Electronic Supplementary Information for

Design, synthesis and application of near-infrared fluorescent probe for in vivo imaging of aminopeptidase N

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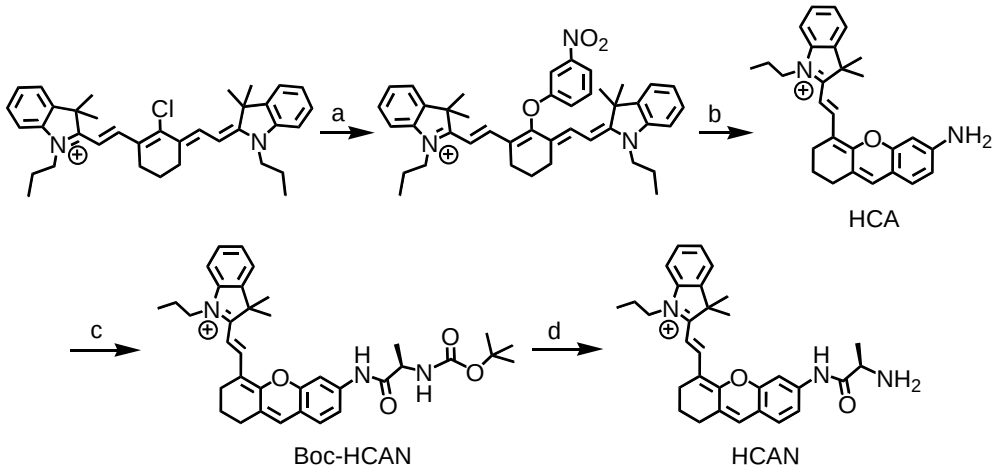
1. Apparatus and reagents

Fluorescence measurements were made on a Hitachi F-4600 spectrophotometer in 10 mm × 10 mm quartz cells (Tokyo, Japan). ^{1}H- and ^{13}C-NMR spectra were measured with a Bruker DMX-400 spectrometer. Electrospray ionization mass spectra were measured on a Shimadzu LC-MS 2010A instrument (Kyoto, Japan). High resolution electrospray ionization mass spectra (HR-ESI-MS) were recorded on an APEX IV FTMS instrument (Bruker, Daltonics). Absorption spectra were recorded in 1-cm quartz cells with a TU-1900 spectrophotometer (Beijing, China). MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] analysis was made on a microplate reader (Molecular Devices SpectraMax i3). Fluorescence imaging was made on an FV 1200-IX83 confocal laser scanning microscope (Olympus). In vivo fluorescence imaging was made on a Kodak In-vivo Imaging System FX Pro.

IR-780 iodide and bestatin were obtained from Sigma-Aldrich. Aminopeptidase N was purchased from R&D systems. Boc-L-alanine was purchased from Beijing InnoChem Science & Technology Co. Ltd. Phosphate buffered saline (PBS: 155 mM NaCl, 2.97 mM Na_2HPO_4, 1.05 mM KH_2PO_4; pH 7.4), Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from Thermofisher. Ultrapure water (over 18 MΩ cm) was employed throughout.

2. Syntheses of compounds



Scheme S1. Synthesis of **HCAN**. Conditions: Conditions: a) 3-nitrophenol, K_2CO_3, CH_3CN, room temperature, 4 h; b) $SnCl_2$, HCl, CH_3OH, 70 ^{o}C, overnight; c) Boc-L-alanine, HATU, DIPEA, CH_2Cl_2, 4 h; d) CF_3COOH, CH_2Cl_2, 3 h.

Synthesis of compound Boc-HCAN. HCA was synthesized following our previous method (He et al. *Chem. Sci.* **2017**, *8*, 3479).

Boc-L-alanine (151.4 mg, 0.8 mmol), HATU (380 mg, 1 mmol) and DIPEA (130 μL, 0.6 mmol) were dissolved in dry CH_2Cl_2 (20 mL) with stirring at 0 ℃ for 40 min. Then, **HCA** (83 mg, 0.2 mmol) in CH_2Cl_2 (2 mL) was introduced, and the reaction mixture was further stirred at room temperature for 4 h. The precipitate was filtered, and the filtrate was evaporated under reduced pressure. Then, the residue was purified by silica gel chromatography with CH_2Cl_2/CH_3OH (v/v, 500:3) as eluent, yielding compound Boc-**HCAN** as dark-blue solid (40 mg, yield 34%). ^1H and ^{13}C NMR spectra are shown in Figures S1 and S2.

^1H NMR (400 MHz, 298 K, $CDCl_3$): δ 8.90 (s, 1H), 8.69 (d, 1H, J = 14.4 Hz), 7.89 (s, 1H), 7.66 (s, 1H), 7.51 (d, 1H, J = 7.6 Hz), 7.44 (t, 1H, J = 7.2 Hz), 7.36 (t, 1H, J = 7.2 Hz), 7.24 (s, 1H), 7.08 (s, 1H), 6.32 (d, 1H, J = 14.8 Hz), 5.34 (s, 1H), 4.35 (t, 1H, J = 6.4 Hz), 4.26-4.14 (m, 2H), 2.69 (s, 2H), 2.61 (t, 2H, J = 5.6 Hz), 1.97-1.88 (m, 4H), 1.80 (s, 6H), 1.49 (d, 3H, J = 7.2 Hz), 1.47 (s, 9H), 1.07 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100 MHz, 298 K, $CDCl_3$): δ 178.4, 172.8, 161.8, 153.5, 146.4, 142.6, 142.5, 141.4, 133.6, 128.9, 128.0, 127.9, 127.3, 123.0, 117.6, 117.6, 114.6, 112.1, 106.5, 103.3, 80.1, 77.2, 51.1, 46.7, 29.1, 28.3, 28.1, 23.9, 21.2, 20.3, 11.5. HR-ESI-MS: m/z calcd. for compound Boc-**HCAN** ($C_{36}H_{44}N_3O_4^+$, M^+), 582.3326; found, 582.3324.

