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Organelle-Targeted H₂S Probes Enable Visualization of the Subcellular Distribution of H₂S Donors

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Abstract

Hydrogen sulfide (H₂S) is an essential biological signaling molecule in diverse biological regulatory pathways. To provide new chemical tools for H₂S imaging, we report here a fluorescent H₂S detection platform (**HSN2-BG**) that is compatible with subcellular localization SNAP-tag fusion protein methodologies and use appropriate fusion protein constructs to demonstrate mitochondrial and lysosomal localization. We also demonstrate the efficacy of this detection platform to image endogenous H₂S in Chinese hamster ovary (CHO) cells and use the developed constructs to report on the subcellular H₂S distributions provided by common H₂S donor molecules AP39, ADT-OH, GYY4137, and diallyltrisulfide (DATS). The developed constructs provide a platform poised to provide new insights into the subcellular distribution of common H₂S donors and a useful tool for investigating H₂S biochemistry.

Graphical abstract

Hydrogen sulfide (H₂S) has emerged as an important biological molecule involved in human health and has motivated significant efforts to develop small-molecule chemical tools to study the different roles of H₂S in biology and medicine. Concomitant with the expanding roles of H₂S in biology, new chemical tools for H₂S detection and delivery have emerged as two pillars of investigative studies.^{1–3} For example, development of fluorescent H₂S reporters

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Supporting Information

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Notes

The authors declare no competing financial interest.

has emerged as an attractive strategy to image H₂S genesis with the potential to provide spatiotemporal feedback on H₂S genesis and action. Complementing detection strategies, the development of H₂S donor molecules has emerged as an important strategy for delivering H₂S at slow, sustained rates, akin to enzymatic H₂S synthesis. These methods offer distinct benefits over direct administration of NaSH, which results in a large bolus of H₂S that is quickly metabolized and detoxified by cellular machinery. Despite these benefits, significant challenges remain including separating the pharmacological and toxicological profiles of H₂S and resolving controversies including the observation that different donors of H₂S exert seemingly paradoxical pro- and anti-inflammatory responses. We view that much of this controversy may stem from different bioavailability and localization profiles from synthetic donors.

One strategy to address key needs related to both H₂S sensing and delivery is to develop a single sensing platform that can be localized to different subcellular organelles and could be used to investigate the subcellular release of H₂S from different donor constructs. In addition, subcellular localization would also increase the dynamic range of the probes by lowering diffuse background signal. One common method to impart subcellular localization to small molecule constructs is to append functional groups that direct the molecules to specific subcellular compartments. For example, triphenylphosphonium and morpholine functionalization are often used to direct molecules to the mitochondria and lysosome, respectively. Although such modifications impart subcellular localization, they also increase the number of compounds that must be prepared and can also change the properties of the reporter, thus, making direct comparisons between different scaffolds difficult. One option to circumvent this problem is to genetically encode sensing motifs, green fluorescent proteins containing unnatural amino acids, into different cell lines. A second strategy is to use one probe construct that is compatible with fusion protein techniques to covalently attach the small molecule to proteins that naturally localize in different cellular compartments. We note that initial applications of this approach for H₂S sensing appeared in the literature during the review process of the present manuscript. Two of the most popular of such techniques include HaloTag, which uses alkyl chlorides in combination with dehalogenase enzymes, and SNAP-tag, which uses benzyguanine-ligated substrates in combination with AGT fusion proteins to generate subcellularly localized probes. Motivated by this need, we report here the development of a genetically registered, organelle-targeted H₂S probe utilizing SNAP-tag methodology and use this developed system to image the differential subcellular donation of H₂S from selected synthetic donors.

EXPERIMENTAL SECTION

Material and Methods

Flash chromatography was performed using silica gel and an automated flash chromatography instrument. Thin-layer chromatography (TLC) was performed on silica gel plates (250 μ m thickness) and viewed by UV illumination. NMR spectra were acquired on either a 500 or 600 MHz spectrometer at 25.0 °C. Chemical shifts are reported in parts per million (δ) and are referenced to residual protic solvent resonances. The following abbreviations are used in describing NMR couplings: (s) singlet, (d) doublet, (t) triplet, (m)

multiplet, and (b) broad. Fluorescence spectra were obtained on an ISS PC1 fluorimeter equipped with a cuvette temperature controller. Aminooxyacetic acid (AOAA) and diallyltrisulfide (DATS) were purchased from Cayman Chemicals, and anhydrous NaSH was purchased from Strem Chemicals. GYY4137, ADTOH, and AP39 were prepared as described in the literature. SNAP tag precursors were prepared as described previously.

