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β-galactosidase-activated Nitroxyl (HNO) Donors Provide Insights into Redox Cross-Talk in Senescent Cells

Laxman R. Sawase,[a] T. Anand Kumar,[a] Abraham B. Mathew,[b] Vinayak S. Khodade,[c] John P. Toscano,[c] Deepak K. Saini[b] and Harinath Chakrapani[a,*]

The cross-talk among reductive and oxidative species (redox cross-talk), especially those derived from sulfur, nitrogen and oxygen, influence several physiological processes including aging. One major hallmark of aging is cellular senescence, which is associated with chronic systemic inflammation. Here, we report a chemical tool that generates nitoxyl (HNO) upon activation by β-galactosidase, an enzyme that is over-expressed in senescent cells. In a radiation-induced senescence model, the HNO donor suppressed reactive oxygen species (ROS) in a hydrogen sulfide (H_2S)-dependent manner. Hence, the newly developed tool provides insights into redox cross-talk and establishes the foundation for new interventions that modulate levels of these species to mitigate oxidative stress and inflammation.

Many physiological processes depend on maintaining a fine balance between oxidative and reductive species in cells. When the oxidative equivalents exceed the cell's antioxidant capacity, cells are under oxidative stress. While this stress is useful for certain processes such as wound healing or immune response to pathogens,[1,2] high levels of reactive oxygen species (ROS) for extended durations can cause harmful and irreversible cellular damage.[3] For example, when exposed to ionizing radiation or during aging, due to the build-up of free radicals, cells are under oxidative stress. They enter a state of senescence, gradually compromising cellular function and eventually cell death. Senescent cells are characterized by increased ROS levels which leads to irreversible damage to biomolecules- DNA, proteins and lipids thus leading to a gradual decrease in cellular function.[4] Another interesting feature of senescent cells is the increase in hydrogen sulfide (H_2S) levels.[5] H_2S is an endogenously produced cell signalling molecule that plays critical roles in diverse physiological processes, including regulation of vasodilation, neuromodulation, anti-inflammatory effects, and oxidative stress regulation. H_2S is produced enzymatically primarily via three enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) (Figure 1A).[6] Protein persulfidation, which is an oxidative post-translational modification of cysteine residues mediated by H_2S is found to be elevated in senescent cells.[5]. It has been suggested that this modification can be protective for protein function.[7] The other gasotransmitter nitric oxide (NO), which is produced by nitric oxide synthase (NOS), is a short-lived intermediate in nitrogen metabolism and is another central player in redox biology. NOS levels appear to diminish during aging and senescent cells are associated with low levels of NO.[8] Although its biosynthesis is not well characterized, the reduced form of NO, nitroxyl (HNO) has distinct properties and has been considered as a therapeutic agent in cardiovascular disease.[9] Despite the unique chemical properties of HNO, its specific role in senescence remains unknown. Based on a report demonstrating the antioxidant properties of HNO,[10] we anticipate that it may exhibit beneficial effects in the context of senescence.

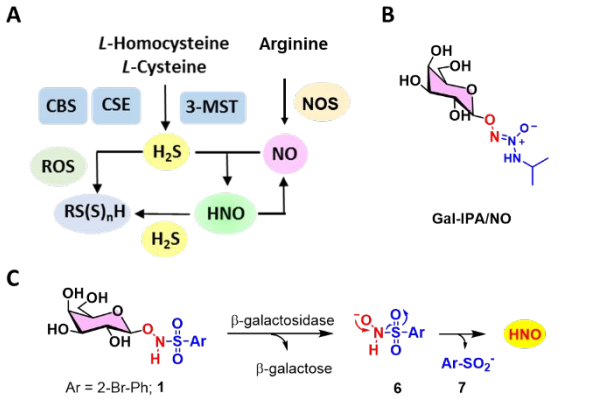

Figure 1. (A) Reaction of H_2S with NO to form HNO. NOS refers to nitric oxide synthases (nNOS/iNOS/eNOS); CBS/CSE/3-MST = H_2S biosynthesing enzymes; (B) Structure of Gal-IPA/NO; (C) Derivatives of **1** can be cleaved by β-galactosidase to produce 2-bromopiloty's acid (Ar = 2-BrPh, **6**), which generates HNO and an arylsulfinate **7**.

