**Specific regulation** **of senescence-associated secretory phenotype by****a** **senescence-targeted** **activatable senomorphic**

Author a,#, Author a,#, Author a, Author a, Author a, Author a, and Author a,\*

#These authors contributed equally to this work.

\*Corresponding Author:

**Abstract**

**Background:** Senomorphics have been considered as an effective alternative senotherapeutics for regulating cellular senescence and the associated disorders by inhibiting the adverse impacts of senescence-associated secretory phenotype (SASP) components secreted by senescent cells without occasioning cell death. However, current senomorphics usually encounter low selectivity towards senescent cells and inevitably cause undesirable side effects, highlighting the demand for developing innovative senomorphic strategies for specific regulation of cellular senescence.

**Methods:** To address this challenging, here we design a novel class of senomorphics with active cell targeting and activatable activities for selective regulation of SASP, by conjugating a senescence-targeted aptamer with a SA-β-gal-activated H2S donor. With senescent BJ cells as the cell model, a series of examinations have been performed to evaluate the performance of the engineered senomorphic (Apt-H2SD) for cell-specified regulation of SASP during cellular senescence.

**Results:** Apt-H2SD demonstrated specific binding and accumulation towards senescent cells over proliferating cells through the aptamer-mediated cell targeting. Upon internalization, Apt-H2SD was efficiently activated by the accumulated SA-β-gal in senescent cells, leading to the release of H2S precursor and subsequently suppressing the expression of three important SASP factors (IL-6, IL-1β and MMP3) at the mRNA level.

**Conclusion:** Our results strongly support the potential of Apt-H2SD as a valuable senomorphic. With rational design of the molecular structure, this study may offer a general strategy to build advanced senescence-targeted activatable senomorphics for precise intervention of cellular senescence and age-related diseases.

**Keywords**

Senescence-associated secretory phenotype, senomorphics, L1CAM, cellular senescence

**Introduction**

Senescence-associated secretory phenotype (SASP), as one of the most important metabolic and signalling features of senescent cells, has long been a hot topic in the field of senescence and aging research[1,2]. SASP can accelerate senescence in an autocrine fashion and trigger senescence of surrounding cells in a paracrine fashion, cumulatively aggravating the development of aging‐related diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders[3,4]. Suppressing SASP has thus been considered an effective way for treatment and/or prevention of age-related diseases[5]. In this regard, development of medications capable of specifically regulating SASP has become a research frontier in both academic and clinical fields.

Of particular significance is senomorphics, a class of senotherapeutics able to maintain the metabolic homeostasis and control over the pathological progress by attenuating the secreted SASP from senescent cells[6,7]. Senomorphics are commonly compounds from natural extraction or chemical synthesis, but most of these compounds lack selectivity to senescent cells and induce adverse effects on normal cells, given the fact that some SASP factors play important roles in many biological processes[7, 8]. Engineering senotherapeutic prodrugs represents an important alternative strategy to overcome this challenge owing to the control over drug activity[9-12]. Previously, researchers have reported a senescence-associated β-galactosidase (SA-β-gal)-activated H2S donner as an effective senomorphic prodrug[13]. It can be cleaved by the accumulated SA-β-gal in senescent cells, triggering the release of H2S to alleviate the levels of SASP and reactive oxygen species in senescent cells. Despite the controlled senomorphic activity, SA-β-gal-activated senomorphics still suffer from nonspecific activation in some non-senescent cells, because of the limitation of using SA-β-gal for distinguishing cellular senescence from other cell states with endogenous expression of β-gal[14]. Therefore, strategic innovations are highly in demand to further improve the senomorphic selectivity during regulation of cellular senescence.

Aiming at achieving this goal, we conceive and report a senescence-targeted activatable senomorphic via combination of the membrane biomarker-mediated active cell recognition with intracellular biomarker-promoted prodrug activation mechanisms. Specifically, an aptamer targeting L1CAM, a transmembrane protein that plays important roles in various cellular processes and overexpressed in various senescent cells, was chosen as the targeting ligand, and SA-β-gal-activated H2S donor was used as the prodrug moiety for constructing senescence-targeted activatable senomorphic (Apt-H2SD). Benefiting from the aptamer moiety, Apt-H2SD demonstrates active targeting and enhanced cellular uptake in senescent cells against proliferating cells. Under the receptor-mediated endocytosis, Apt-H2SD could be in-situ activated by the accumulated SA-β-gal in lysosomes of senescent cells, triggering the release of H2S for regulating the SASP signature. Our results have suggested that Apt-H2SD holds the potential as a useful molecular tool for precise intervention of cellular senescence and the associated disorders.