Plasmid Construction of Human O⁶-Alkylguanine-DNA Alkyltransferase (AGT) Fusion Proteins

The full length *cox8*, *LAMP1*, and *EYFP:SNAP* plasmid inserts were purchased from Integrated DNA Technologies. The mammalian expression plasmid pSNAPf (New England BioLabs) was digested with *NheI*-HF/*NotI*-HF and ligated to the gBlock *EYFP:AGT* (*EYFP:AGT* backbone). All fragments were cloned into the N-terminus of *EYFP:AGT* backbone.

Cell Culture and Fluorescence Labeling of AGT Fusion Proteins

Chinese hamster ovary (CHO-K1) cells, which are AGT-deficient, were cultured in F12K media (LifeTech) supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% penicillin/streptomycin. Cells were seeded on a 22 mm diameter glass coverslip at $\sim 2 \times 10^6$ cells per well in a six-well culture dish and allowed to adhere for 24 h. Transient expression of the AGT-fusion protein was performed by following the standard Lipofectamine LTX and Plus Reagent protocol. CHO-K1 cells were incubated for 72 h at 37 °C, 5% CO₂ to allow for high efficacy protein expression.

For AGT labeling, the cells were washed with 1× Dulbecco's phosphate buffered saline (DPBS) and incubated with 1 mL of labeling media (OptiMEM) containing 5 μM of the HSN2 benzyl-guanine conjugate (**HSN2-BG**) for 30 min at 37 °C. The labeling media was then removed and cells were washed with 1× DPBS twice to remove excess probe. For colocalization studies, fresh OptiMEM media containing Far-Red MitoTracker (300 nM) or Far-Red LysoTracker (90 nM) was added to the cells, after which they were incubated at 37 °C with 5% CO₂ for an additional 30 min. For nuclear staining, four drops of NucLIVE stain was added to each well after **HSN2-BG** treatment and incubated for another 30 min at 37 °C with 5% CO₂. Cells were then washed three times with 1× DPBS and fixed for 15 min with 3.7% formaldehyde in 1× DPBS at 37 °C followed by two rinses and two washes with 1× DPBS. Coverslips containing fixed cells were mounted in Vectashield Hardset Mounting Medium (Vector Laboratories).

For inhibition of endogenously produced H₂S studies, transiently transfected CHO-K1 cells were treated with aminooxyacetic acid (AOAA, 20 μM) for 45 min at 37 °C with 5% CO₂, cells were then washed with 1× DPBS twice to remove any excess AOAA. **HSN2-BG** (5 μM) was then introduced to the media and incubated at 37 °C with 5% CO₂ for 30 min. Cells were then washed three times with 1× DPBS and fixed for 15 min with 3.7% formaldehyde in 1× DPBS at 37 °C followed by two rinses and two washes with 1× DPBS. Coverslips containing fixed cells were mounted in Vectashield Hardset Mounting Medium (Vector Laboratories).

Mitochondria-targeted H₂S donor studies were performed by treating transiently transfected CHO-K1 cells with AP39 for 1 h incubation at 37 °C and 5% CO₂. Cells were washed with 1× DPBS and then treated with **HSN2-BG** (5 μM) for 30 min at 37 °C and 5% CO₂. Excess substrate was washed off with 1× DPBS and fixed for 15 min with 3.7% formaldehyde in 1× DPBS at 37 °C, followed by two rinses and two washes with 1× DPBS. Coverslips containing fixed cells were mounted in Vectashield Hardset Mounting Medium (Vector Laboratories).

Fluorescence Microscopy and Statistical Analysis

Images were acquired on a confocal microscope (Olympus Fluoview 1000) using an oil-immersion 40× (1.3 NA) or 60× (1.4 NA) objective and laser excitation sources (405, 488, 515, 635 nm). All images were processed with ImageJ. Fluorescent data was analyzed using ImageJ software and all statistical comparisons were performed using Prism. (One-way ANOVA with Dunnett's posttest was performed using GraphPad Prism version 5.01 for Windows; GraphPad Software: San Diego, CA www.graphpad.com). For fluorescence quantification, regions of interest (ROIs) were defined by the EYFP signal. This treatment provided identical results to whole-image analysis, but was employed to ensure that only colocalized HSN2-BG fluorescence was used in the fluorescence quantification.

