Electronic Supplementary Information

Enzyme-activated near-infrared fluorogenic probe with high-efficiency intrahepatic targeting ability for visualization of drug-induced liver injury

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Materials and Methods

Reagents and Apparatus.

All chemicals were purchased from Sigma Aldrich and used without further purification. Leucine aminopeptidase (LAP) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. All cell culture media, fetal bovine serum (FBS) and phosphate buffered saline (PBS, pH 7.4) were purchased from HyClone. The ultra-pure water (18 Ω) used in all experiments was obtained from a Milli-Q system (Millipore, USA). High resolution mass spectrometry (HRMS) was obtained on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, USA). The MS parameters are positive ion mode, electrospray ionization (ESI), and the resolution is 30000 (FWHM). $^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker Advance spectrometer with TMS as an internal standard. Column chromatography was performed using silica gel (200-300 mesh, Qingdao Ocean Chemicals, Qingdao, China). Spectroscopic data was measured on a FS5 Spectrofluorometer (Edinburgh Instruments Ltd., UK) or SpectraMax M4 by Molecular Device. Photostability data was measured on a FS5 Spectrofluorometer with an intermittent excitation of xenon lamp (150 W, once per min, 180 times). High-performance liquid chromatography (HPLC) chromatograms were performed on Agilent, Infinity 1260 with a C18 column (100 Å, 30 × 4.6 mm). Fluorescence images of cells were carried out on a Nikon Ti-e confocal laser scanning microscope. The in vivo fluorescence images were acquired with the IVIS spectrum system (PerkinElmer, USA). The fluorescence intensity of region of interest was analyzed by Living Image 4.5 Software.
**Determination of the fluorescence quantum yield.**

Quantum yields for all the fluorescent compounds were measured by dividing the integrated emission area of their fluorescent spectrum against the area of ICG in PBS excited at 730 nm ($\Phi_{ICG} = 0.13$). Quantum yields where then calculated using equation (1), where $F$ represents the integrated emission area of fluorescent spectrum, $\eta$ represents the refractive index of the solvent, and Abs represents absorbance at excitation wavelength selected for standards and samples. Emission was integrated from 640 nm to 800 nm.

$$\Phi_{sample} = \Phi_{reference} \left( \frac{F_{sample}}{F_{reference}} \right) \left( \frac{\eta_{sample}}{\eta_{reference}} \right) \left( \frac{Abs_{reference}}{Abs_{sample}} \right)$$

(1)

**Cell Culture and Cytotoxicity Assay.**

Human normal liver cell line (LO2) was purchased from iCell Bioscience Inc. (Shanghai, China) and human hepatocarcinoma cell line (HepG2) was purchased from Cell Resource Centre, Peking Union Medical College. LO2 cells were incubated in Roswell Park Memorial Institute (RPMI)-1640 culture medium containing 20% (v/v) FBS and 1% (v/v) penicillin-streptomycin at 37°C in a 5% CO$_2$ humidified atmosphere. HepG2 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) culture medium containing 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin at 37°C in a 5% CO$_2$ humidified atmosphere. The cytotoxicity of hCy-CA-LAP and hCy-LAP to LO2 and HepG2 cells was measured by using standard Cell Counting Kit-8 system (CCK-8) assays. LO2 and HepG2 cells were respectively plated in a 96-well plates at $1 \times 10^5$ cells/well and incubated overnight in the cell incubator before exposure to hCy-CA-LAP and hCy-LAP. Then, different concentrations of hCy-CA-LAP or hCy-LAP
probes were incubated with cells for 24 hours. After removing the above solution, CCK-8 reagent diluted with RPMI-1640 medium (20% FBS) or DMEM (10% FBS) was added to each well and incubated for 30 min. Finally, the absorbance was finally measured at 450 nm by a microplate reader.

