



Cite this: *Chem. Commun.*, 2015, 51, 3653

Received 6th December 2014,
Accepted 20th January 2015

DOI: 10.1039/c4cc09771h

www.rsc.org/chemcomm

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

De-Tai Shi,^a Dan Zhou,^{ab} Yi Zang,^b Jia Li,^{*b} Guo-Rong Chen,^a Tony D. James,^c Xiao-Peng He^{*a} and He Tian^a

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 lysosome¹⁶ and mitochondria targeting¹⁷ abilities have been developed, fluorogenic probes that address hepatocyte-selective imaging of the species are rare.

Recently, we^{18–21} and others^{22–26} 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 glyco-probes by the cells. Here we show that a galactosyl azidonaphthalimide probe has the ability to image H₂S selectively in hepatocytes among other cells, due to galactoside receptor-promoted 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 μM (3σ_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

^a Key Laboratory for Advanced Materials & Institute of Fine Chemicals, East China University of Science and Technology, 130 Meilong Rd., Shanghai 200237, P. R. China. E-mail: xphe@ecust.edu.cn

^b National Center for Drug Screening, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 189 Guo Shoujing Rd., Shanghai 201203, P. R. China. E-mail: jli@simm.ac.cn

^c Department of Chemistry, University of Bath, Bath, BA2 7AY, UK

† Electronic supplementary information (ESI) available: Experimental section and additional figures. See DOI: 10.1039/c4cc09771h



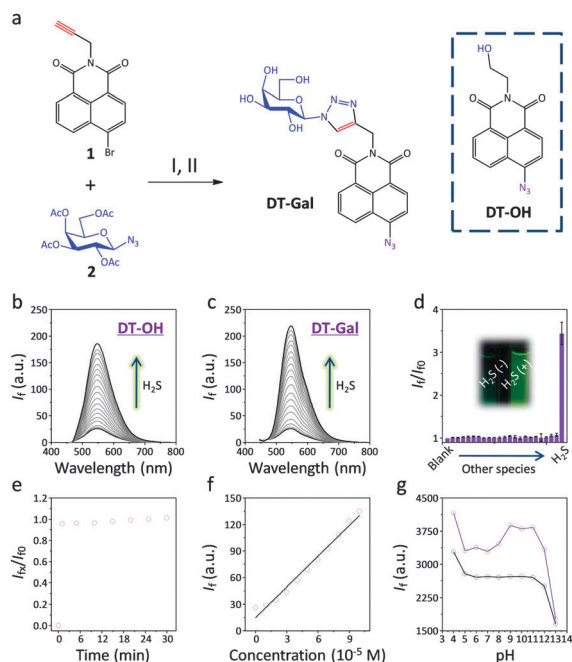


Fig. 1 (a) Synthesis of **DT-Gal** and the structure of **DT-OH**. Reagents and conditions: (i) $\text{CuSO}_4 \cdot \text{H}_2\text{O}$, Na ascorbate in $\text{H}_2\text{O}-\text{CH}_2\text{Cl}_2$. (ii) NaN_3 in DMF, and then Et_3N . Fluorescence titration of (b) **DT-OH** (10 μM) and (c) **DT-Gal** (10 μM) in the presence of increasing H_2S (0–200 μM). (d) Fluorescence change of **DT-Gal** (10 μM) 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^- , $\text{S}_2\text{O}_3^{2-}$, PO_4^{3-} , HPO_4^{2-} , H_2PO_4^- , Phe, Ser, Thr, Pro, Val, His, Gly, Cys, GSH and H_2S (100 μM), where I_f and I_{f0} 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 H_2S). (e) Plotting the fluorescence change of **DT-Gal** (10 μM) with H_2S (200 μM) as a function of time, where I_{f_t} and I_{f_0} are the fluorescence intensity of the probe with H_2S at different time points and that of the probe alone, respectively. (f) Plotting the I_f of **DT-Gal** (10 μM) in the presence of increasing H_2S . (g) Plotting the I_f of **DT-Gal** (10 μM) without H_2S (black) and with H_2S (200 μM) (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 H_2S 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 H_2S 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 ASGPr–galactose 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