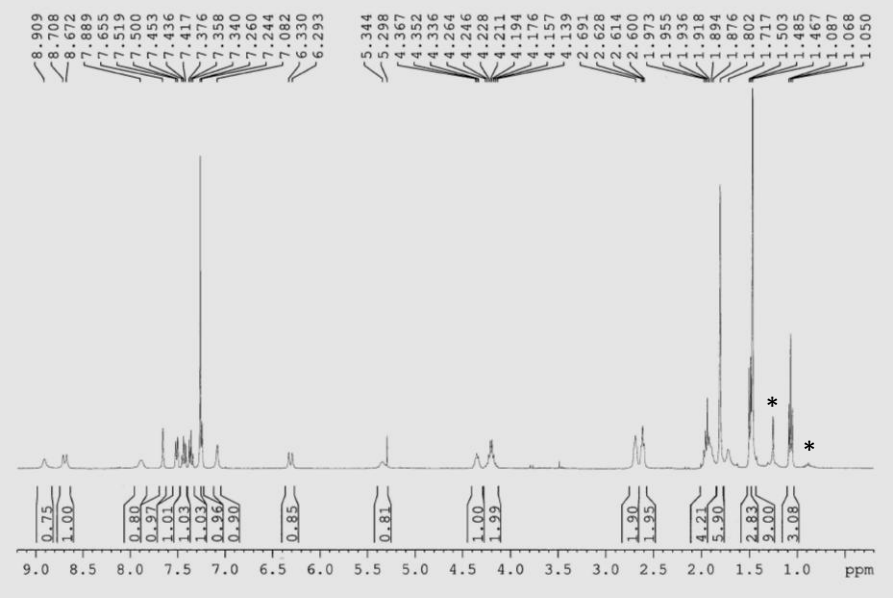


Figure S1. ^1H NMR spectrum of Boc-**HCAN** (400 MHz, $CDCl_3$, 298 K). The label "*" indicates grease peaks from purification (Gottlieb et al, *J. Org. Chem.* 1997, **62**, 7512-7515).

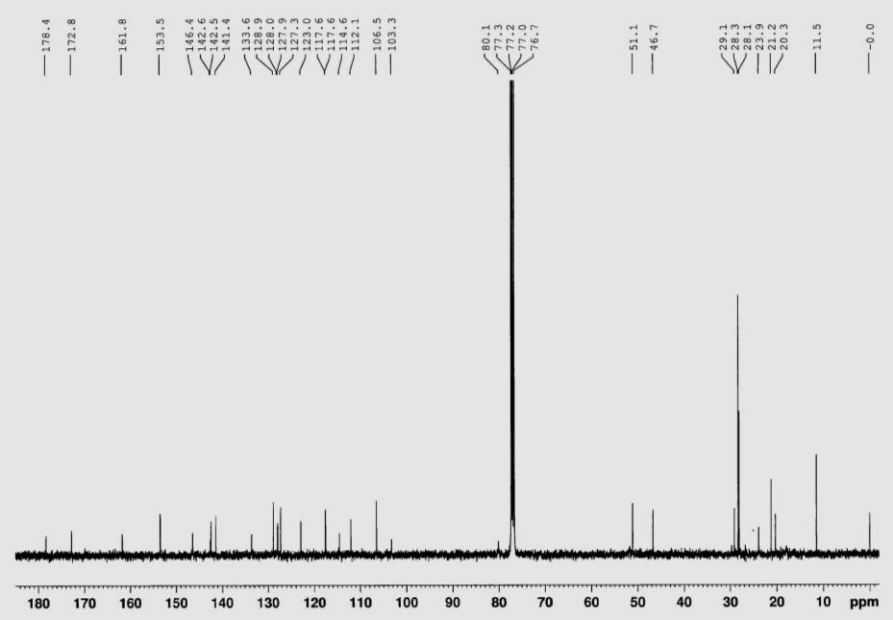


Figure S2. ^{13}C NMR spectrum of Boc-**HCAN** (100 MHz, $CDCl_3$, 298K).

Synthesis of probe HCAN. Compound Boc-**HCAN** (35 mg, 0.06 mmol) was stirred in CH_2Cl_2 (4 mL) containing trifluoroacetic acid (0.6 mL) at room temperature for 3 h. The solvent was removed by evaporation under reduced pressure, and the crude product was purified by silica gel chromatography eluted with CH_2Cl_2/CH_3OH (v/v, 10:1), affording probe **HCAN** as blue solid (20.3 mg, yield 70%). ^{1}H and ^{13}C NMR spectra are shown in Figures S3 and S4.