[a] *Department of Chemistry, Indian Institute of Science Education and Research, Pune, Pune 411 008, Maharashtra, India. E-mail: harinath@iiserpune.ac.in*
[b] *Department of Molecular Reproduction, Development and Genetics, Indian Institute of Science Bangalore 560012 Karnataka India*
[c] *Department of Chemistry, Johns Hopkins University, Baltimore, MD USA*
† Footnotes relating to the title and/or authors should appear here.
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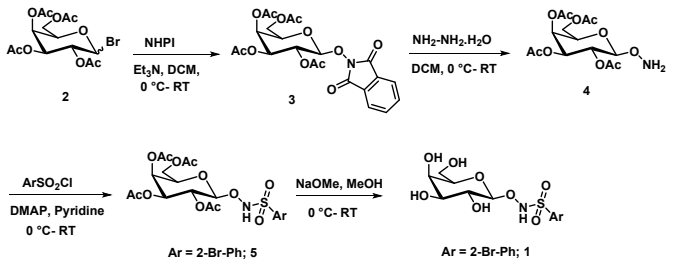
HNO is an unstable molecule, necessitating the use of donor molecules for its application. Numerous HNO-releasing compounds have been developed,[11] but there remains a need for selective HNO donors targeting senescent cells. Senescent cells are known to exhibit increased expression of β-galactosidase,[12] which provides an opportunity to develop small molecules specifically activated in these cells for HNO release. Piloty's acids have demonstrated potential as HNO donors due to their spontaneous decomposition under physiological conditions, releasing HNO.[13] To control HNO release, we have functionalized Piloty's acid with a β-galactose moiety (Figure 1B). By utilizing the β-galactose functional group as a substrate for the overexpressed enzyme, this modified HNO donor is designed to activate and release HNO selectively within senescent cells. Here, we report a small molecule that is cleaved under conditions associated with cellular senescence to generate HNO. Furthermore, we examine the HNO reaction with H_2S to produce polysulfides, which are important antioxidants in cells.[14] Additionally, we explore the antioxidant effects of the HNO prodrug in a radiation-induced senescence model.

As depicted in Scheme 1, compound **1** is synthesized in five steps. Starting from β-galactose pentaacetate, using a reported protocol, the 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl bromide **2** is prepared.[15] Reaction of **2** with *N*-hydroxy phthalimide in the presence of triethylamine gives **3**, which is treated with hydrazine hydrate to afford **4**.[16] *N*-sulfonation of **4** is carried out with the 2-bromobenzene sulfonyl chloride in the presence of dimethylaminopyridine yielding **5**.[13] The deprotection of **5** is accomplished using sodium methoxide in methanol to produce the desired 2,3,4,6-tetrahydroxy-β-D-galactopyranosyl derivative **1**. (Scheme 1).[17]



Scheme 1. Synthesis of **1**

Next, we quantified HNO generation from **1** by measuring nitrous oxide (N_2O) utilizing GC head-space analysis. This is a reliable method for measuring HNO[18] since HNO undergoes dimerization, followed by loss of H_2O, resulting in the production of N_2O. We initially measured the yield of N_2O from 2-bromopiloty's acid, which served as a positive control (Figure 2A). We then investigated whether the presence of β-galactosidase affects the measured HNO yield by potentially reacting with the released HNO. However, we observed similar levels of N_2O in the presence of the enzyme. Under similar conditions, the yield of N_2O from **1** in the presence of β-galactosidase is found to be nearly quantitative (94%), confirming its ability to efficiently release HNO.

Further, to evaluate HNO production from **1**, the HNO sensitive fluorescence probe, **PCM 8** was used. **1** was independently incubated in the presence of β-galactosidase in buffer pH 7.4 for 4 h and fluorescence enhancement was monitored (370 nm excitation; 460 nm emission). **1** showed comparable HNO generation with a known HNO donor, 2-bromopiloty's acid **6** (Figure 2B). In the absence of β-galactosidase, we did not observe a significant fluorescence increment (Figure 2B, ESI, Figure S1). Taken together, these data suggest that **1** was an excellent source of HNO generation.

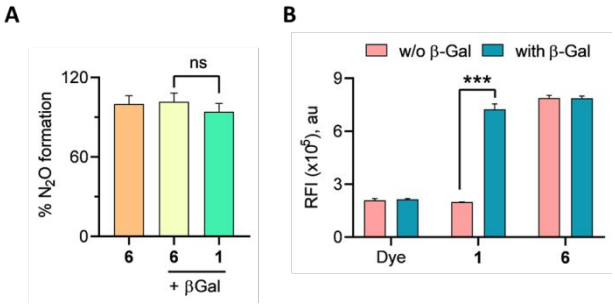


Figure 2. (A) N_2O detection. **6** and **1** were incubated in the presence of β-galactosidase (10 U/mL) in buffer (pH 7.4) for 15 h; (B) HNO detection using dye. **1** and **6** were incubated with dye in the presence and absence of β-galactosidase (10 U/mL) in buffer (pH 7.4) for 4 h; Dye refers to PCM **8**.

