript and the authors should address about these to improve the manuscript.*

We sincerely thank Reviewer #1 for these positive comments. The point-by-point responses are given below.

**Major concern,**

1. *This aptamer is highly specific to senescent cells (Fig. 3E). SA-β-gal is in lysosome. Is this aptamer specifically localized in lysosome? The authors should show the aptamer is localized in lysosome in senescent cells.*

**Response:** We thank the reviewer for this thoughtful suggestion. As suggested, the lysosomal colocalization analysis was performed. Senescent BJ cells were treated with Cy5-labled Apt-H2SD and stained with lysotracker Green, followed by confocal fluorescence imaging. Results showed a large overlap between the green and red fluorescence channels (Figure 3F), confirming the lysosomal accumulation of Apt-H2SD in senescent cells. The data and discussion have now been provided in the revised manuscript (***Please see Figure 3F and the highlights on page 7 in the revised manuscript***).

2. *Treatment of this aptamer in senescent cells suppressed the gene expression relating to SASP without killing cells. This suppression phenotype of the gene expression by the aptamer is promising. However, this seems to be transient. If so, this might be disadvantage for developing a new strategy. The authors should examine whether the gene expression relating to SASP is recovered after removing the aptamer in the culture medium.*

**Response:** We thank the reviewer for this important comment. As the reviewer indicated, the engineered Apt-H2SD suppressed the secretion of SASP in a transient manner. Distinct from senolytics that directly eliminate senescent cells via apoptosis, senomorphics are a class of agents that can suppress the aggravation and propagation of senescence through SASP blocking without killing senescent cells. Senomorphics are considered clinically significant, as they can reduce the detrimental effects of senescent cells while preserving them in the original niches for sustained tissue structures and physiological integrity (e.g., *Nat Rev Drug Discov.* 2018, 17, 377; *Nat Rev Cancer.* 2019, 19, 439; and *Nat Rev Chem.* 2019, 3, 426). Of note, since many SASP factors participate and play important regulating roles in many important biological processes, which requires the SASP regulating strategies to have high selectivity to minimize the off-target toxicities. Aiming at achieving this goal, in this study we designed a new type of senomorphics (Apt-H2SD) by combining the aptamer-mediated active cell targeting and senescence-associated enzyme-switchable drug activity for specific regulation of SASP secretion. On one hand, the active targeting and cell-specified activation could significantly improve the regulation selectivity. On the other hand, we envisioned that such transient regulation of SASP, instead of permanent manipulation of the SASP-related genes via genetic modulation methods, may largely avoid the unwanted adverse effects to normal cells and tissues. In future studies, we will perform more in-depth studies to reveal the exact mechanisms underlying the SASP regulation of the present senomorphic. This part has been discussed in the revised manuscript (***Please see the highlights on pages 8-9 in the revised manuscript***).

*3. This phenotype to the senescent cells most likely appears via H2S production. The authors need to discuss why and how H2S produced from the aptamer inhibits SASP related gene expression without killing senescent cells.*

**Response:** We thank the reviewer for this important suggestion.Hydrogen sulfide (H2S) is an endogenous signaling molecule with broad therapeutic effects including anti-inflammatory and anti-oxidative effects (e.g., *Nat Rev Drug Discov*. 2007, 6, 917; Nat *Rev Drug Discov*. 2015, 14, 329; and *Anal. Chem*. 2021, 93, 4894). Recently, it has been demonstrated that H2S can ameliorate the secretion of multiple SASP without inducing cell apoptosis (*Aging*. 2016, 8, 2264 and *Aging*. 2018, 10, 1666). Nevertheless, the the exact mechanism underlying the SASP regulation of H2S remains unclear. More in-depth studies will be carried out in the future. This limitation has been discussed in the revised manuscript.

**Minor points**

1. *Unspecified abbreviations are too many to easily understand, especially in Materials and Methods section.*

**Response:** We thank the reviewer for this important comment. The full names of these abbreviations have been provided in the revised manuscript.

2. *Page 5, senescence induction and characterization part, the authors indicated that cells were treated for 6 days to induce senescence but the authors used 24 h-treated cells for* *SA-β-gal staining. Is it correct? If yes, the authors need SA-b-gal staining for 6 day-treated cells.*

**Response:** We thank the reviewer for this comment. In our study, the cellular senescence model was established by treatment of BJ cells with H2O2 for 6 days. SA-β-gal staining was then performed in these senescent cells to prove the successful senescence introduction. For SA-β-gal staining, senescent BJ cells were cultured for 24 h to allow adherence, followed by washing, fixing, and staining. This duration (24 h) was referred to the staining time, not the time for senescence establishment. We have now added more descriptions in the revised manuscript to avoid any misunderstanding (***Please see the highlights in the experimental section***).