^{1}H NMR (400 MHz, 298 K, CD_3OD): δ 8.83 (d, 1H, J = 14.4 Hz), 8.10 (s, 1H), 7.69 (d, 1H, J = 7.2 Hz), 7.62-7.55 (m, 2H), 7.51 (d, 2H, J = 8.4 Hz), 7.42 (dd, 1H, J = 6.8, 10.0 Hz), 7.38 (s, 1H), 6.60 (d, 1H, J = 14.8 Hz), 4.38 (t, 2H, J = 7.2 Hz), 3.79 (s, 1H), 2.82 (t, 2H, J = 5.6 Hz), 2.75 (t, 2H, J = 5.6 Hz), 2.02-1.94 (m, 4H), 1.86 (s, 6H), 1.48 (d, 3H, J = 6.8 Hz), 1.14 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100 MHz, 298 K, CD_3OD): δ 179.8, 162.7, 155.0, 147.4, 143.6, 143.3, 143.0, 134.1, 130.4, 130.2, 129.4, 128.7, 123.9, 119.6, 118.2, 116.7, 115.9, 114.2, 107.2, 105.6, 52.2, 47.8, 30.2, 28.4, 25.1, 22.4, 21.7, 20.6, 11.6. HR-ESI-MS: m/z calcd. for probe **HCAN** ($C_{31}H_{36}N_3O_2^+$, M^+), 482.2802; found, 482.2801.

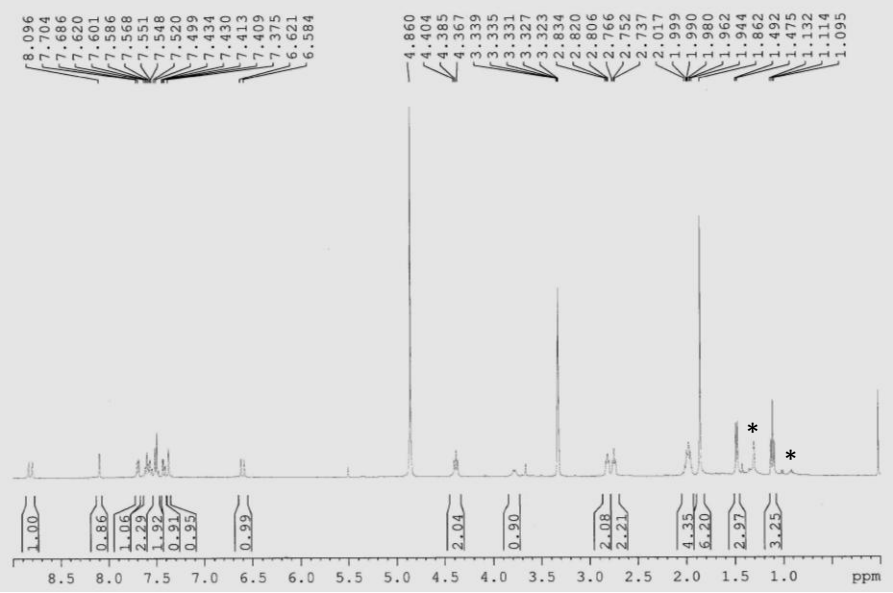


Figure S3. ^{1}H NMR spectrum of **HCAN** (400 MHz, CD_3OD, 298 K). The label "*" indicates grease peaks from purification (Gottlieb et al, *J. Org. Chem.* 1997, **62**, 7512-7515).

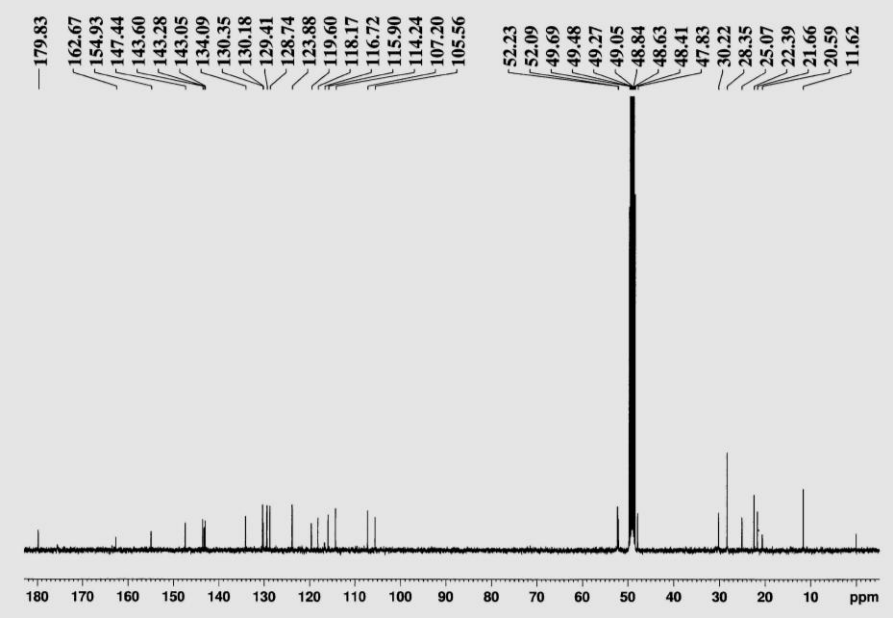


Figure S4. ^{13}C NMR spectrum of **HCAN** (100 MHz, CD_3OD, 298K).