Syntheses

2-(4-((2-Amino-9*H*-purin-6-yl)oxy)methyl)-benzyl)-6-azido-1*H*-benzo[de]isoquinoline-1,3(2*H*)-dione (**HSN2-BG**). Amine **2** (200 mg, 0.739 mmol) was suspended in ~11 mL of dioxane and 6-azidobenzo[de]isochromene-1,3-dione (**1**) was added to the solution. The reaction was refluxed under nitrogen in the dark for 7 h. The reaction mixture was then allowed to cool to room temperature, and the solvent was evaporated under vacuum. The resultant crude product was purified by SiO₂ chromatography (5% MeOH in DCM) to yield the desired product (80.0 mg, 22% yield). ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.57 (m, *J* = 4.13 Hz, 1H, ArH), 8.56 (d, *J* = 1.15 Hz, 1H, ArH), 8.52 (d, *J* = 3 Hz, 1H, ArH), 8.45 (d, *J* = 9.73 Hz, 1H, ArH), 8.25 (t, *J* = 8.2 Hz, 1H, ArH), 7.89 (t, *J* = 7.9 Hz, 1H, ArH), 7.77 (d, *J* = 8.13 Hz, 1H, ArH), 7.33 (d, *J* = 10.27 Hz, 2H, ArH), 7.25 (d, *J* = 7.95 Hz, 2H, ArH), 5.20 (s, 2H, CH₂), 5.11 (bs, 2H, NH₂), 4.42 (s, 2H, CH₂). ¹³C{¹H} NMR (150 MHz, DMSO-*d*₆) δ 163.8, 163.5, 163.4, 163.3, 143.6, 141.9, 136.2, 136.0, 133.4, 132.4, 132.0, 130.4, 129.9, 128.9, 127.9, 127.0, 124.1, 123.2, 122.5, 118.5, 116.5, 63.1, 43.3.

RESULTS AND DISCUSSION

We chose to use the SNAP-tag methodology due to its compatibility with the **HSN2** H₂S detection platform (vide infra) as well as its previous and effective application for other small bioinorganic analytes including Zn²⁺, Ca²⁺, and H₂O₂. To develop a SNAP-tag compatible H₂S probe, we modified the **HSN2** system, which we reported previously and which has been used by other groups to investigate H₂S-mediated cytoprotection and differentiation in cancer cell lines, as well as the role of CBS in breast cancer cells.[−] We expected that simple modification of the **HSN2** platform with SNAP-tag compatible benzylguanine moiety would require only simple synthetic modifications but would increase

the efficacy of the **HSN2** platform for organelle-specific detection of endogenous H₂S and could provide insight into subcellular release patterns of synthetic H₂S donors. To prepare the required **HSN2** conjugate, we treated azidonaphthalic anhydride (**2**) with benzyl guanine precursor **1** to afford benzylguanine ligated **HSN2-BG** (Scheme 1).

With **HSN2-BG** in hand, we confirmed that **HSN2-BG** functioned as a viable H₂S reporter by incubating **HSN2-BG** with purified AGT protein, isolated from *E. coli*, in a 1:1 ratio for 30 min at 37 °C (Scheme 2), followed by the addition of 10 equiv of NaSH. Under these in vitro conditions, we observed an 8-fold fluorescence turn-on response (Figure S1). This lower fluorescence enhancement by comparison to the parent **HSN2** is likely due to the shorter reaction time of 30 min, lower NaSH concentration used, and the presence of 1 mM DTT required for in vitro AGT ligation. We also observed that ligation of **HSN2-BG** to AGT was required for fluorescence activation by H₂S. For example, treatment of **HSN2-BG** with NaSH in the absence of AGT failed to produce a fluorescence response, suggesting that the guanine moiety of **HSN2-BG** may effectively quench the naphthalimide fluorescence, which has been observed previously for common fluorophores.

