Supporting Information

NAG-targeting fluorescence based probe for precision diagnosis of kidney injury

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Experimental Procedures

Materials and Method

Materials

Lysozyme (Ls), α -glucosidase (α -Glc), N-acetyl- β -D-glucosaminidase (NAG), carbonic anhydrase (Cas), proteinase K (Pak) and carboxylesterases (CE), including CE1b, CE1c and CE2, β -galactosidase (β -GLa), GLU, β -glucosidase (β -Glc) from bovine liver, glutamate, Cisplatin were all obtained from Sigma-Aldrich (St Louis, MO). Bovine serum albumin (BSA), Myristic acid, Serine, Tryptophan, Tyrosine, Glutanic acid, Glycine, L-Arginine, L (+)-Cysteine, Lysine, Glutamine, Glutathione and Glucose were all purchased from Shanghai yuanye (Shanghai, China). HK-2 cells, NRK-52e cells and MCT cells were purchased from American Type Culture Collection (Manassas, VA). NAG antibodies, β -actin antibodies were obtained from Proteintech Group. Blood urea nitrogen kits, NAG kits and creatinine kits were purchased from Nanjing jiancheng biological engineering research institute (Nanjing, China). 4', 6diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories, Burlingame, CA, USA, Hoechst 33342 was purchased from Abcam. Anti-fluorescence quenching sealant was purchased from Beyotime Biotechnology (Shanghai, China). All fluorescence tests performed on Synergy Neo Microplate Reader (Bio-Tek). NMR spectra were obtained using a Bruker 500. Accurate mass detection was measured on G6224A TOF MS. 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.

The UV-vis spectra and fluorescence emission/excitation spectra were measured on a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). A Waters 2695 system equipped with a PDA detector was used to determine **NHPO** and its metabolites.

Methods

Synthesis pathway for NHPO

To a solution of **HHPO** (53.2 mg, 0.25 mmol) in 10 mL of dry CH₃CN were added 2-acetamido-3, 4, 6tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl chloride (478.0 mg, 2 mmol), Ag₂O (577.5 mg, 2.5 mmol), NaI (300 mg, 2 mmol) and a sufficient amount of anhydrous Na₂SO₄ to remove water. The reaction mixture was stirred at room temperature (RT) for 24 h, filtered, and evaporated. The residue was dissolved in 10 mL of CH₃OH, and CH₃ONa (108 mg, 2 mmol) was added. The mixture was stirred at RT for 2 h, neutralized with 1M HCl, filtered, and evaporated. The residue was purified by HPLC (a linear gradient formed from CH₃CN and H₂O: isocratic at 12/88 for 55 min, linear gradient to 100/ 0 in 3 min, then isocratic for 20 min) to afford 10.2 mg of orange powder. Yield: 9.8% in 2 steps.

¹H NMR (500 MHz, DMSO-*d*6) δ 7.82 (d, *J* = 8.9 Hz, 1H), 7.77 (d, *J* = 8.8 Hz, 1H), 7.53 (d, *J* = 9.8 Hz, 1H), 7.09 (d, *J* = 2.5 Hz, 1H), 7.02 (dd, *J* = 8.8, 2.5 Hz, 1H), 6.79 (dd, *J* = 9.8, 2.0 Hz, 1H), 6.27 (d, *J* = 2.0 Hz, 1H), 5.19 (d, *J* = 8.5 Hz, 1H), 5.13 (d, *J* = 5.3 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 1H), 4.61 (t, *J* = 5.7 Hz, 1H), 5.19 (d, *J* = 8.5 Hz, 1H), 5.13 (d, *J* = 5.3 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 1H), 4.61 (t, *J* = 5.7 Hz, 1H), 5.19 (d, *J* = 8.5 Hz, 1H), 5.13 (d, *J* = 5.3 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 1H), 4.61 (t, *J* = 5.7 Hz, 1H), 5.19 (d, *J* = 8.5 Hz, 1H), 5.13 (d, *J* = 5.3 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 1H), 4.61 (t, *J* = 5.7 Hz, 1H), 5.19 (d, *J* = 5.5 Hz, 1H), 5.13 (d, *J* = 5.3 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 1H), 5.13 (d, *J* = 5.7 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 1H), 5.13 (d, *J* = 5.7 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 1H), 5.13 (d, *J* = 5.7 Hz, 1H), 5.09 (d, *J* = 5.5 Hz, 1H), 5.13 (d, *J* = 5.7 Hz, 1H), 5.13 (d, *J* = 5.5 Hz, 1H), 5.13 (d, J = 5.5