3. General procedure for spectroscopic detection

The stock solution (1.0 mM) of probe **HCAN** was prepared in DMSO. In a test tube, 4 mL of PBS (pH 7.4) and 25 μL of 1 mM probe were mixed, followed by addition of APN solution. The final volume was adjusted to 5 mL with PBS. After incubation at 37 ℃ for 45 min in a thermostat, the reaction solution was transferred to a quartz cell of 1-cm optical length to measure absorbance or fluorescence with $\lambda_{ex/em}$ = 665/705 nm.

4. Cell incubation and fluorescence imaging

Cells (LO2 and HepG2) were grown on glass-bottom culture dishes (MatTek Co.) in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin at 37 ℃ in a humidified 5% CO_2 incubator. Before use, the adherent cells were washed three times with FBS free DMEM. For APN imaging, the cells were incubated with **HCAN** (5 μM) in DMEM at 37 ℃ for 30 min. Then cell imaging was conducted on an FV 1200-IX83 confocal laser scanning microscope with 635 nm excitation and 650-750 nm emission through a 100×1.4 NA objective. The pixel intensity of the cells in the fluorescence image was determined by using ImageJ software (version 1.45s, NIH). The cells were taken as a Region of Interest based on their periphery. For comparison, the intensity from at least five cells in each fluorescence image was measured and averaged in this work. Moreover, the cytotoxicity of **HCAN** to HepG2 cells was evaluated using standard MTT assay (Song et al, *J. Mater. Chem.* **2012**, *22*, 12568).

5. Scratch assay

The cell monolayer was scraped in a straight line with a pipet tip. The debris was removed, and the edge of the scratch was smoothed by washing the cells once with 1 ml of the growth medium (Liang et al, *Nat. Protoc.*, **2007**, *2*, 329). After incubation at 37 ^{o}C for 24 h in a humidified atmosphere containing 5% CO_2, the cells were observed with a 10× 0.4 NA objective on an FV 1200-IX83 confocal laser scanning microscope through DIC channel.

6. siRNA transfection into HepG2

The target DNA sequence is 5'-AAC GAT CTC TTC AGC ACA TCA-3'. The siRNA sequence is 5'-AAC GAU CUC UUC AGC ACA UCA-3' (Fukasawa et al. *Cancer Letters* **2006**, *243*, 135). The HepG2 cells were incubated with the siRNA/oligofectamine (Invitrogen) mixture for 4 h at 37 ^{o}C in a humidified atmosphere with 5% CO_2. After the incubation, the transfection medium was replaced with standard medium.

7. In vivo imaging of mice

To establish HepG2 tumor model (He et al, *Chem. Sci.* DOI: 10.1039/c6sc05712h; Cui, et al, *Food Funct.* **2016**, *7*, 455), 1×10^{6} HepG2 cells suspended in PBS were injected on the shoulder of each mouse. After three weeks, tumor-bearing mice were injected with **HCAN** in saline for in vivo imaging. At different time intervals, the mice were anesthetized and photographed using an in vivo imaging system with a 650 nm excitation

filter and a 700 nm emission filter. All animal operations were in accord with the institutional ethics committee regulations and guidelines on animal welfare.

8. Supplementary figures

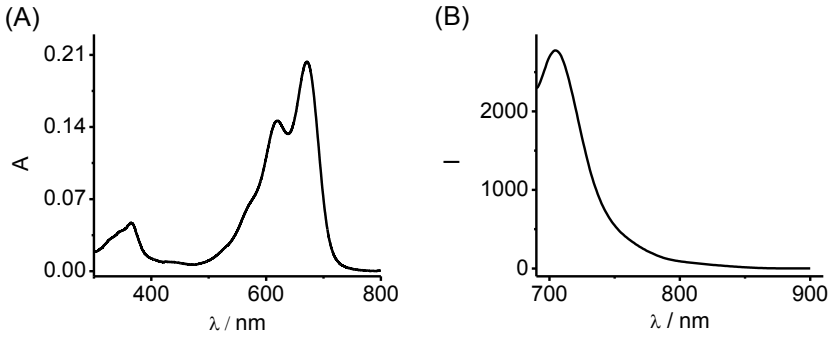


Figure S5. (A) Absorption and (B) fluorescence emission spectra of **HCA** (5 μM) in pH 7.4 PBS. λ_{ex} = 665 nm.

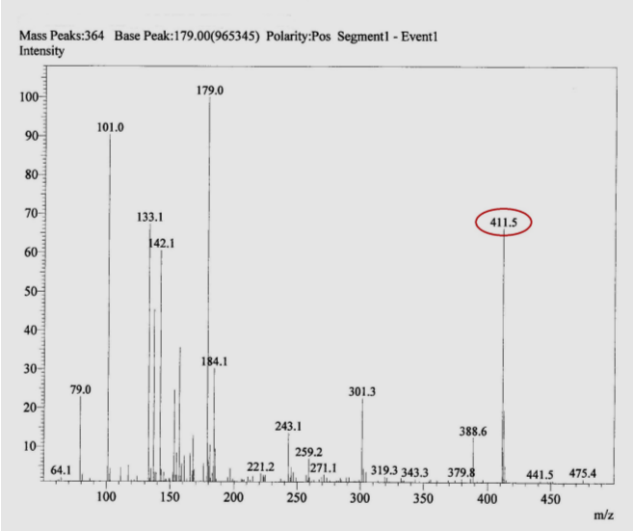


Figure S6. ESI-MS of the reaction solution of **HCAN** (50 μM) with APN (2 μg/mL).