In a previous study, a significant amount of NO generation (29%) was observed from the β-galactosidase-cleavable donor Gal-IPA/NO (an HNO/NO prodrug).[19] Hence, we also examined NO generation from **1** using a Griess assay.[20] Briefly, **1** and **6** were independently incubated in the presence of β-galactosidase in buffer for 4 h. As expected, diminished NO generation is observed with **1** compared to **6** (ESI, Figure S2). This result is consistent with our previous report, which demonstrated that proper substitution at the *O*-atom of Piloty's acid derivatives can help to improve selectivity towards the generation of HNO over NO following deprotection.[21] Simultaneous generation of HNO and NO can complicate interpretations of biological studies since these entities are known to have opposite biological functions in certain models.[22] Moreover, HNO has the potential to interact with NO to further generate potentially toxic reactive nitrogen species.[23]

Next, we studied the decomposition of **1** in the presence of β-galactosidase in buffer pH 7.4 by both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (Figure 3, ESI, Figures S3 and S4). Upon treatment of **1** with β-galactosidase, we observed complete disappearance of **1** (RT 6.9 min) along with the formation of **7** (RT 8.9 min), and rate constants for decomposition of **1** as well as formation of **7** were found to be 0.039 h^{-1} and 0.018 h^{-1} (Figure 3). In the absence of β-galactosidase, no decomposition of **1** was observed. Docking analysis further supported this finding and showed a favourable interaction between the glycosidic moiety of **1** with the catalytic residue Q537 (3.7 Å) allowing the cleavage of the glycosidic bond to generate **7** (ESI, Figure S5-S7, Table S1-S2).

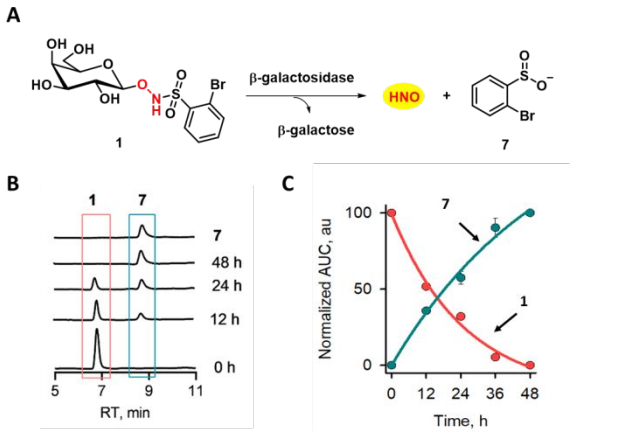


Figure 3. (A) Reaction scheme illustrating the generation of HNO and **7** from HNO prodrug **1**. (B) HPLC analysis showing the decomposition of **1** (50 μM) mediated by β-galactosidase (10 U/mL) and the formation of **7** (2-bromobenzene sulfinate) in pH 7.4 buffer at 37 °C. (C) Kinetics of decomposition of **1** and the generation of byproduct **7**. The data represent the average ± standard derivation (SD) (n = 3).

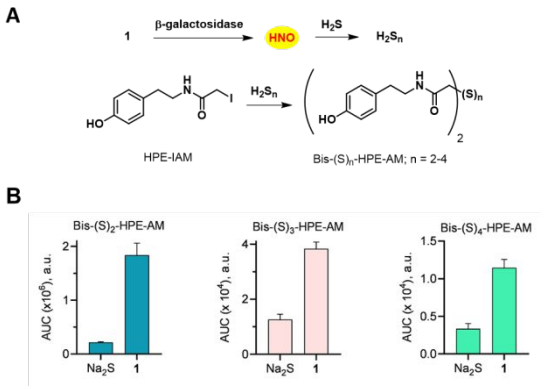


Figure 4: (A) Reaction scheme illustrating polysulfide formation through the reaction of HNO released from **1** with H_2S, followed by polysulfide trapping with HPE-IAM to generate Bis-$(S)_n$-HPE-AM. (B) Measurement of polysulfide formation by detecting trapped HPE-AM species after incubation of Na_2S (200 μM), and **1** (50 μM) + Na_2S (200 μM) with β-galactosidase (10 U/mL) in pH 7.4 buffer at 37 ˚C for 15 min. A comparison is made between polysulfides (e.g., H_2S_2, H_2S_3, and H_2S_4) detected by trapped HPE-AM species from H_2S alone and H_2S reaction with HNO released from **1**. The formation of the following species is observed: Bis-SS-HPE-AM (expected: m/z = 421.1250 $[M + H]^+$; observed: m/z = 421.1151), Bis-SSS-HPE-AM (expected: m/z = 453.0971 $[M + H]^+$; observed: m/z = 453.0865), and Bis-SSSS-HPE-AM (expected mass = 485.0692; observed mass = 485.0714).

