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A highly selective ratiometric fluorescent probe for real-time imaging of β-glucuronidase in living cells and zebrafish

Xiaokui Huo[a,1], Xiangge Tian[b,1], Yannan Li[a,1], Lei Feng[a,c], Yonglei Cui[a], Chao Wang[a], Jingnan Cui[c], Chengpeng Sun[a], Kexin Liu[a,*], Xiaochi Ma[a,*]

[a] Academy of Integrative Medicine, College of Pharmacy, Dalian Medical University, Dalian, 116044, China
[b] College of Basic Medical Science, Dalian Medical University, Dalian, 116044, China
[c] State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian, 116024, China

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ABSTRACT

β-Glucuronidase (GLU) plays a vital role in the lysosomal degradation of glycosaminoglycans and the enterohepatic circulation of endogenous and exogenous substances. In present study, a sensitive ratiometric fluorescent probe NH-Glu was developed for highly selective sensing of GLU for the first time, using 4-hydroxy-1, 8-naphthalimide (NH) platform. The ratios (I_{556}/I_{445}) displayed brilliant GLU-dependent performance and responded linearly to GLU activity. The probe showed much higher selectivity and sensitivity for GLU than other hydrolases. Common endogenous substance (amino acids and metal ions) gave slight interference to the detection of GLU. NH-Glu exhibited ideal reactivity and kinetic behaviours with low cytotoxicity. Our probe was successfully used for bioimaging of endogenous GLU in human cancer cell lines HepG2 and LoVo, which indicated HepG2 cells had a much higher GLU activity than that of LoVo cells. Furthermore, the probe was also utilized for the real-time visualization of GLU in cancer tissues and zebrafish. These findings demonstrated that NH-Glu could serve as a highly selective and sensitive molecular tool for investigating GLU-mediated biological functions *in vitro* and *in vivo*.

1. Introduction

β-Glucuronidase (GLU) is a member of the lysosomal glycosidase family, which is found in cell membranes and extracellular matrix. It mainly catalyzes the degradation of glucuronic acid from exogenous drugs and some vital endogenous substances including conjugated bilirubin and estrogen metabolites [1,2]. GLU is widely distributed in various tissues, especially abundant in the intestinal tract, and it plays a key role in the enterohepatic circulation of drugs and endogenous substances [2]. Furthermore, it was found to be overexpressed in different tumor types such as liver cancer, colon carcinoma, prostate cancer, renal carcinoma and particularly in necrotic areas, which is closely related to the invasion, metastasis and proliferation of tumor cells [3–7]. Nowadays, GLU has been regarded as a tumor biomarker [1,8,9]. Therefore, on the basis of broad biological functions of GLU, it is very necessary to real-time detect endogenous GLU in living cells and animals.

Currently, several methods were available to detect GLU bioactivity. Among them, fluorescence technique has been paid considerable attention due to its many advantages such as high sensitivity, real-time detection, high spatiotemporal resolution, especially its noninvasive monitoring capability and usability in live cells and animals [10–14]. Until now, only several "Turn-On" fluorescent probes were developed for detecting GLU [15–17]. However, "Turn-On" fluorescent probe could be easily influenced by the difference of biological samples, inducing significant variations in excitation intensity and emission collection efficiency [18–20]. In contrast, ratiometric fluorescent probes are based on the ratio of fluorescence intensities at two wavelengths and can alleviate most of problems mentioned above and obtain a more accurate determination [21,22]. 4-hydroxy-1,8-naphthalimide (**NH**) and its derivatives, with excellent sensing properties, are commonly used in the design and synthesis of ratiometric fluorescent probes [23–25]. So far, there is no ratiometric fluorescent probe for detecting endogenous GLU. As an important diagnostic biomarker of many human diseases, a ratiometric fluorescent probe for accurate detection of endogenous GLU is very urgent.

* Corresponding authors.
E-mail addresses: kexinliu@dlmedu.edu.cn (K. Liu), maxc1978@163.com (X. Ma).
[1] These authors contributed equally to this work.