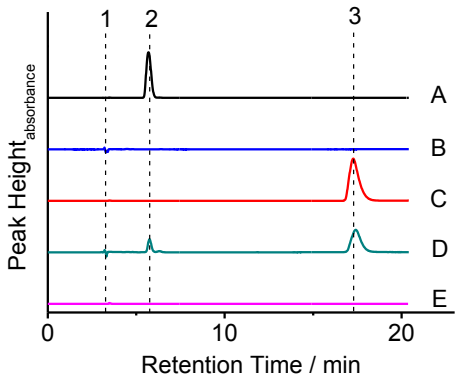


Figure S7. Chromatograms of different reaction systems. (A) **HCAN** (50 μM); (B) APN

(5 μg/mL); (C) **HCA** (50 μM); (D) the reaction solution of 50 μM **HCAN** with 2 μg/mL APN for 45 min; (E) PBS (blank). The assignments of the peaks: (1) 3.47 min, APN; (2) 5.70 min, **HCAN**; (3) 17.27 min, **HCA**.

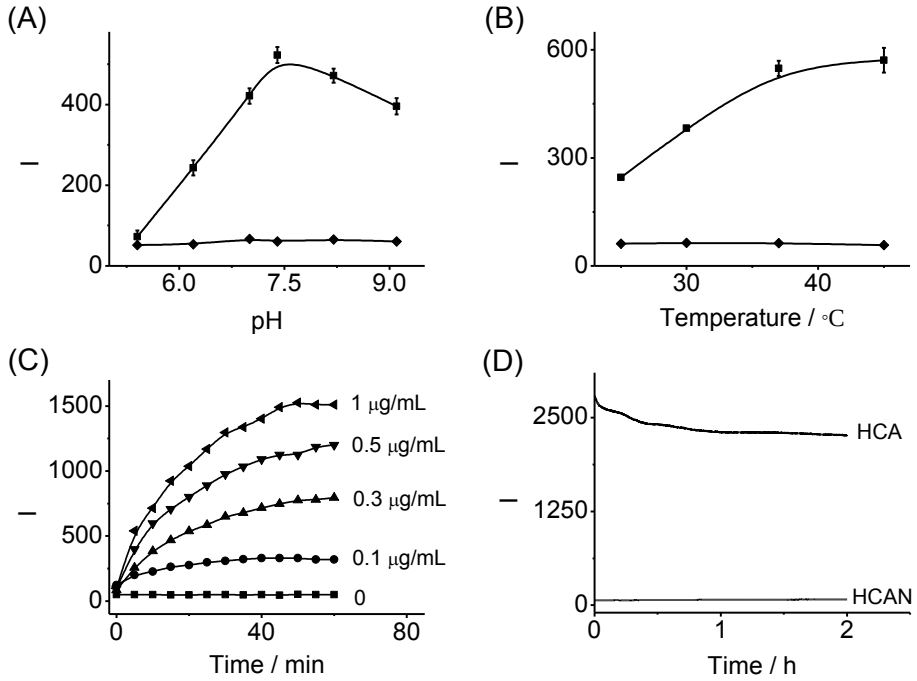


Figure S8. Effects of pH (A) and temperature (B) on the fluorescence of **HCAN** (5 μM) with (■) and without (◆) APN (0.2 μg/mL). (C) Plots of fluorescence intensity vs. the reaction time of **HCAN** (5 μM) with varied concentrations of APN (0-1 μg/mL). (D) Photostability of **HCAN** and **HCA** under the irradiation of xenon lamp. $\lambda_{ex/em}$ = 665/705 nm.

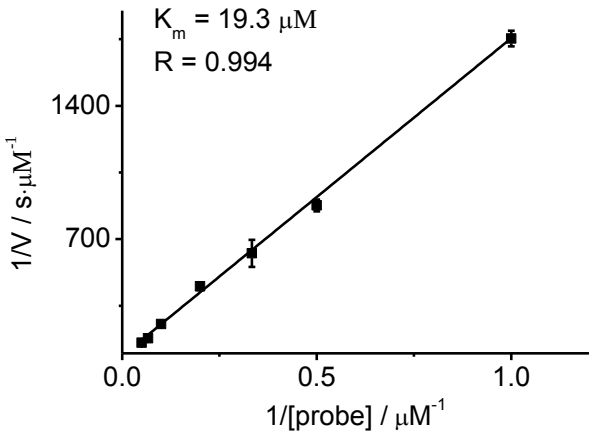


Figure S9. Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V = V_{max}$ [probe]/(K_m + [probe]), where V is

the reaction rate, [probe] is the probe concentration (substrate), and K_m is the Michaelis constant. Experiments were repeated three times at each probe concentration. Conditions: 200 ng/mL APN, and 1-20 μM of **HCAN**. $\lambda_{ex/em}$ = 665/705 nm.