Hz, 1H), 3.75 - 3.66 (m, 2H), 3.52 - 3.46 (m, 1H), 3.43 (dd, J = 9.8, 5.6 Hz, 2H), 3.20 (td, J = 9.2, 5.3 Hz, 1H), 1.80 (s, 3H). ¹³C NMR (125 MHz, DMSO-*d6*) δ 185.33, 169.30, 160.80, 149.62, 145.90, 144.91, 134.94, 133.94, 131.26, 128.59, 114.84, 105.75, 102.71, 98.71, 77.34, 70.11, 62.76, 55.28, 23.00. HRMS (ESI negative) calcd for [M+HCOO⁻] ⁻ 461.1202, found 461.1216. (**Fig. S11-14**)

The fluorescence property of NHPO toward NAG

Based on the following procedure, all the evaluations of NAG activity were performed in 100 mM potassium phosphate buffer (pH 7.4), with a final incubation volume of 0.2 mL. The system contained potassium phosphate buffer, and 2 μ L of stock solution of **NHPO** (the final concentration was 10 μ M) and NAG was mixed. After incubation at 37 °C for 1 hour, the reaction was terminated by the addition of 100 μ L of acetonitrile with strong stirring. The mixtures were centrifuged at 20,000 × g for 20 min at 4 °C. Then, aliquots of supernatant were used for further fluorescence analysis. Control incubations without enzyme or without substrate were used to ensure that metabolite formation was enzyme-dependent. Furthermore, the sample after incubating with NAG was analyzed and identified by HPLC and ESI-MS/MS analysis. The HPLC method was as follows: the mobile phase consisted of acetonitrile (A) and 0.1% formic acid aqueous solution (B) at a flow rate of 0.8 mL min⁻¹. The following gradient condition was used: 0–10 min 90%B; 10–25 min 90%- 30% B; 25–30 min 30% B. All assays were performed in duplicate. The final concentration of DMSO did not exceed 1% (ν/ν), to avoid interfering with the enzymes' catalytic activity.

Screening the selectivity of NHPO

In order to verify the selective sensing ability of **NHPO**, diverse hydrolase including Ls, α -Glc, NAG, Cas, Pak, CE1b, CE1c and CE2, β -GLa, β -Glc, GLU were screened in our standard incubation system. The final concentrations of all hydrolase were 1 µg mL⁻¹. Furthermore, the fluorescence stability of **NHPO** with some endogenous and exogenous substances such as common metal ions and anions (Mn²⁺, Ca²⁺, Mg²⁺, Ni²⁺, Zn²⁺, Sn⁴⁺, K⁺, Cu²⁺, Fe³⁺, Na⁺, Ba²⁺, NO₃⁻, CO₃²⁻ and SO₄²⁻) and aminoacids (Myristic acid, Serine, Tryptophan, Tyrosine, Glutamic acid, Glycine, L-Arginine, L(+)-Cysteine, Lysine, Glutamine, Glutathione and Glucose) were also evaluated using our standard incubated at 37 °C for 1 hour. All statistical analysis was conducted using the Prism software package (GraphPad Software 6.0, LaJolla, CA).

The application of NHPO in cisplatin-induced acute kidney injury model

Cisplatin-induced acute kidney injury model was established in C57BL/6 mice (6-8 weeks, 18-22 g) purchased from the Experimental Animal Center of Dalian Medical University, and housed under diurnal lighting condition with 12 hours of light. The mice were given a standard normal diet with free access to water, and randomly divided into two groups, a control group and a cisplatin group. All animal

experiments were performed according to the animal experimental guidelines approved by the Committee of Animal Experimentation of the Dalian Medical University. Mice in the cisplatin group were administered intraperitoneally (i. p.) with cisplatin dissolved in normal saline at a concentration of 1.0 mg mL⁻¹, and the dosage of administration is 20 mg kg⁻¹. Mice in the control group were administrated i. p. with normal saline. The urine of experimental animals was collected using metabolic cages for 24 hours. Blood and kidneys from the experimental animals were harvested 72 hours after cisplatin injection.

Blood urea nitrogen (BUN) and serum creatinine (sCr) were respectively measured by commercial kits and high performance liquid chromatography (HPLC). The evaluation of NAG activity was performed in the mixed system of 80 μ L mouse urine, 100 mM potassium phosphate buffer (pH 7.4), and 2 μ L of stock solution of **NHPO** with a final incubation volume of 0.2 mL. After incubation at 37 °C for 1 hour, the reaction was terminated by the addition of 100 μ L of acetonitrile and strong stirring. The mixtures were centrifuged at 20,000 × g for 20 min at 4 °C. Then, aliquots of the supernatant were used for further fluorescence analysis. Mouse urine was replaced with the same volume potassium phosphate buffer in control incubations. The urine protein level was checked by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was stained with coomassie brilliant blue.