While the precise mechanisms underlying the aging process remain elusive, accumulating evidence emphasizes the crucial roles of various reactive species in regulating cellular senescence. Notably, protein persulfidation, a posttranslational modification, has emerged as a significant player in aging.[24] Protein persulfidation involves the covalent modification of cysteine residues, resulting in the formation of cysteine hydropersulfides (Cys-SSH). Initially attributed primarily to H_2S, recent studies have revealed the ability of reactive sulfur species such as persulfides and polysulfides to persulfidate cysteine residues efficiently.[25] Hence, several approaches to directly enhance persulfide in cells using persulfide donors are in development.[26] Furthermore, recent investigations have demonstrated that the interaction between HNO and H_2S leads to polysulfide production.[27,21]

We measured the ability of prodrug-derived HNO to generate polysulfides in the presence of H_2S. Specifically, **1** was incubated with β-galactosidase in the presence of sodium sulfide in buffer for 15 min, followed by the addition of the trapping agent, β-(4-hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) and LC/MS analysis (Figure 4, Figure S8-10). [28] Results of this experiment support the ability of the HNO donor to enhance polysulfides in the presence of H_2S. Lastly, to understand if HNO generation was selective to activation by β-galactosidase, human embryonic kidney (HEK) 293 cell lysate was treated with **1** and the HNO fluorescent probe **9**. However, no detectable HNO generation was observed from prodrug **1**. (ESI, Figure S11; **6** was the positive control). HNO was detected when β-galactosidase was added exogenously.

Expanding upon these findings, we investigated the role of HNO in senescence using lung carcinoma A549 cells by employing prodrug **1**. Senescence was induced in A549 cells by exposing with 8 Gy of ionizing radiation.[5] Prior to conducting further experiments, we performed a standard cell viability assay to establish the tolerability of prodrug **1**, revealing that it is well tolerated up to a concentration of 100 μM (Figure 5A).

We then examined the effects of enhancing HNO levels on ROS in senescent cells. ROS levels were measured using DCF-H_2DA, a ROS-responsive fluorescence turn-on probe.[5] Specifically, A549 cells alone or treatment with **1** were incubated for 48 h followed by treatment with the DCF-H_2DA probe for 40 min in the dark. The fluorescence signal (excitation, 492 nm and emission, 525 nm) corresponding the formation of fluorescein was monitored. Compared to basal ROS levels, cells treated with **1** exhibit a lower fluorescence response, suggesting the ability of the prodrug **1** to mitigate ROS in senescent cells (Figure 5B). To understand if there is any role for H_2S in this antioxidant effect, we carried out a similar experiment with β-cyano-*L*-alanine (BCA), a cystathionine gamma-lyase (CSE) inhibitor.[29] The inhibitor alone do not show a significant effect on ROS levels. However, when the cells were treated with BCA and **1**, we do not observe reduction in ROS levels, supporting a role for H_2S in the observed antioxidant effect of HNO.

While the findings presented in this study are preliminary and require further extensive investigation, they suggest the potential involvement of polysulfides as mediators of the antioxidant effects of HNO. To the best of our knowledge, this study represents the first report examining the effects of HNO in senescence. This study opens new avenues for understanding the role of this reactive species in the aging process. This finding is significant, as it aligns with the emerging understanding of the regulatory roles of hydrogen polysulfides and hydropersulfides in redox biology. These reactive species possess antioxidant properties, including the ability to directly scavenge lipid peroxides,[30] and can activate crucial antioxidant pathways such as nuclear factor erythroid 2-related factor 2 (Nrf2) activation. [31] Moreover, our study revealed that prodrug **1** effectively reduced ROS in senescent cells, and this reduction was dependent on the presence of H_2S. Overall, our study opens up

new avenues for the development of innovative antioxidants that harness the redox cross-talk between H_2S and HNO, holding promise for novel therapeutic interventions.

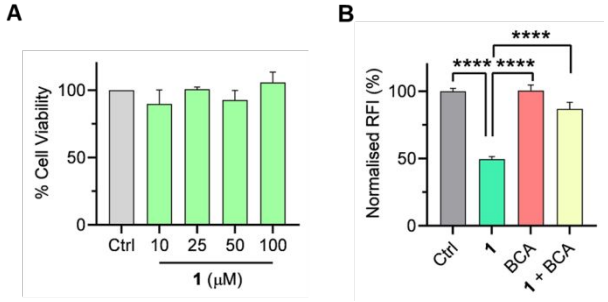


Figure 5. (A) Cell viability assay. Senescent A549 cells were treated with varying concentrations of **1** for 48 h; (B) ROS quenching assay was conducted on senescent A549 cells with **1** and BCA for 48 h; Ctrl refers to DMSO treated cells; BCA refers to CSE inhibitor.

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Conflicts of interest

There are no conflicts to declare.

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