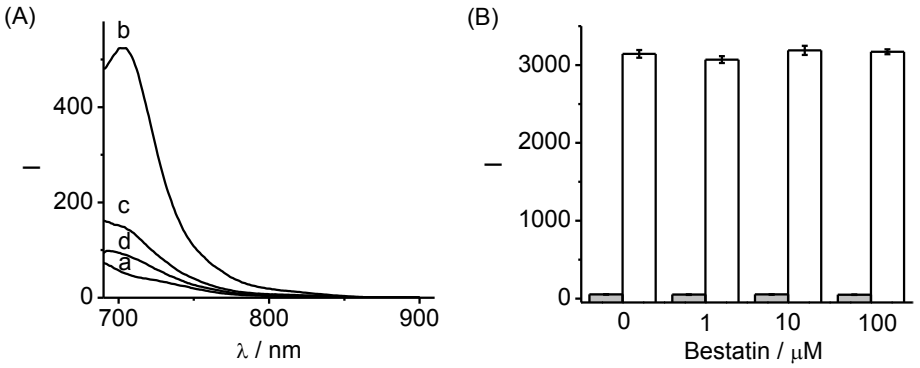


Figure S10. The inhibitor experiments. (A) Fluorescence emission spectra of different reaction systems. (a): **HCAN** (5 μM); (b): system (a) + APN (200 ng/mL); (c): system (a) + bestatin (1 μM) + APN (200 ng/mL); (d): system (a) + bestatin (10 μM) + APN (200 ng/mL). (B) Effects of bestatin at varied concentrations on the fluorescence intensity of 5 μM **HCAN** (gray) or 5 μM **HCA** (white). $\lambda_{ex/em}$= 665/705 nm.

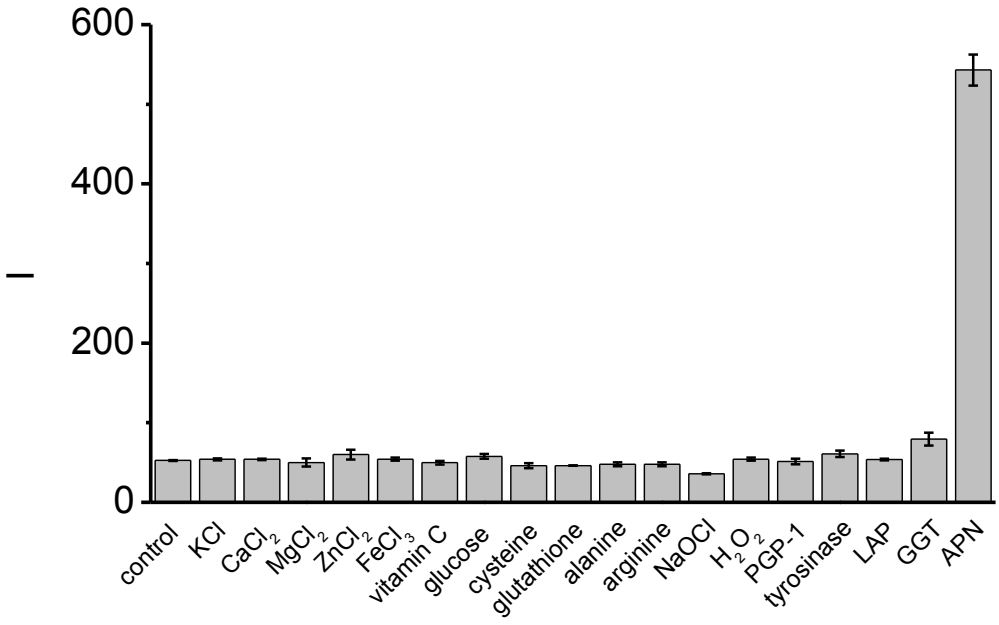


Figure S11. Fluorescence responses of probe **HCAN** (5 μM) to various potential interfering substances: (1) control; (2) KCl (150 mM); (3) $CaCl_2$ (2.5 mM); (4) $MgCl_2$ (2.5 mM); (5) $ZnCl_2$ (100 μM); (6) $FeCl_3$ (10 μM); (7) vitamin C (1 mM); (8) glucose (10 mM); (9) cysteine (1 mM); (10) glutathione (1 mM); (11) alanine (1 mM); (12) arginine (1 mM);

(13) NaOCl (100 μM; (14) H_2O_2 (100 μM); (15) PGP-1 (pyroglutamate aminopeptidase 1, 1 μg/mL); (16) tyrosinase (200 U/L); (17) LAP (leucine aminopeptidase, 1 μg/mL); (18) GGT (γ-glutamyl transpeptidase, 1 μg/mL); (19) APN (200 ng/mL). $\lambda_{ex/em}$ = 665/705 nm.

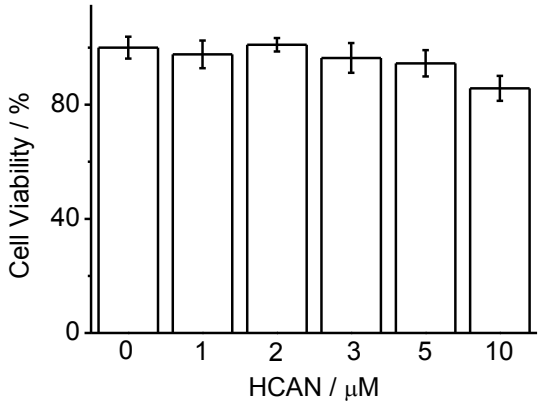


Figure S12. Effects of **HCAN** at varied concentrations (0-10 μM) on the viability of HepG2 cells. The viability of cells without **HCAN** is defined as 100%. The results are the mean ± standard deviation of five separate measurements.