3. *Legend for Figure 1 is not reader friendly. Please re-write this legend as the readers can understand it more easily.*

**Response:** As suggested, the legend for Figure 1 has been revised.

**Reviewer #2:**

*The role of cellular senescence in many diseases has been clearly established. Therefore, the synthesis of new compounds to target/modulate the senescent phenotype is important. Nevertheless, the article should improve the characterization of the new compound, since it is poorly characterized, such a co-culture of cells and maybe checking a tumoral cell line. In addition, the title is somehow confusing.*

**Response:** We thank the reviewer for this comment. Specific regulation of the senescence-associated secretory phenotype (SASP) is vital to block senescence-induced detrimental cellular plasticity. However, since many SASP factors participate and play important regulating roles in many important biological processes, specific regulation of SASP in senescent cells is highly desirable, but remains challenging. Despite many senomorphics have been developed to modulate SASP, effective SASP regulating strategies with cell targeting and controllable activities are still lacking. The aim of this work is therefore to design a new type of senomorphics (Apt-H2SD) by combining the aptamer-mediated active cell targeting and senescence-associated enzyme-switchable drug activity for specific regulation of SASP secretion in senescent cells.

It is also worth noting that, in this study, the feasibility of the engineered Apt-H2SD for regulating SASP has been examined in oxidative stress-induced senescent BJ cells. BJ cells, a normal human fibroblast cell line, were selected as the model cell to induce cellular senescence, given the fact that fibroblast senescence contributes to the organic aging and the pathology of many important diseases, such as pulmonary fibrosis, cancer, neurodegeneration, and cardiac disorders (e.g., *J Am Coll Cardiol*. 2016, 67, 2018; *Cancer Res*. 2006, 66, 794; *Trends Mol Med*. 2022, 28, 97; and *Circulation*. 2018, 138, 809). Senescent BJ cells have been widely recognized as one of the most representative cell models in the field of senescence (e.g., *Nat Cell Biol*. 2006, 8, 877; *Nucleic Acids Res*. 2018, 46 5664; *Anal Chem*. 2017, 89, 2937; and *Aging Cell*. 2004, 3, 103).

The synthesis and enzyme-responsive H2S release were characterized by spectrum methods (Figure 2 and Figures S1-S9). To assess the performance of Apt-H2SD for SASP regulation in senescent cells, we first tested the cell targeting capability by using confocal imaging and flow cytometry analysis. Results showed that Apt-H2SD could selectively recognize and accumulate in senescent BJ cells against in proliferating cells (Figure 3A-E). Moreover, we have performed supplementary fluorescence colocalization experiments in the revised manuscript to confirm the lysosomal accumulation of Apt-H2SD. As shown in Figure 3F, a large overlap between the fluorescence signal from Apt-H2SD and that from lysotracker was observed. Next, the ability of Apt-H2SD for suppressing SASP was determined at the mRNA level. Treatment with Apt-H2SD could mitigate the upregulation of proinflammatory interleukins (IL-6 and IL-1β) and matrix metalloproteinases 3 (MMP3) in senescent cells (Figure 4), without causing unwanted cytotoxicity (Figure S11).

We agree with the reviewer that evaluations of the engineered senomorphic in tumor cells are important, which will be performed in future investigations. Also, our study still has some other limitations. More in-depth studies, such as its influence on the secretion of other types of SASP, the expression of SASP at the genetic level, and the duration of action, are still needed to better evaluate the performance of Apt-H2SD and reveal the exact mechanisms underlying the SASP regulation. Although the research we present here is relatively preliminary, its scientific applications are potentially broad, as cellular senescence not only contributes to aging, but is highly implicated in the initiation and progress of many important diseases. Such aptamer-prodrug conjugating strategy may also pave the way for design and construction of various senomorphics by changing the drug moiety, which is expected to generate profound impacts in the treatment and prevention of age-related diseases. The limitations of this work have been discussed (***Please see the highlights on pages 8-9 in the revised manuscript***).

In addition, the title of this manuscript has been revised, as suggested by the reviewer.

**Reviewer #3:**

*In this manuscript, Yuqi Xie et al. developed a novel senomorphic called Apt-H2SD and confirmed that it could bind and accumulate in senescent cells over proliferating cells through aptamer-mediated cell targeting. I think their result is amazing and makes an excellent contribution to the field. This manuscript can be accepted if the author can address the following concerns.*

We sincerely thank Reviewer #1 for these positive comments. The point-by-point responses are given below.

1. *Why did the author choose BJ cells to study cellular senescence in this study? This could be clarified in the Discussion section.*

**Response:** We appreciate the reviewer for this constructive suggestion. The reason for the selection of BJ cells as the cell model has been added in the discussion section (***Please see the highlights on pages 8-9 in the revised manuscript***).

2. *A reference should be added in the Methods section to support the H2O2-induced senescent cell model.*

**Response:** As suggested, the references related to the establishment of H2O2-induced senescent cell model have been cited in the Methods section.

3. *Could the author add the levels of cell cycle-related factors, such as p16, p21, p53, RB, or Ki67, to confirm the cellular senescence in BJ cells?*

**Response:** We thank the reviewer for this important comment. Evaluations on the levels of cell cycle-related factors including p16 and p21 have been added (***Please see Figure S10 in the revised support information***).

4. *A* *graphical abstract could be added to clearly show the results of this study.*

**Response:** As suggested, the graphical abstract has been added in the revised manuscript.

Once again, we thank the reviewers and editors for the helpful comments and suggestions, which we believe have produced an improved manuscript. We hope that our responses have addressed all the issues raised by the reviewers and editors.

Sincerely,

Yanlan Liu