**Materials and methods**

**Synthesis of compounds**

Compounds 2, and 3 were synthesized according to previously published procedure[9]. To synthesize compound 4, NaH (45 mg, 1.12 mmol) was added into a solution containing compound 3 200 mg,0.37 mmol) and p-tolylisothiocyanate (67 mg, 0.45 mmol) in anhydrous THF (20 mL) at 0°C. The mixture was stirred for 30 min at 0°C, then at room temperature for another 4 h, after which the reaction was quenched with H2O, extracted with ethyl acetate. The solvent was removed by a rotary evaporator, and the residue was purified by column chromatography to obtain compound 4 in 48% yield.

For compound 5 (H2D), compound 4 (123 mg, 0.18 mmol) was dissolved in a mixture of methanol and dichloromethane (5:3, w/w). Thereafter, sodium methoxide (65 mg, 1.2 mmol) was added at 0°C. The mixture was stirred at 0 °C for 30 min, and then the solution was neutralized with amberlite IRC 50 for 10 min, after which the solvent was removed by a rotary evaporator. Finally, the residue was purified by column chromatography to give compound 5 in 78% yield.

For compound 6(Apt-H2SD),Azide-modified anti-L1CAM aptamer (50 nmol) was dissolved in 50 µL of ddH2O and mixed with 50 µL of the TEAA buffer (2 M). 150 mL of DMF containing 500 nmol compound 5 was added into the above mixture. Thereafter, 25 µL of Cu-TBTA complex (10 mM in 55 vol % DMSO) and 25 µL of ascorbic acid (5 mM) were mixed and added. After stirring for 4 h at 37 °C, the mixture was purified by HPLC to obtain Apt-H2SD. HPLC procedures and the DNA sequences used in this study were summarized in Table S1 and Table S2, respectively.

**H2S release studies.** Therelease of H2S from H2SD was determined by a methylene blue (MB) colorimetric assay according to the previous method[13]. Briefly, a 5 mM stock solution of H2SD was added into PBS containing β -gal (0.5 U/mL) and CA (25 μg/mL), followed by incubation at 37°C under shaking. MB colorimetric assay was performed, and the absorbance at 670 nm was determined for calculation of the release of H2S. For reference, a blank solution without the addition of β-gal was also investigated.

**Cell culture.** BJ cells were purchased from ATCC, and cultured in DMEM with the addition of 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) in an incubator with a humidified atmosphere of 5% CO2 at 37°C.

**Senescence introduction and characterizations**

For establishing H2O2-induced senescent cell model, BJ cells were seeded at 6 × 105cells per well and cultured for 24 h. Then, the cells were cultured with DMEM containing H2O2 (600 µM) for 6 days, followed by replacement of the medium with fresh DMEM for the following experiments.

To prove the successful senescence introduction, the activity of SA-β-gal in cells was first tested with the senescence β-galactosidase staining kit. Briefly, 1×105 BJ cells with or without H2O2 treatment were plated in the 6-well plate for 24h, respectively. Then these cells were washed and fixed, followed by SA-β-gal staining according to the manufacture’s procedures. Finally, the cells were imaged using an inverted microscope (Olympus).

Meanwhile, the cell proliferation ability was examined with EdU assay. Briefly, 1×105 BJ cells per well treated with or without H2O2 were plated in the 6-well plates. After adherence, the cell proliferation ability was examined as the manufacturer’s protocols described and imaged using an inverted microscope.

**Cytotoxicity studies**

Cell viability was determined with the CCK-8 assay. Briefly, 4000 cells per well of proliferating/senescent BJ cells were plated in 96-well plates and cultured for 24h, the cells were incubated with freshly prepared DMEM with different concentrations of Apt-H2SD (0, 0.125, 0.25, 0.5, 1 and 2 μM) for 24 h. Subsequently, the cell viability was tested according to the CCK-8 detection assay.

**Cell binding analysis.** The targeted binding capability of Apt-H2SD towards senescent cells was evaluated by flow cytometry and confocal fluorescence imaging. For flow cytometry, proliferating/senescent BJ cells were digested with 0.2% EDTA and washed twice by DPBS. Apt-H2SD and Con-H2SD labeled with Cy5 (250 nM) were respectively incubated with 1.5×105 cells in a serum-free DMEM for 45 min at 4°C. After that, the cells were centrifuged and washed for flow cytometry (BD FACS Verse).