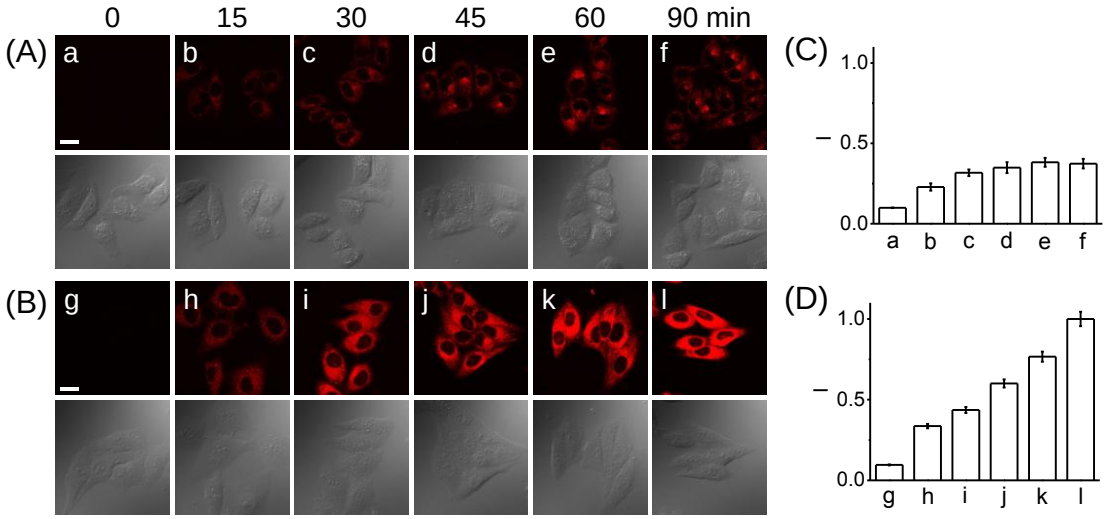


Figure S13. Fluorescence images of LO2 (A) and HepG2 (B) cells incubated with **HCAN** (5 μM) for different periods of time (0, 15, 30, 45, 60 and 90 min for images a-f and g-l, respectively). The differential interference contrast (DIC) images of the corresponding samples are shown below. Scale bars, 20 μm. Relative fluorescence intensities of the corresponding fluorescence images in panel A and panel B are shown in panel C and D, respectively. The maximum intensity of image l is defined as 1.0. The results are the mean ± standard deviation of three separate measurements.

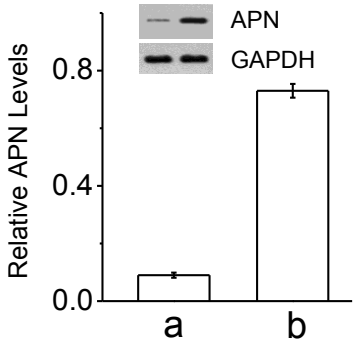


Figure S14. The APN levels in (a) LO2 and (b) HepG2 cells by western blot analyses. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein standard.

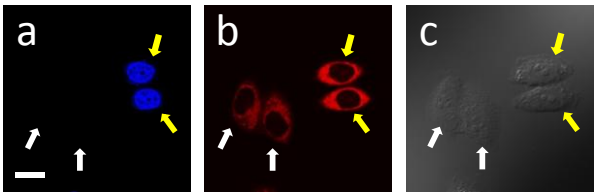


Figure S15. Confocal fluorescence images of the cell mixture (LO2 and Hoechst-33342 stained HepG2 cells) with probe **HCAN**. Image a: blue channel for Hoechst-33342 (λ_{ex} = 405 nm, λ_{em} = 430 - 530 nm); image b: red channel for **HCAN** (λ_{ex} = 635 nm, λ_{em} = 650 - 750 nm); image c: the corresponding DIC image. The yellow arrows indicate the HepG2 cells that were pre-stained with Hoechst-33342, and white arrows indicate the LO2 cells. Scale bar, 20 μm.

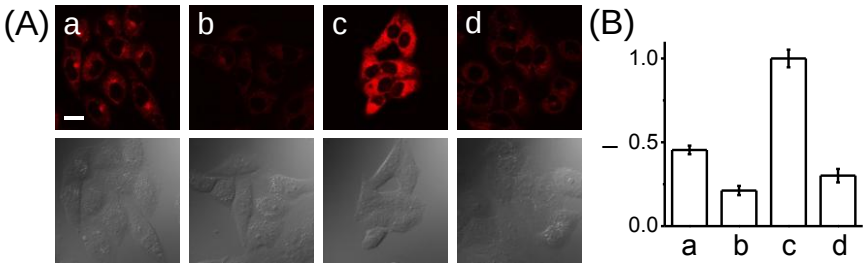


Figure S16. (A) Confocal fluorescence images of cells with probe **HCAN**. (a) LO2 cells; (b) LO2 cells pretreated with bestatin (100 μM) for 1 h; (c) HepG2 cells; (d) HepG2 cells pretreated with bestatin (100 μM) for 1 h. The DIC images of the corresponding samples are shown below. Scale bar, 20 μm. (B) Relative fluorescence intensity of the corresponding fluorescence images in panel A (the intensity from image c is defined as 1.0). The results are the mean ± standard deviation of three separate measurements.