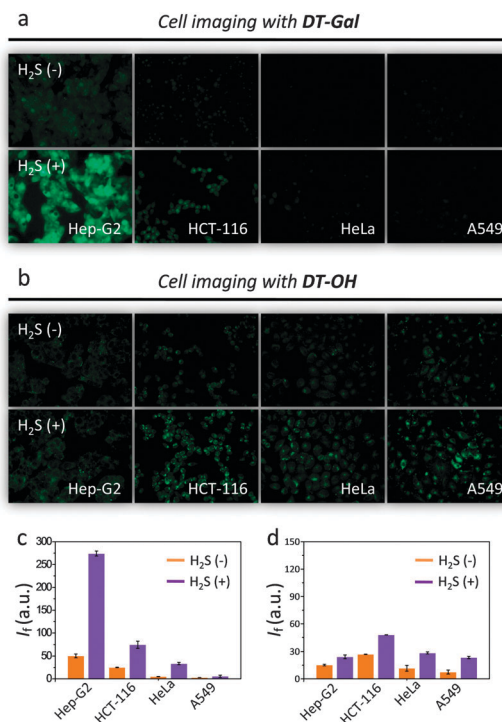


Fig. 2 Cell imaging (a for **DT-Gal** and b for **DT-OH**) and quantification of the fluorescence intensity (I_f) (c for **DT-Gal** and d for **DT-OH**) in the absence (–) and presence (+) of H_2S (Hep-G2: human liver cancer; HCT-116: human colon cancer; HeLa: human cervix cancer; A549: human lung cancer).

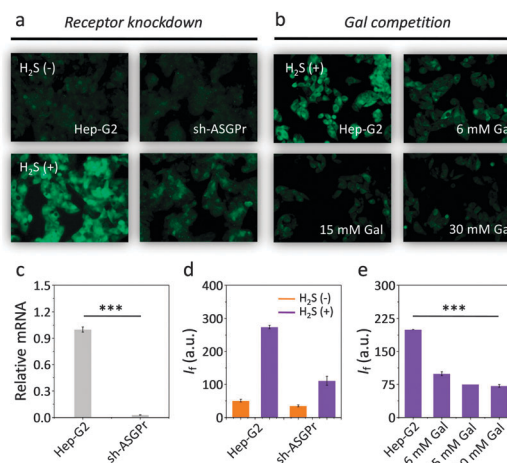


Fig. 3 Cell imaging (a) and fluorescence quantification (d) of **DT-Gal** for Hep-G2 and sh-ASGPr with reduced ASGPr expression. Cell imaging (b) and fluorescence quantification (e) of **DT-Gal** with Hep-G2 in the absence and presence of increasing free galactose. (c) ASGPr mRNA level of Hep-G2 and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



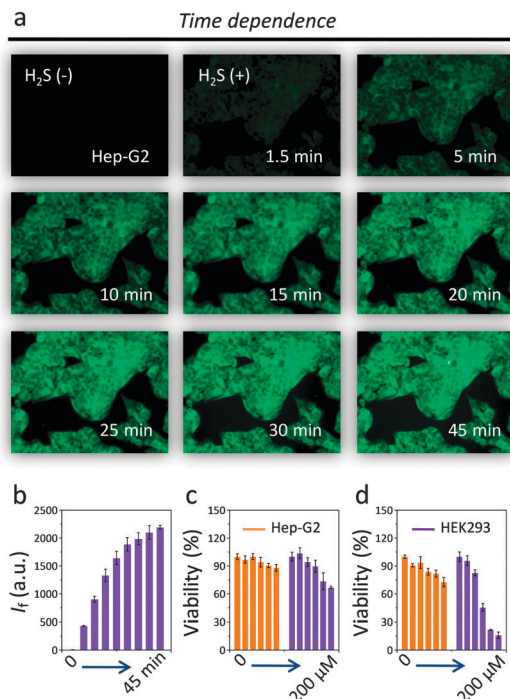


Fig. 4 Cell imaging (a) and fluorescence quantification (b) of **DT-Gal** for Hep-G2 with increasing incubation time. Cytotoxicity of (c) **DT-Gal** (0–200 μM) and (d) **DT-OH** (0–200 μM) for Hep-G2 and HEK293.

We also tested the kinetics of **DT-Gal** for H_2S 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 H_2S 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 H_2S results in significantly enhanced fluorescence. This clearly demonstrates that the fluorescence enhancements observed here are due to H_2S recognition within the cells.

In conclusion, a galactosyl azidonaphthalimide based fluorogenic probe for hepatocellular-selective imaging of H_2S was developed, which sets a basis for the target-specific imaging of H_2S in the liver, the main organ that produces this gaseous transmitter.

This research is supported by the 973 project (2013CB733700), the National Science Fund for Distinguished Young Scholars (81125023), the National Natural Science Foundation of China (21176076, 21202045 and 91213303), the Program of Shanghai Subject Chief Scientist (13XD1404300), the Key Project of Shanghai Science and Technology Commission (13NM1400900) and the Fundamental Research Funds for Central Universities. The Catalysis And Sensing for our Environment (CASE) network is thanked for research exchange opportunities.

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