For confocal fluorescence imaging, proliferating/senescent BJ cells were plated in the confocal dish at 1.0×105 cells per well. After cultured for 24 h, the cells were washed twice with DPBS and cultured with 200 μL of serum-free DMEM containing 250 nM Cy5-Apt-H2SD/Con-H2SD for 45 min at 4°C. Then the cells were washed and stained with Hoechst. The cellular fluorescence was tested by confocal laser scanning microscope (FV1000 confocal microscope, Olympus).

**SASP inhibition analysis.** The expression of SASP in senescent BJ cells treated with or without Apt-H2SD were investigated on the mRNA level using RT-qPCR. After treatment with Apt-H2SD, all the experimental manipulations were the same as previously described[9]. The primers for this experiment are summarized in Table S3.

**Results**

The synthesis of Apt-H2SD was illustrated in Figure 1. Glycosylated intermediate (compound 3) was first synthesized according to previous work[9], which is an ideal platform for conjugating the H2S donating moiety at the benzylic position and the targeting ligand on the terminal alkyne. The following conjugation with p-tolylisothiocyanate and deprotection of the hydroxy groups gave rise to the cleavable compound 5 (H2SD). In the presence of SA-β-gal, H2SD could release COS, which would be rapidly converted to H2S by the ubiquitous enzyme carbonic anhydrase (CA) inside the senescent cells to execute the SASP regulation. Finally, anti-L1CAM aptamer was conjugated at the tail via click chemistry to yield the senescence-targeted activatable molecular senomorphic. All the intermediates were characterized by NMR spectra, and Apt-H2SD was characterized by MS (Figure S1-S9).



**Figure 1.** Synthetic route for Apt-H2SD and its H2S release mechanism. (I) Propargyl bromide, Al, HgCl2, THF. (II) Ag2CO3, 1,1,4,7,10,10-hexamethyltriethylenetetramine, 2,3,4,6-tetra-O-acetyl-α-d-galactopyranosyl bromide, CH3CN, rt. (III) P-tolylisothiocyanate, NaH, THF. (IV) CH3OH, CH3ONa. (V) CuSO4, sodium ascorbate, N3-labeled anti-L1CAM aptamer, DMF/H2O.

With H2SD in hand, we proceeded to investigate its enzyme responsive and H2S releasing capabilities. In the presence of β-gal and carbonic anhydrase via methylene blue assay. As shown in Figure 2, H2SD demonstrated rapid release of H2S upon incubation in the PBS buffer containing β-gal and CA, and the reaction was almost completed within 2 h, with the release efficiency of about 50%. In sharp contrast, no H2S production was detected in the absence of β-gal within the same time period, suggesting that only the presence of CA did not induce the cleavage of thiocarbamates. Collectively, these results confirmed the β-gal-activated property of H2SD.



**Figure 2.** (A)The standard curve for measurement of H2S with the methylene blue (MB) method and Na2S as the standard.(B)Time-dependent H2S release from H2SD in the presence vs absence of β-gal, as measured by the MB assay.

After having confirmed the controllable activity, we next wondered whether Apt-H2SD could achieve targeted recognition of senescent cells. As a proof-of-concept illustration, oxidative stress-induced cellular senescence was established with BJ cells as the model and H2O2 as the stress. SA-β-gal staining results showed that after stimulation of H2O2, BJ cells exhibited obvious enlargement in the cell volume, coupled with blue staining (Figure 3A), indicating the accumulation of SA-β-gal. 5-Ethynyl-2′-deoxyuridine (EdU)-based cell proliferation assay further confirmed that these stressed BJ cells lost the proliferating capability, as evidenced by the negative EdU staining (Figure 3B). On the other hand, we have also examined the levels of three senescence-associated markers including p16, p21, and IL-1β in these stimulated cells. Results showed a significant elevation in the expression of these markers at the mRNA level (Figure S10). Moreover, the upregulation of L1CAM in senescent BJ cells was verified through western blot analysis (Figure 3C).

Next, the cell recognition ability of Apt-H2SD towards senescent cells was evaluated by flow cytometry and confocal microscopy. As depicted in Figure 3D, H2O2-induced senescent BJ cells demonstrated a significant shift in fluorescence intensity after incubation with Cy5-labled Apt-H2SD, as compared to that in proliferating BJ cells. Moreover, such fluorescence shift in senescent BJ cells treated with Cy5-labled Apt-H2SD was much larger than that in senescent cells treated with Cy5-labled nontargeting Con-H2SD, suggesting that Apt-H2SD could selectively recognize senescent cells over proliferating cells. Consistent with results from flow cytometry, confocal imaging further confirmed the senescent cell-targeting capability of Apt-H2SD. Among different treatment groups, only senescent cells treated with Cy5-labled Apt-H2SD displayed bright red fluorescence (Figure 3E).