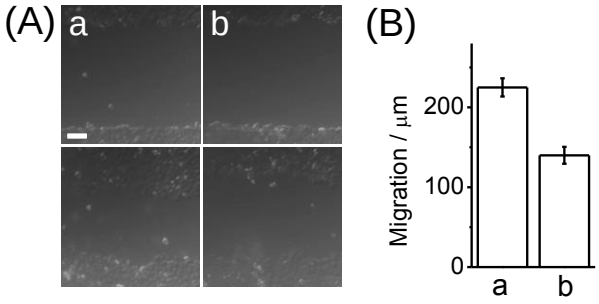


Figure S17. (A) Cellular morphology of HepG2 (a) and LO2 cells (b) of immediately (up) and at 24 h (down) after scratch. (B) Cell migration distance in the corresponding images in panel A. Scale bar, 100 μm.

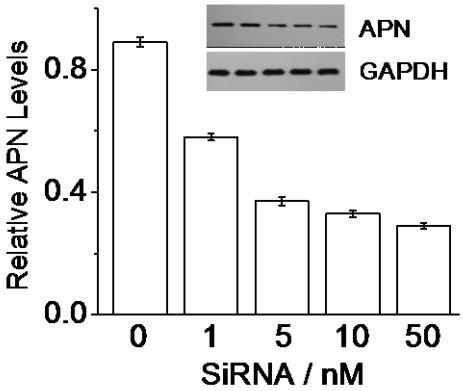


Figure S18. APN levels in the siRNA-transfected HepG2 cells by western blot assay. HepG2 cells were pretreated with varied concentrations of siRNA (0, 1, 5, 10, and 50 nM) for 4 h.

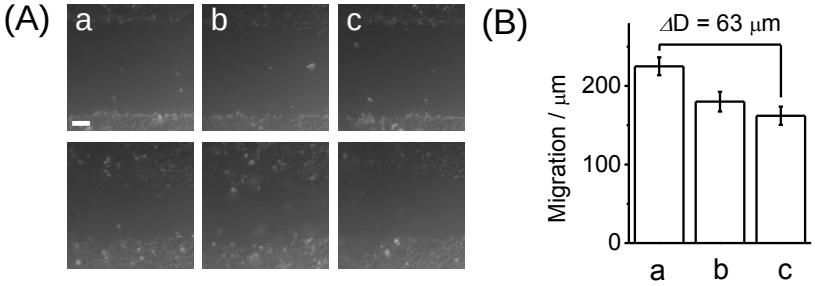


Figure S19. (A) Cellular morphology of HepG2 incubated with 0 (a), 50 (b), 100 μM (c) of bestatin immediately (up) and at 24 h (down) after scratch. (B) Cell migration distance in the corresponding images in panel A. Scale bar, 100 μm.

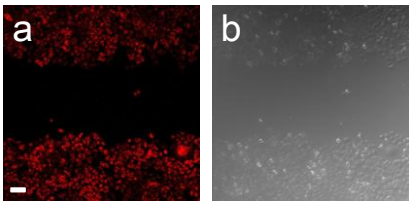


Figure S20. Confocal fluorescence image (a) and the corresponding DIC image (b) of HepG2 cells. After scratch, the cells were cultured for 24 h, and then stained with **HCAN** (5 μM). Scale bar, 100 μm.

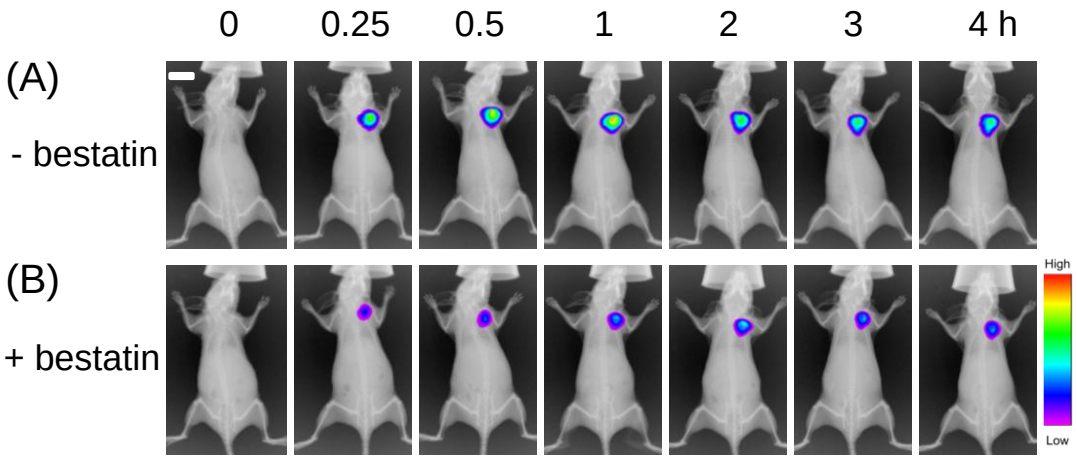


Figure S21. In vivo fluorescence imaging of HepG2 tumor-bearing mice. The mice were intratumorally preinjected with 50 μL of (A) saline (control) and (B) bestatin (100 μM in saline) for 1 h, and then subjected to the intratumoral injection of 50 μL **HCAN** (50 μM) in saline for different periods of time (0, 0.25, 0.5, 1, 2, 3 and 4 h). Scale bar, 1 cm.