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Selective fluorogenic imaging of hepatocellular H₂S by a galactosyl azidonaphthalimide probe†

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We have developed a galactosyl azidonaphthalimide probe for the selective fluorogenic imaging of hepatocellular H2S, an important gaseous transmitter produced in the liver.

Despite its toxicity, hydrogen sulfide (H2S) has been identified as an important gaseous transmitter in many cellular signalling pathways. 1 Cystathionine β-synthase (CBS) and cystathionine γ -lyase (CSE) are the two major enzymes responsible for the production of H₂S with L-(homo)cystein as the substrate. Because of the tissue-specific distribution of these enzymes, H₂S is predominantly generated in the liver.² However, this metabolic organ is vulnerable to damage from the aberrant expression of redox-active species including H2S, leading to hepatic diseases such as liver cirrhosis.³ As a result, selective detection of hepatocellular H2S represents an important goal to aid not only cell biology research but also disease diagnosis.

Conventional techniques for H₂S detection depend on chromatographic and electrochemical sensors, which are not applicable for live cell imaging.4 To address these issues, numerous fluorescence small-molecule probes for H2S have been developed over recent years. The basis for the fluorogenic response (or fluorescence "OFF-ON") of the probes relies on H₂S-mediated reduction of azides,^{5,6} copper sulfide precipitation^{7,8} and nucleophilic addition strategies. 9-11 All these probe prototypes address sensitive and selective detection of the species, even in live cells and in vivo. 12-14 Nevertheless, although elegant H2S probes with cellular trapping, 15 lysosome 16 and mitochondria targeting 17 abilities have been developed, fluorogenic probes that address hepatocyte-selective imaging of the species are rare.

Recently, we¹⁸⁻²¹ and others²²⁻²⁶ determined that the introduction of a glycosyl moiety (as a targeting agent) to a fluorescence probe can greatly enhance its selective internalisation by cells derived from a certain tissue. This could be ascribed to the selective, sugar-receptor-mediated endocytosis of the glycoprobes by the cells. Here we show that a galatosyl azidonaphthalimide probe has the ability to image H2S selectively in hepatocytes among other cells, due to galactoside receptorpromoted endocytosis.

The desired DT-Gal was synthesised by a click-coupling of alkynyl naphthalimide 1 with azido galactoside 2, followed by a sequential azidation and deacylation (Fig. 1a). The presence of the azide weakens the fluorescence of naphthalimide by an ICT process, and a subsequent reduction by H2S produces aminonaphthalimide, enhancing the fluorescence. 14 DT-OH which is a known H₂S probe that lacks the galactosyl targeting agent was used as a control.²⁷

With these probes in hand, we tested their fluorescence response to H2S in an aqueous solution (PBS, pH 7.4). The presence of increasing H2S caused a gradual fluorescence increase of both DT-OH (Fig. 1b) and DT-Gal (Fig. 1c) with the latter being more sensitive. This might be a result of the better water solubility of the latter glyco-probe. Among a range of anions (Fig. 1d) and amino acids (Fig. S1, ESI†), both probes showed good selectivity. Mass spectroscopic analysis indicated that the aminonaphthalimide derivative of DT-Gal was produced upon reaction with H₂S (Fig. S2, ESI†).

A kinetic investigation determined that DT-Gal showed rapid fluorogenic response to H2S (Fig. 1e), and good linearity was observed by plotting the fluorescence intensity of the probe as a function of increasing H2S (Fig. 1f). The limit of detection of **DT-Gal** for H₂S was determined to be 0.78 μ M (3 σ _b/k). Additionally, we observed that the probe functioned well over a wide pH range (4-12, Fig. 1g), suggesting its applicability for cellular analysis.

Subsequently, we tested the ability of the probes to image H₂S in live cells. We used the human hepatoma cell line (Hep-G2) with over-expressed asialoglycoprotein receptor (ASGPr) that

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Communication

١, ١١ DT-OH DT-Gal 150 150 Wavelength (nm) Wavelength (nm) 1.0 0.8 0.6 0.4

/f (a.u.) 2250 0.2 7 8 9 1011121314 6 12 18 24 Concentration (10-5 M) Fig. 1 (a) Synthesis of DT-Gal and the structure of DT-OH. Reagents and

conditions: (I) CuSO₄·H₂O, Na ascorbate in H₂O-CH₂Cl₂. (II) NaN₃ in DMF, and then Et_3N . Fluorescence titration of (b) **DT-OH** (10 μM) and (c) **DT-Gal** (10 μ M) in the presence of increasing H₂S (0-200 μ M). (d) Fluorescence change of **DT-Gal** (10 μ M) in the absence (blank) and presence of, from left to right, F⁻, Cl⁻, Br⁻, I⁻, CO_3^{2-} , HCO_3^{-} , SO_4^{2-} , HSO_4^{-} , $S_2O_3^{-}$, PO_4^{3-} , HPO₄²⁻, H₂PO₄⁻, Phe, Ser, Thr, Pro, Val, His, Gly, Cys, GSH and H₂S (100 μ M), where I_f and I_{fo} are the fluorescence intensity of the probe in the presence and absence of analyte, respectively (inset: photographed fluorescence change of DT-Gal in the presence of H2S). (e) Plotting the fluorescence change of DT-Gal (10 μ M) with H₂S (200 μ M) as a function of time, where I_{fx} and I_{f0} are the fluorescence intensity of the probe with H₂S at different time points and that of the probe alone, respectively. (f) Plotting the I_f of **DT-Gal** (10 μ M) in the presence of increasing H₂S. (g) Plotting the I_f of DT-Gal (10 μ M) without H₂S (black) and with H₂S (200 μ M) (blue) at different pH. All fluorescence spectra were recorded in PBS (0.05 M, pH 7.4 or varied pH as indicated) with excitation at 426 nm.