**Cell imaging.**

For cell fluorescence imaging, LO2 or HepG2 cells were plated in a glass-bottom dish for 24 h before the experiment. Fluorescence images of the cells were captured by a Nikon Ti-e microscope ($\lambda_{ex} = 639$ nm, $\lambda_{em} = 663-738$). Cell fluorescence imaging of hCy-CA-LAP in normal LO2 cells can be divided into five groups. hCy-CA-LAP (1 mM in DMSO) was diluted with culture media and reached a concentration of 10 μM in cell culture wells. In group hCy-CA-LAP, hCy-CA-LAP (10 μM) was incubated with LO2 or HepG2 cells for 30 min. In group Bestatin + hCy-CA-LAP, LO2 or HepG2 cells were pertreated with bestatin (100 μM) for 1 h before incubating with hCy-CA-LAP. In group APAP + hCy-CA-LAP, LO2 or HepG2 cells were pertreated with APAP (1 mM) for 12 h followed by bestatin (100 μM) for 1 h before incubating with hCy-CA-LAP. In group NAC + APAP + hCy-CA-LAP, LO2 or HepG2 cells were pertreated with NAC (100 μM) for 1 h followed by APAP (1 mM) for 1 h before incubating with hCy-CA-LAP. The cells were washed 3 times with PBS buffer before cell imaging.
In vivo fluorescence imaging of acetaminophen (APAP) induced acute liver injury.

All animal procedures were performed in accordance with the Ethics Committee of Beijing University of Technology, China. Female BALB/c mice (body weight, around 18-20 g) were purchased from Beijing HFK Bioscience Co., Ltd., and maintained under standard conditions. The mice were housed in sterile cages and fed water and food ad libitum. For in vivo imaging APAP induced acute liver injury, the mice were divided into four groups, three in each group, and the mice were fasted overnight before the experiment. In group PBS + hCy-CA-LAP, mice were intraperitoneally injected with PBS (200 µM) for 1 h, and then intravenously injected with hCy-CA-LAP (50 µM, 100 µL). In group APAP + hCy-CA-LAP, mice were intraperitoneally injected with APAP (300 mg/Kg, 200 µL) for 1 h, and then intravenously injected with hCy-CA-LAP (50 µM, 100 µL). In group PBS + hCy-LAP, mice were intraperitoneally injected with PBS (200 µM) for 1 h, and then intravenously injected with hCy-LAP (50 µM, 100 µL). In group APAP + hCy-LAP, mice were intraperitoneally injected with APAP (300 mg/Kg, 200 µL) for 1 h, and then intravenously injected with hCy-LAP (50 µM, 100 µL). Subsequently, the mice were immediately performed whole body optical imaging at predetermined time points (0, 1, 2, 3, and 4 h) via IVIS imaging system (Perkin Elmer) equipped with fluorescent filter sets ($\lambda_{ex} = 640$ nm, $\lambda_{em} = 740$ nm). Data were analyzed with the Living Image 4.5 Software and ROIs of equal area in the regions of interest were obtained for further analysis. The above four groups of mice were all sacrificed 3 h after intravenously injecting probes. Major organs (heart, liver kidney,
spleen, lung) were collected and washed with saline. The images of the organs were taken using IVIS imaging system as described above.

**In vivo fluorescence imaging of rifampicin (RFP) induced cholestatic liver injury.**

For in vivo imaging of RFP-induced cholestatic liver injury, the mice were divided into three groups, three in each group, and the mice were fasted overnight before the experiment. Mice were with RFP at a hepatotoxic dosage (dissolved in aqueous solution of 0.5% carboxymethylcellulose sodium, 300 mg·Kg⁻¹·d⁻¹) for one or three days. For the control group, the mice were intragastrically administrated with 200 μL of aqueous solution of 0.5% carboxymethylcellulose sodium. The mice were then intravenously injected with hCy-CA-LAP (50 μM, 100 μL) at 6 h after the last drug treatment. Subsequently, the mice were immediately performed whole body optical imaging at predetermined time points (0, 25, 50, 75, and 100 min) via IVIS imaging system equipped with fluorescent filter sets (λₑₓ = 640 nm, λₑᵐ = 740 nm). ROIs of equal area in the regions of interest were obtained for further analysis. The three groups of mice were sacrificed 1 h after intravenously injecting probes. Major organs (heart, liver, kidney, spleen, lung) were collected and washed with saline. The images of the organs were taken using IVIS imaging system as described above.