****

**Figure 3.** (A)SA-β-gal staining and (B) EdU staining images of proliferating BJ cells and H2O2-induced senescent BJ cells, respectively. (C) Western blot analysis of the expression of L1CAM in proliferating BJ cells and senescent BJ cells. (D) Flow cytometry analysis and (E) confocal imaging of proliferating BJ cells and senescent BJ cells after incubation with Cy5-labeled Apt-H2SD or Con-H2SD, respectively.

After having identified the cell targeting property, we next evaluate the senomorphic activity of Apt-H2SD in suppressing the secretion of SASP in senescent cells. When BJ cells were stimulated with H2O2, the expression of proinflammatory interleukins (IL-6 and IL1β) and matrix metalloproteinases 3 (MMP3) were upregulated as compared to non-treated cells. Intriguingly, Apt-H2SD could mitigate the expression of these SASP factors. On the other hand, Apt-H2SD demonstrated good biocompatibility, without causing adverse effects on the proliferating and growth in non-senescent cells at the tested concentrations (Figure S11).



**Figure 4.** Expression of IL-6, IL-1β, and MMP3 at the mRNA level in proliferating BJ cells and H2O2-induced senescent BJ cells after exposure to Apt-H2SD. Data are demonstrated as mean ± SD (n = 3). \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

**DISCUSSION**

Cellular senescence is a complex cellular stress response triggered by endogenous and/or exogenous stimuli[15]. Despite loss of the proliferative capability, senescent cells are still metabolically active, preserving the potential to secrete different types of SASP (e.g., pro-inflammatory cytokine, growth factors, chemokines, and matrix degrading enzymes)[16]. These signalling molecules can reprogram the tissue microenvironment and trigger inflammation, which in return promotes second senescence in surrounding cells, cumulatively driving aging and aging-related diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders[17, 18]. Thus, SASP has recently emerged as therapeutic targets for age-related diseases, and studies have found that selective regulation of SASP with senomorphics is an important strategy to delay the aging process and age-related diseases or to improve disease treatment[19]. Traditionally, senomorphics are discovered by high-throughput screening of large libraries composed of small chemicals and natural products, which may affect the signalling pathways of normal cells, resulting in unwanted side-effects[8, 20]. Although SA-β-gal-activated senomorphics have been developed to enhance the selectivity of senescent cells, precise intervention of cellular senescence is still a big challenge, because β-gal is expressed not only in senescent cells but also in certain normal cells and proliferating cancer cells. Hence, it is urgent to develop targeted approaches with enhanced specificity and efficacy towards SASP regulation during senescence.

This work therefore aimed to improve the applicability of senomorphic-based anti-senescence methods, and reported a novel class of senomorphics, denoted by Apt-H2SD, with active cell recognition and activatable senomorphic activity, by taking advantage of aptamer-prodrug conjugating strategy. The senomorphic activity of Apt-H2SD can be switched by SA-β-gal-catalysed cleavage of molecular structure, leading to the release of H2S donor and subsequently conversion of H2S by carbonic anhydrase (Figure 2), a ubiquitous enzyme in different mammalian cells. As a proof of concept, the feasibility of Apt-H2SD for regulating SASP has been examined, with oxidative stress-induced cellular senescence in BJ cells as the cell model. Cell-binding studies have shown that Apt-H2SD could selectively target and accumulate in senescent BJ cells over proliferating cells through the aptamer-mediated cell recognition (Figure 3). More impressively, treatment with Apt-H2SD could downregulate the expression of three types of important SASP factors in senescent cells (Figure 4), suggesting its potential for cell-specified 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 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.

**Authors’ contributions**

Author, Author, and Author conceived this work; Author, Author, Author, Author, Author, and Author collected and analyzed the data; Author, Author, and Author drafted the manuscript. The final submitted version of manuscript was reviewed and approved by all the authors.

**Availability of data and materials**

The data that support the findings of study are available from the corresponding author upon reasonable request.

**Financial support and sponsorship**

This study was supported by the National Key Research and Development Program of China (grant no. 2020YFA0210802), the National Natural Science Foundation of China (grant nos. NSFC22274044 and 21877031), and the Science and Technology Innovation Program of Hunan Province (grant no. 2018RS3043).

**Conflicts of interest**

The authors declare no competing financial interest.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**References**

1. Acklin S, Zhang M, Du W, Zhao X, Plotkin M, Chang J, et al. Depletion of senescent-like neuronal cells alleviates cisplatin-induced peripheral neuropathy in mice. Sci Rep, 2020, 10(1): 14170.