recognises galactosides 18-21 as well as cancer cells without the receptor expression such as human colon cancer HCT116, cervix cancer HeLa and lung cancer A549. 18 We observed that, the addition of DT-Gal to the cell lines pre-treated with H₂S led to fluorescence enhancement for Hep-G2, but only a slight fluorescence increase for other cells (Fig. 2a and c). In sharp contrast, co-incubation of **DT-OH** with the cells led to slight, unselective fluorescence enhancement (Fig. 2b and d). These results suggest that the presence of the galactosyl warhead facilitates the selective internalisation of the H₂S probe probably by ASGPr-mediated endocytosis. 18-21 Notably, the assumed endocytosis led to much stronger fluorescence generation of DT-Gal than the DT-OH without a targeting group.

To corroborate that the imaging was a result of selective ASGPrgalactose interaction, we used a Hep-G2 cell line with a reduced ASGPr expression level (sh-ASGPr)21 to incubate with DT-Gal. We observed that addition of the galactosyl probe to sh-ASGPr resulted in a much weaker fluorescence compared to Hep-G2 (Fig. 3a and d). This correlates with the distinct ASGPr expression level of the two cell

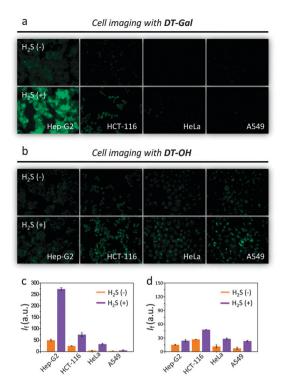


Fig. 2 Cell imaging (a for DT-Gal and b for DT-OH) and quantification of the fluorescence intensity (\emph{I}_{f}) (c for **DT-Gal** and d for **DT-OH**) in the absence (-) and presence (+) of H₂S (Hep-G2: human liver cancer; HCT-116: human colon cancer; HeLa: human cervix cancer; A549: human lung cancer).

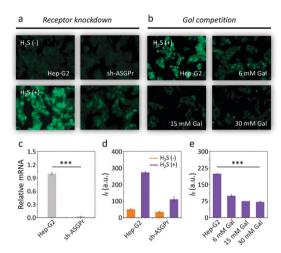


Fig. 3 Cell imaging (a) and fluorescence quantification (d) of DT-Gal for Hep-G2 and sh-ASGPr with reduced ASGPr expression. Cell imaging (b) and fluorescence quantification (e) of DT-Gal with Hep-G2 in the absence and presence of increasing competing free galactose. (c) ASGPr mRNA level of Hep-G2 and sh-ASGPr measured by PCR (***P < 0.001).

lines (Fig. 3c). Moreover, pre-incubation of increasing free galactose with Hep-G2 inhibited the fluorescence enhancement of DT-Gal in a concentration-dependent manner (Fig. 3b and e). These data support the assumption that the strong and selective fluorogenic signal produced by DT-Gal in raw Hep-G2 cells is facilitated by ASGPr-mediated endocytosis.

ChemComm Communication

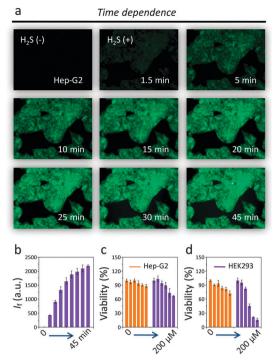


Fig. 4 Cell imaging (a) and fluorescence quantification (b) of DT-Gal for Hep-G2 with increasing incubation time. Cytotoxicity of (c) DT-Gal $(0{-}200~\mu\text{M})$ and (d) DT-OH $(0{-}200~\mu\text{M})$ for Hep-G2 and HEK293.

We also tested the kinetics of DT-Gal for H₂S imaging in the hepatocellular cell line. We observed a time-dependent fluorescence enhancement of the imaging experiments and equilibrium was achieved in about 30 min (Fig. 4a and b). Note that this cellular kinetics is slower than that in solution probably because of the complexity of the cellular environment. Interestingly, DT-Gal (Fig. 4c) was much less toxic towards Hep-G2 as well as a human kidney cell line (HEK293) than DT-OH (Fig. 4d). This suggests that, in addition to its targeting ability, the presence of the galactosyl moiety might also mitigate the intrinsic cytotoxicity of naphthalimide-based compounds.²⁸

We note that a similar azidonaphthalimide probe for H₂S has been previously reported.²⁹ However, unlike our DT-Gal probe, that probe does not contain a cellular targeting unit, which is vital for target-specific imaging in vivo. Since azidonaphthalimide can be reduced to the fluorescent amino derivative within cells, 25 we carried out a time-dependent imaging assay with Hep-G2 and just DT-Gal (Fig. S3, ESI†). While the fluorescence increased slightly, the co-existence of H₂S results in significantly enhanced fluorescence. This clearly demonstrates that the fluorescence enhancements observed here are due to H₂S recognition within the cells.

In conclusion, a galactosyl azidonaphthalimide based fluorogenic probe for hepatocellular-selective imaging of H₂S was developed, which sets a basis for the target-specific imaging of H₂S in the liver, the main organ that produces this gaseous transmitter.

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