**Histology.**

Liver tissues of mice were excised and then rinsed with normal saline, and fixed in 10% formaldehyde solution for paraffin embedding. The embedded liver tissues were processed into 10 μm section. Histological examination was performed by staining with hematoxylin and eosin (H&E). All images were obtained from a Nikon ECLIPSE Ti2-U
inverted fluorescence microscope equipped with a IXON-L-888 cameras (ANDOR, UK).

**Synthesis and Characterizations**

**Scheme S1** Synthetic route of hCy-LAP and hCy-CA-LAP.

**Synthesis of hCy7-LAP-Fmoc.** NIR fluorophore hCy7 and compound Fmoc-Leu-Br were prepared by the reported methods.\(^2\)\(^,\)\(^3\) A mixture of hCy7 (28.1 mg, 0.063 mmol), Ag\(_2\)CO\(_3\) (34.7 mg, 0.126 mmol) and Na\(_2\)SO\(_4\) (17.9 mg, 0.126 mmol) in 3 mL MeCN was stirred at room temperature for 30 min, and then Fmoc-Leu-Br (32.8 mg, 0.063 mmol) was added under a nitrogen atmosphere. The mixture was stirred at 40 °C for 3 h. The solvent was evaporated under reduced pressure and the crude product was purified by column chromatography (DCM: MeOH = 25:1) to afford hCy7-LAP-Fmoc as a blue solid (18 mg, 32%). \(^1^H\) NMR (600 MHz, CDCl\(_3\)) \(\delta\) 10.82 (s, 1H), 8.36 (d, \(J = 14.8\) Hz, 1H), 8.09 (d, \(J = 8.0\) Hz, 2H), 7.89 – 7.85 (m, 2H), 7.64 (dd, \(J = 13.3, 6.6\) Hz,
4H), 7.35 (dt, J = 15.3, 7.4 Hz, 2H), 7.29-7.27 (m, 2H), 7.26-7.18 (m, 7H), 6.97 (s, 1H), 6.68 (s, 1H), 6.25 (d, J = 14.8 Hz, 1H), 5.17 (q, J = 13.3 Hz, 2H), 4.19-4.16 (m, 3H), 4.05 (t, J = 7.5 Hz, 1H), 4.02 – 3.97 (m, 1H), 2.61 (s, 2H), 2.52 (s, 2H), 1.85-1.81 (m, 2H), 1.78 (m, 4H), 1.72 (s, 6H), 0.98 (t, J = 7.3 Hz, 3H), 0.90 (t, J = 6.0 Hz, 6H). $^{13}$C NMR (151 MHz, CDCl$_3$) δ 178.07, 172.91, 160.81, 156.94, 156.56, 152.43, 145.84, 144.27, 141.98, 141.14, 140.11, 131.92, 129.83, 129.06, 128.40, 128.15, 127.79, 127.51, 127.48, 127.21, 127.03, 125.94, 125.84, 124.01, 120.97, 120.59, 119.68, 115.86, 114.63, 112.28, 104.19, 102.37, 72.17, 66.78, 56.40, 51.17, 47.23, 47.10, 41.76, 29.81, 29.19, 28.19, 25.09, 23.17, 22.02, 21.39, 20.22, 11.63. HRMS m/z (C$_{56}$H$_{57}$Cl$_3$N$_3$O$_5$)$^+$ calculated (M)$^+$: 886.3981, found (M)$^+$: 886.4012.