Having verified that AGT-conjugated **HSN2-BG** is an effective H₂S reporter in cuvettes, we next used Chinese hamster ovarian-K1 (CHO) cells to translate our investigation into cellular systems. We chose to use CHO cells because they do not express AGT endogenously, which ensures that only intentionally transfected AGT is present. To confirm bioconjugation of **HSN2-BG** to AGT in CHO cells, we transiently transfected CHO cells with fusion construct containing both EYFP and AGT (EYFP:AGT), followed by treatment with **HSN2-BG**. Additionally, the excitation profiles of **HSN2** ($\lambda_{\text{max}} = 435$ nm) and EYFP ($\lambda_{\text{max}} = 515$ nm) are sufficiently separated to ensure efficient but separate excitation of each fluorophore. Indeed, in the absence of the **HSN2-BG** conjugate, excitation in the **HSN2-BG** channel failed to produce a fluorescence response in the EYFP channel, confirming signal separation under our experimental conditions. We note that, although EYFP is not required for AGT function, incorporation of the genetically encoded fluorophore allows for simple tracking of transfection efficiency (which we determined to be >85%), and it also serves as an internal standard to confirm colocalization of the EYFP fluorescence with **HSN2-BG**. For each fusion construct, use of the raw fluorescence intensities or those scaled to the EYFP internal standard provided equivalent results, which is consistent with both high transfection and AGT labeling efficiencies. In these experiments, colocalization of EYFP:AGT and **HSN2-BG** fluorescence was observed. By contrast, treatment of CHO cells that had not been transfected with the EYFP:AGT construct with **HSN2-BG** failed to generate a fluorescent response, confirming that ligation to AGT is required for signal generation (Figure 1).

We next constructed organelle-specific conjugates by appending EYFP and AGT to the N-terminus of different proteins that naturally localize to different subcellular compartments. Because 3-mercaptopyruvate sulfurtransferase (3-MST), one of the primary enzymes in H₂S synthesis, resides in the mitochondria, we chose to generate a fusion protein targeted to this cellular compartment. We also reasoned that the small-molecule H₂S donors may accumulate in the lysosome, so generated fusion proteins using Cox8 (cytochrome c-oxidase VIII) and LAMP1 (lysosomal-associated membrane protein I) to generate constructs localized to the mitochondria and lysosome, respectively. After transfection, we monitored

the EYFP and **HSN2-BG** fluorescence in combination with organelle-specific small molecule fluorescent markers and established that the AGT fusion proteins labeled the desired organelles (Figure 2). To further establish this colocalization, we measured the Pearson coefficient for the different conjugates. In the Cox8 and LAMP1 systems, Pearson coefficients of 0.89 and 0.87, respectively, between the **HSN2** and organelle-specific signals confirmed excellent colocalization.

Having established that **HSN2-BG** is a suitable substrate for intracellular labeling of different AGT constructs, we next investigated whether the localized constructs were sufficiently sensitive to detection endogenous, enzymatically produced H₂S. Our expectation was that covalent ligation of **HSN2-BG** to specific subcellular locales would increase the dynamic range of the probe by significantly reducing the background fluorescence due to diffuse probe localization and, thus, facilitate endogenous H₂S detection. To test this hypothesis, we compared the observed fluorescence from the three organelle-specific conjugates with cells that had been treated with 20 μ M AOAA, a cystathionine γ -lyase (CSE) and cystathionine β -synthase (CBS) inhibitor. We observed that, for both lysosomal LAMP1:AGT, a significant reduction in fluorescence was observed. By contrast, mitochondrial H₂S levels imaged by Cox8:AGT were statistically unaltered. The differential response to AOAA in the mitochondria and the lysosome is consistent with AOAA inhibition of cytosolic CBS and CSE rather than the mitochondrial 3-MST. Taken together, these results suggest that the **HSN2-BG** platform is sufficiently sensitive to detect endogenous H₂S produced in individual organelles (Figures 3 and 5).