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In present study, a highly sensitive and selective ratiometric fluorescent probe **NH-Glu** was developed for the real-time determination of endogenous GLU activity and *in vivo* imaging of GLU. To the best of our knowledge, it was the first time to report a ratiometric fluorescence assay for sensing endogenous GLU, which would allow us to effectively detect the living cells, tumor tissues and zebrafish.

2. Experimental section

2.1. Materials

Lysozyme, *N*-acetyl glucosaminidase (NAG), Carbonic anhydrase (Cas), Proteinase K (Pak), Carboxylesterases CE1 and CE2, β-Galactosidase (Gla), β-Glucosidase (Glc), and GLU were all obtained from Sigma-Aldrich. Bovine serum albumin (BSA), Glutamate, Glutamine, Glycine, Serine, Glutathione, Arginine, lysine, Cysteine, Tryptophan, Glucose, Tyrosine, Bilirubin, and Myristic acid were purchased from Shanghai yuanye (Shanghai, China). Bispnitrophenyl phosphate (BNPP), loperamide (LPA), α-galactose, Epicatechingallate (ECG) and baicalein were purchased from J&K Chemicals. LoVo and HepG2 cell lines were purchased from ATCC (Manassas, VA). All fluorescence tests performed on Synergy neo Microplate Reader (Bio-Tek). NMR spectra were required using a Bruker 501. Accurate mass detection was measured on fourier transform ion cyclotron resonance mass spectrometer (LTQ Orbitrap XL). The hydrolysis supernatants were determined by HPLC-UV analysis (Waters e2695 equipped with 2998 PDA Detector). All other reagents and solvents used were of the highest grade commercially available.

2.2. Synthesis of NH-Glu

2.2.1. Synthesis of compound NH-Gla

NH (54 mg, 0.2 mmol), bromo-2, 3, 4-tri-*O*-acetyl-α-D-glucopyranuronic acid methyl ester (400 mg, 1 mmol) Ag_2O (463 mg, 2 mmol), NaI (150 mg, 1 mmol) and a sufficient amount of anhydrous Na_2SO_4 were dissolved in dry CH_3CN (10 mL) in a 25 mL round bottom flask and stirred at room temperature under a nitrogen atmosphere for 48 h, filtered, and evaporated. The residue was purified by chromatography (silica gel, EtOAc–hexane as eluent, 1:5, v/v) to afford 69 mg **NH-Gla** as a yellow solid (Yield 58.8%) (Scheme 1). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.61 (dd, J = 7.3, 0.9 Hz, 1H), 8.55 (d, J = 8.2 Hz, 1H), 8.41 (dd, J = 8.4, 1.0 Hz, 1H), 7.74 (dd, J = 8.3, 7.5 Hz, 1H), 7.22 (d, J = 8.3 Hz, 1H), 5.77 (d, J = 7.8 Hz, 1H), 5.54 (dt, J = 11.5, 5.7 Hz, 1H), 5.50–5.46 (m, 1H), 5.46–5.42 (m, 1H), 4.35 (d, J = 9.1 Hz, 1H), 4.16 (t, J = 7.0, 2H), 3.71 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H) 2.03 (s, 3H), 1.74–1.66 (m, 2H), 1.44 (td, J = 14.9, 7.4 Hz, 2H), 0.97 (t, J = 7.4 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.92, 169.89, 169.40, 169.33, 164.23, 163.65, 157.05, 132.57, 131.84, 129.33, 128.14, 126.85, 123.57, 122.61, 117.66, 109.09, 98.35, 73.02, 71.85, 70.64, 68.96, 53.00, 40.20, 30.23, 20.75, 20.54, 20.50, 20.37, 13.82. HRMS (ESI positive) calcd. for $C_{29}H_{32}NO_{12}^+$ $[M+H]^+$ 586.1919, found 586.1917.