To evaluate the renal histopathologic features, the kidney specimens from the two groups were fixed in 10% buffered formalin for 24 hours. Then the half of the kidney specimens were embedded in paraffin for 72 hours. The kidney specimens at a thickness of 10 µm sections were subjected to Hematoxylin and Eosin (H&E) staining according to the manufacturer's instructions. The stained kidney specimens were photographed using an Axioplan 2 Imaging system (Carl Zeiss Micro-Imaging, Inc. Thornwood, NY). The other half specimens after fixed in 10% buffered formalin were sectioned directly. The 10 µm sections were stained with periodic acid–Schiff (PAS) reagents. The proximal glomerular tubule damage in PAS stained sections was observed under the light microscope. The histological examinations were conducted blind.

Correlation study

Urine samples were collected from graduate students (healthy individuals) and patients (people with varying degrees of kidney injury) to confirm **NHPO** activity of NAG in human urine. The NAG activity and creatinine of urine were determinated using commercial kits according to the manufacturer's instructions. The evaluations of the probe were performed in a mixed system of 198 μ L urine and 2 μ L of stock solution of **NHPO** with a final concentration was 10 μ M. After incubation at 37 °C for 1 hour, the reaction was terminated by the addition of 100 μ L of acetonitrile and strong stirring. The mixtures were centrifuged at 20,000 × *g* for 20 min at 4 °C. Then, aliquots of supernatant were used for fluorescence analysis and the concentration was determined according to the standard curve of the **HHPO**. Urine was replaced with the same volume potassium phosphate buffer in control incubations. Next, 30 urine samples

from healthy individuals and 28 urine samples from patients were measured by **NHPO** in our standard incubation system.

The fluorescence imaging of NAG in different cells

HK-2 cells and NRK-52e: rat renal proximal tubule epithelial cell line were cultured in DME/F-12 medium, MCT: mouse renal proximal tubule cell line were cultured in DMEM medium, both with 10% (ν/ν) fetal bovine serum (FBS) and 1% (ν/ν) antibiotics (penicillin/streptomycin) at 37 °C in 5% CO₂. Cytotoxicity was evaluated by the CCK-8 assay (Roche Diag-nosis, Indianapolis, IN). In brief, HK-2, NRK-52e and MCT cells were seeded in a 96-well plate at a concentration of 8 × 10⁴ mL⁻¹ in culture medium and incubated overnight. Then FBS free culture medium containing various concentrations of **NHPO** was added (0, 1, 2, 5, 10, 20, 50 µM). After 24 hours incubation, discarded culture medium and FBS free culture medium with 10% (ν/ν) CCK-8 was added. After one hour incubation in an incubator, cells were detected at 450 nm using the Synergy Neo Microplate Reader (Bio-Tek). At least three independent biological replicates were performed. Cell viability in the absence of **NHPO** was considered as 100%.

Cells were seeded on 20-mm glass polylysine-coated confocal cell culture dishes and then cultured overnight. After discarded culture medium, the adherent cells were incubated with/without 50 μ M of **NHPO** (prepared in culture medium) in incubator at 37 °C for 1 hour. After the incubation cells were washed with 37 °C PBS (pH 7.4) three times. Then cells were imaged using a confocal microscope (Leica TCS SP8).

Then, we inhibited the expression of NAG in the HK-2 cells using c-DNA according to the manufacturer's instruction. HK-2 cells were cultured in DME/F-12 medium with 10% FBS and 1% antibiotics and incubated overnight in a humidified atmosphere of 5% CO₂ at 37 °C. After starving cells with FBS free culture medium for 4 hours, we added prepared lipo 2000 with c-DNA into FBS free culture medium. Six hours later, we stopped the transfection process, replaced the transfection culture medium with DME/F-12 medium with 10% FBS and 1% antibiotics. We knocked down the HK-2 cells on 20-mm glass polylysine-coated confocal cell culture dishes to image on a confocal microscope. We knocked down the HK-2 cells on 60-mm cell culture dishes to determine the levels of protein expression.

In order to determine the levels of protein expression of NAG in c-DNA transfected HK-2 cells, cells were lysed with an appropriate cold lysis buffer supplemented with proteinase inhibitor. Proteins (30 μ g) were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). Membranes were blocked with 5% skim milk in TBST buffer for 2 hours at RT with swaying gently, and then probed with specific antibodies. Protein bands were detected using an enhanced chemiluminescence system. Bands were normalized to β -actin. At least three independent biological replicates were performed.

The fluorescence imaging of NAG in tissues

The tissues slices of kidney specimens were cut using a cryostat microtome at a thickness of 10 μ m for the experiment of fluorescence imaging. Firstly, the slices were incubated with/without **NHPO** (20 μ M) for 2 hours at 37 °C. Secondly, washing with phosphate buffered saline (PBS) three times, then the slices was fixed for 10 min at 4 °C with acetone. Thirdly, washing with PBS for three times and then blocked with 5% bovine serum albumin (BSA) in PBS buffer for 1 hour at RT and kept in dark place. Subsequently, after washing with PBS three times, primary antibody AQP1 in the 1% blocking solution were added to the slices and incubated overnight at 4 °C. After washing with PBS gently, the slices incubated with Fluorescein rhodamine conjugated secondary anti-rabbit antibodies diluted by 1% blocking solution for 1 hour at RT and kept in dark place. After washing with PBS three times, the slices were mounted with DAPI for 15 min at RT. Finally, the slices were blocked by anti-fluorescence quenching sealant and were analyzed with a Leica DM 14000B confocal microscope. LeicaSP8 DIVE confocal microscope at individual rational fluorescent conditions.