2. Childs BG, Durik M, Baker DJ, Van Deursen JM. Cellular senescence in aging and age-related disease: From mechanisms to therapy. Nat Med, 2015, 21(12): 1424-1435.

3. Chen H, Ruiz PD, Mckimpson WM, Novikov L, Kitsis RN, Gamble MJ. MacroH2A1 and ATM play opposing roles in paracrine senescence and the senescence-associated secretory phenotype. Mol Cell, 2015, 59(5): 719-731.

4. Martel J, Ojcius DM, Wu CY, Peng HH, Voisin L, Perfettini JL, et al. Emerging use of senolytics and senomorphics against aging and chronic diseases. Med Res Rev, 2020, 40(6): 2114-2131.

5. Tchkonia T, Zhu Y, Van Deursen J, Campisi J, Kirkland JL. Cellular senescence and the senescent secretory phenotype: Therapeutic opportunities. J Clin Invest, 2013, 123(3): 966-972.

6. Lozono-Torres B, Estepa-Fernández A, Rovira M, Orzáez M, Serrano M, Martínez-Máñez R, et al. The chemistry of senescence. Nat Rev Chem, 2019, 3(7): 426-441.

7. Zhang L, Pitcher LE, Prahalad V, Niedernhofer LJ, Robbins PD. Targeting cellular senescence with senotherapeutics: Senolytics and senomorphics. FEBS J, 2023, 290(5): 1362-1383.

8. Lagoumtzi SM, Chondrogianni N. Senolytics and senomorphics: Natural and synthetic therapeutics in the treatment of aging and chronic diseases. Free Radic Biol Med, 2021, 171: 169-190.

9. Xia Y, Li J, Wang L, Luo X, Xie Y, Liu Y. Spatially confined intervention of cellular senescence by a lysosomal metabolism targeting molecular prodrug for broad-spectrum senotherapy. Angew Chem Int Ed Engl, 2022, 61(12): e202115764.

10. González-Gualda E, Pàez-Ribes M, Lozano-Torres B, Macias D, Wilson I I I, González-López C, et al. Galacto-conjugation of navitoclax as an efficient strategy to increase senolytic specificity and reduce platelet toxicity. Aging Cell, 2020, 19(4) : e13142.

11. Guerrero A, Guiho R, Herranz N, Uren A, Withers DJ, Martínez-Barbera JP, et al. Galactose-modified duocarmycin prodrugs as senolytics. Aging Cell, 2020, 19(4): e13133.

12. Cai Y, Zhou H, Zhu Y, Sun Q, Ji Y, Xue A, et al. Elimination of senescent cells by beta-galactosidase-targeted prodrug attenuates inflammation and restores physical function in aged mice. Cell Res, 2020, 30(7): 574-589.

13. Li J, Xie Y, Wang J, Wang L, Xia Y, Liu Y, et al. Activatable and self-monitoring hydrogen sulfide-based molecular senomorphics for visualized regulation of cellular senescence. CCS Chemistry, 2023, DOI: 10.31635/ccschem.023.202302714.

14. Li J, Wang L, Luo X, Xia Y, Xie Y, Liu Y, et al. Dual-parameter recognition-directed design of the activatable fluorescence probe for precise imaging of cellular senescence. Anal Chem, 2023, 95(8): 3996-4004.

15. Hernandez-Segura A, Nehme J, Demaria M. Hallmarks of cellular senescence J.. Trends Cell Biol, 2018, 28(6): 436-453.

16. Hayakawa T, Iwai M, Aoki S, Takimoto K, Maruyama M, Maruyama W, et al. Sirt1 suppresses the senescence-associated secretory phenotype through epigenetic gene regulation. PloS One, 2015, 10(1): e0116480.

17. Freund A, Orjalo A V, Desprez P Y, Campisi J. Inflammatory networks during cellular senescence: Causes and consequences. Trends Mol Med, 2010, 16(5): 238-246.

18. Niedernhofer LJ, Robbins PD. Senotherapeutics for healthy ageing. Nat Rev Drug Discov, 2018, 17(5): 377.

19. Guo J, Huang X, Dou L, Yan M, Shen T, Tang W, et al. Aging and aging-related diseases: From molecular mechanisms to interventions and treatments. Signal Transduct Target Ther, 2022, 7(1): 391.

20. Raffaele M, Vinciguerra M. The costs and benefits of senotherapeutics for human health. Lancet Healthy Longev, 2022, 3(1): e67-e77.