2.2.2. Synthesis of compound NH-Glu

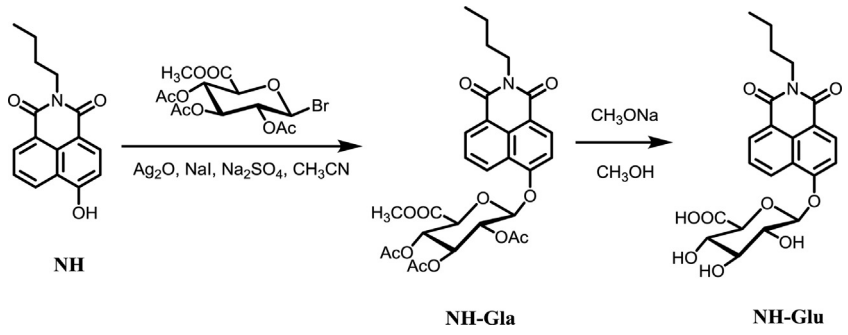
NH-Gla (29 mg, 0.05 mmol) was dissolved in 10 mL of MeOH, and MeONa (108 mg, 2 mmol) was added, the mixture was stirred at room temperature for 3 h, then evaporated, and the resulting residue was purified by HPLC (a isocratic chromatographic condition with phase 55% A (A: 0.03% trifluoroacetic acid water; B: acetonitrile), the flow rate was set at 1.0 mL/min). Finally we afford 11 mg **NH-Glu** as a light yellow solid (Yield 49.6%) (Scheme 1). ^{1}H NMR (500 MHz, DMSO – *d 6*) δ 8.69 (d, J = 8.3 Hz, 1H), 8.51 (d, J = 7.2 Hz, 1H), 8.44 (d, J = 8.3 Hz, 1H), 7.85 (t, J = 7.8 Hz, 1H), 7.47 (d, J = 8.3 Hz, 1H), 5.67 (s, 1H), 5.38 (d, J = 7.6 Hz, 1H), 5.30 (d, J = 20.8 Hz, 1H), 4.03 (t, J = 7.1 Hz, 2H), 3.94 (d, J = 8.3 Hz, 1H), 1.66–1.56 (m, 2H), 1.35 (dd, J = 14.7, 7.3 Hz, 2H), 0.92 (t, J = 7.3 Hz, 3H). ^{13}C NMR (125 MHz, DMSO – *d 6*) δ 170.98, 164.01, 163.34, 158.36, 133.25, 131.63, 129.03, 126.93, 123.43, 122.31, 115.90, 110.05, 100.49, 76.19, 75.54, 73.47, 72.02, 40.50, 30.17, 20.29, 14.21. HRMS (ESI positive) calcd for $C_{22}H_{24}NO_9^+$ $[M+H]^+$ 445.1446, found 445.1443.

2.3. Incubation and determination system of GLU

All the measurements of GLU activity were carried out in 100 mM potassium phosphate buffer (pH 6.0) system with a final incubation volume of 0.2 mL. Briefly, the incubation system for the probe reaction included potassium phosphate buffer, GLU (50 μg/mL), and 2 μL stock solution of **NH-Glu** which was incubated at 37 °C for 60 min, then the reaction was terminated by the addition of 100 μL ice acetonitrile. The mixtures were then centrifuged at 20,000 × g for 20 min at 4 °C. Aliquots of supernatant were taken for further fluorescence analysis. Control incubations without enzyme or without substrate were used to ensure that metabolite formation was enzyme-dependent. All assays were performed in duplicates.

2.4. Selectivity assays of NH-Glu

The selectivity of **NH-Glu** for GLU was evaluated among various hydrolase including lysozyme (Ls), *N*-acetyl glucosaminidase (NAG), carbonic anhydrase (Cas), Proteinase K (Pak), carboxylesterase 1 (CE1) and carboxylesterase 2 (CE2), β-galactosidase (GLa), β-glucosidase (GLc) and GLU in our incubation system. The final concentrations of all hydrolase were 10 μg/mL. Furthermore, the fluorescence properties stability of **NH-Glu** in some endogenous and exogenous substances such as common metal ions (Ca^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, Cu^{2+}, K^+, Ni^{2+}, Ba^{2+}, Na^+ and Sn^{4+}) and amino acids (Glutamic acid, Glutamine, Glycine, Serine, Glutathione, Arginine, lysine, Cysteine, Tryptophan, Tyrosine, Glucose, bilirubin and myristic acid) were also performed in our standard incubation sys-



Scheme 1. The synthetic route of **NH-Glu**.