The application of NHPO in the diagnosis of human kidney injury

We collected some urine samples from " The First Affiliated Hospital of Dalian Medical University" including healthy individuals and patients with various kidney diseases; the patient ill-1 was diagnosed with renal insufficiency and the patient ill-2 was diagnosed with acute pyelonephritis and both of them had a renal dysfunction. Briefly, the urine was filtered through 300 mesh nylon mesh filter to remove urinary sediment. Then the samples were centrifuged at $1500 \times r$ for 5 min and resuspended by 37 °C PBS, the suspension were centrifuged at $850 \times r$ for 5 min and resuspended the cell again, next the suspension were centrifuged at $850 \times r$ for 5 min and resuspended the cell again, next the suspension were divided evenly into two sterile eppendorf tubes, cultured with/without **NHPO** (50 µM) added Hoechst 33342 with the final concentration of 2 µM for 2 hours at 37 °C in 5% CO₂. Finally, the cell suspension were centrifuged at $850 \times r$ for 5 min and resuspended by 37 °C PBS for fluorescence imaging with a Leica confocal microscope.

Author Contributions

Participated in the research design: Xiaochi Ma, Lei Feng and Tony D. James; Conducted experiments: Fei Yan, Xiangge Tian; Data analysis: Fei Yan, Xiangge Tian and Lei Feng; Writing of the manuscript: Fei Yan, Xiaochi Ma and Tony D. James.



Fig. S1 (a) Absorption spectra and (b) fluorescence emission response of **NHPO** (10 μ M) after incubation with NAG; (c) Fluorescence spectral changes of **NHPO** (10 μ M) with increasing concentration of NAG (0 – 7 μ g mL⁻¹) with 30 min incubation. (d) linear relationship between the fluorescence intensity changes of **NHPO** (10 μ M) and enzyme concentration after incubation with NAG in a standard incubation system, phosphate buffer-acetonitrile v/v = 2:1, $\lambda_{ex} = 550$ nm).



Fig. S2. Influence of pH on the fluorescence intensity of the substrate **NHPO** (red line) and metabolite **HHPO** (blue line) in buffer– acetonitrile (v/v = 2:1, pH = 7.4) at 37 °C; the results represent the mean \pm SD (n = 3). $\lambda_{ex/em}$ =550/598 nm.



Fig. S3. The influence of pH on the fluorescence response of **NHPO** after incubation with (Blue line) and without NAG (Red line) in buffer– acetonitrile (v/v = 2:1, pH = 7.4) at 37 °C for 30 min; the results represent the mean \pm SD (n = 3). $\lambda_{ex/em}$ =550/598 nm.



Fig. S4. HPLC analysis of **NHPO**, the metabolite **HHPO** and the sample after incubating with NAG at 37 °C for 30 min.



Fig. S5 (a) The selectivity of NHPO (10 μ M) with different metabolic enzymes; (b) the influence of different common metal ions and endogenous substances on the response of NHPO towards NAG isoform.



Fig. S6. The Periodic Acid Schiff (PAS) staining of paraffin section kidney tissues in normal (**a**) and cisplatin treatment group (**b**), respectively.



Fig. S7 Fluorescence imaging of NAG in HK2 cells; (a-c) the fluorescence background and bright field of HK-2 cells; (d-f) the fluorescence imaging of HK-2 cells after incubating with **NHPO** (50 μ M) for 1 h. The scale bar is 50 μ m.



Fig. S8. (a, d) Confocal fluorescence images of NRK-52e cells, treated with **NHPO** (50 μ M) at 37 °C for 60 min; (b, e) bright fields of NRK-52e cells; (c, f) merge images of fluorescence field and bright field.



Fig. S9. (a, d) Confocal fluorescence images of MCT cells, treated with **NHPO** (50 μ M) at 37 °C for 60 min; (b, e) bright fields of MCT cells; (c, f) merge images of fluorescence field and bright field.



Fig. S10. (a-d) Confocal fluorescence images of the cells separated from healthy individual-1, (e-h) confocal fluorescence images of cells separated from healthy individual-2; all the cells were incubating with **NHPO** (50 μ M) at 37 °C for 60 min.



Fig. S 11. The synthetic pathway of NHPO.







Fig. S14. HRMS of NHPO.