On the basis of the endogenous H₂S detection by **HSN2-BG** with AGT fusion constructs, we next used **HSN2-BG** to investigate the differential localization of H₂S release from common synthetic donors in specific organelles. In choosing representative donor molecules, we focused on compounds that have been used widely in different biological studies, which included phosphinodithioate donor GYY4137, anethole dithiolethione (ADT-OH), diallyltrisulfide (DATS), and mitochondrially targeted AP39, as well as NaSH for comparison (Figure 4). We note that other useful small molecule donors are also available and refer the interested reader to a recent review on this topic. Although it was not possible to test each donor at identical concentrations, we elected to use donor concentrations that have been used commonly in previous reports focusing on the biological and pharmacological effects of these donor molecules. These concentrations included 100 nM for AP39, 30 μ M ADT-OH, 400 μ M GYY4137, 10 μ M DATS, and 50 μ M NaSH. The primary goal of these studies was not to measure or refine the efficacies of the different donor motifs, but rather to demonstrate the benefits of subcellular localization in differentiating H₂S donation patterns from different common donors. Of these selected donors, AP39 was designed to be a mitochondrially targeted H₂S donor, but little is known about how other donors localize within cells.

To investigate the distribution of sulfide released from the selected donor molecules, we treated CHO cells transfected with the different localized EYFP:AGT constructs and **HSN2-BG** with each of the selected donors and monitored the fluorescence (Figure 5). Addition of AP39, ADT-OH, and GYY4137 resulted in significant increases in sulfide levels in the mitochondria. Highlighting the efficacy of targeted donors, treatment with 100 nM of AP39

elicited a similar fluorescence response to 30 μM of ADT-OH or 400 μM GYY4137, which are consistent with previous reports of the higher efficiency of AP39 in increasing cellular H_2S levels than other common donors. By contrast to the mitochondria studies, different H_2S signals were observed in the lysosome. Of these, only GYY4137 provided an increase in observed sulfide levels, which is consistent with the increased rate of H_2S release from GYY4137 under acidic conditions. By comparison to the altered H_2S levels observed with slow-releasing donor molecules, we note that 50 μM NaSH failed to elicit a significant fluorescence response within the cells, which is consistent with the rapid metabolism of high levels of free sulfide in biological milieu and highlights the efficacy of synthetic H_2S donors in delivering H_2S . Taken together, these results highlight the efficacy of using subcellular-localized H_2S reports as a viable method for investigating the H_2S release distribution and efficiency of different types of H_2S donors.

CONCLUSIONS

In conclusion, we have demonstrated that the use of AGT fusion proteins with a H_2S selective sensor (**HSN2-BG**) allows for detection of endogenous H_2S levels within specific cellular organelles. We leveraged the sensitivity and subcellular localization of these probes to investigate different commonly used H_2S donating molecules and observed significantly different localization patterns from the different constructs. Taken together, these results provide new insights into the subcellular H_2S release profiles of different H_2S donors, which may contribute to the different observed physiological outcomes of using different H_2S donor molecules in different contexts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

AGT fusion protein is required for **HSN2-BG** bioconjugation. (a–c) Transiently transfected CHO cells with EYFP:AGT and **HSN2-BG** (5 μ M). Scale bars = 10 μ m.

Figure 2.

Subcellular labeling of H_2S with **HSN2-BG** and fusion proteins. Columns include the EYFP signal, **HSN2** fluorescence, organelle label, and colocalization between the **HSN2-BG** signal and the organelle dye. (a–d) EYFP:Cox8:AGT construct and (e–h) EYFP:LAMP1:AGT construct. Scale bars = 10 μm .

Figure 3. Detection of endogenously produced H_2S within organelles of the cell. (top panels) Transiently transfected CHO cells with EYFP:AGT and **HSN2-BG** treatment ($5\ \mu\text{M}$). (bottom panels) CHO cells treated under identical conditions but with AOAA ($20\ \mu\text{M}$). Scale bars = $10\ \mu\text{m}$.

Figure 4.
Chemical structures of common slow-release H₂S donors investigated with **HSN2-BG**.

Figure 5.

Quantification of fluorescence in CHO-K1 cells from **HSN2-BG** transiently transfected with (a) Cox8:EYFP:AGT and (b) LAMP1:EYFP:AGT. Cells were treated with 5 μ M **HSN2-BG** and 20 μ M AOAA, 100 nM AP39, 30 μ M ADT-OH, 400 μ M GYY4137, 10 μ M DATS, or 50 μ M NaSH. * $p < 0.05$, ** $p < 0.01$.

Scheme 1.
Synthesis of HSN2-BG

Scheme 2.

Treatment of Protein Constructs, Labelled with EYFP and AGT, with HSN2-BG Results in Covalent Ligation of the H₂S Sensing Moiety