Synthesis of hCy7-LAP. Compound hCy7-LAP-Fmoc (18 mg, 0.02 mmol) was added to 20% piperidine in DMF (1.25 mL) and stirred at room temperature for 1 h. Then the mixture was extracted with DCM and washed with saturated brine. The crude product was purified by column chromatography (DCM: MeOH = 10:1) to afford hCy7-LAP as a blue solid (8 mg, 60%). $^1$H NMR (400 MHz, CDCl$_3$) δ 10.41 (s, 1H), 8.51 (d, J = 14.9 Hz, 1H), 7.79 (d, J = 8.5 Hz, 2H), 7.69 (d, J = 7.0 Hz, 1H), 7.47 – 7.38 (m, 2H), 7.34 (t, J = 4.2 Hz, 4H), 7.03 (s, 1H), 6.86 (s, 1H), 6.43 (d, J = 14.9 Hz, 1H), 5.22 (s, 2H), 4.33 (t, J = 7.1 Hz, 2H), 3.80 (dd, J = 8.2, 5.1 Hz, 1H), 2.71 – 2.55 (m, 4H), 1.98 – 1.89 (m, 2H), 1.88 – 1.81 (m, 2H), 1.77 (d, J = 1.5 Hz, 6H), 1.56 – 1.46 (m, 1H), 1.03 (t, J = 7.4 Hz, 3H), 0.88 (t, J = 6.0 Hz, 6H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 178.18, 173.01, 160.78, 156.90, 152.62, 145.97, 142.11, 141.39, 138.89, 131.80, 130.61, 129.25, 128.60, 127.90, 127.83, 127.66, 123.29, 120.87, 120.21, 115.97, 114.98,
Synthesis of hCy7-CA. NIR fluorophore hCy7-NH₂ was prepared by the reported method.⁴ A mixture of hCy7-NH₂ (54 mg, 0.117 mmol), cholic acid (47.8 mg, 0.117 mmol), HBTU (53 mg, 0.14 mmol) and DIPEA (40.8 μL, 0.234 mmol) in 2 mL DMF was stirred at room temperature for 2 h. Then, excess petroleum ether was added to remove solvent, and the crude product was purified by column chromatography (DCM: MeOH = 10:1) to afford hCy7-CA as a blue solid (26 mg, 26%).¹ H NMR (600 MHz, DMSO) δ 8.17 (d, J = 13.8 Hz, 1H), 7.98 (s, 1H), 7.65 (s, 1H), 7.59 (s, 1H), 7.54 (d, J = 7.4 Hz, 1H), 7.36 (t, J = 7.6 Hz, 1H), 7.29 (d, J = 7.7 Hz, 1H), 7.18 (t, J = 7.3 Hz, 1H), 6.55 (d, J = 2.9 Hz, 1H), 6.03 (d, J = 13.8 Hz, 1H), 4.08 (s, 2H), 3.73 (s, 1H), 3.20 – 3.10 (m, 3H), 2.66 (s, 2H), 2.61 (s, 2H), 2.18 (dd, J = 25.5, 13.2 Hz, 2H), 2.12-2.08 (m, 2H), 2.00-1.91 (m, 2H), 1.83-1.78 (m, 4H), 1.77 – 1.69 (m, 3H), 1.66 (s, 6H), 1.61-1.58 (m, 2H), 1.43-1.37 (m, 4H), 1.34 – 1.27 (m, 3H), 1.26 – 1.08 (m, 8H), 0.89 (dd, J = 13.6, 6.2 Hz, 3H), 0.87 – 0.81 (m, 1H), 0.79 (t, J = 5.6 Hz, 1H), 0.74 (s, 3H), 0.57 (s, 1H), 0.50 (s, 3H). ¹³C NMR (151 MHz, DMSO) δ 174.94, 172.75, 159.77, 155.78, 155.75, 142.83, 142.49, 140.29, 140.28, 128.33, 127.77, 124.11, 123.75, 122.28, 118.91, 115.03, 114.36, 110.07, 109.87, 102.65, 71.00, 70.42, 66.22, 48.59, 48.14, 48.12, 46.13, 45.70, 41.50, 41.33, 41.11, 36.00, 35.25, 34.99, 34.84, 34.33, 32.56, 31.71, 30.37, 28.52, 27.97, 27.60, 27.25, 26.95, 26.18, 22.74, 22.59, 22.51, 20.54, 17.11, 12.30. HRMS m/z (C₅₂H₆₈ClN₂O₆⁺) calculated (M)⁺: 851.4760, found (M)⁺:
Synthesis of hCy7-CA-LAP. A mixture of hCy7-CA (26 mg, 0.03 mmol), Ag₂CO₃ (16.5 mg, 0.06 mmol) and Na₂SO₄ (8.5 mg, 0.06 mmol) in a mixed solution of 1 mL MeOH and 3 mL MeCN was stirred at room temperature for 30 min, then Fmoc-Leu-Br (16 mg, 0.03 mmol) was added under nitrogen atmosphere. The mixture was stirred at 40°C overnight. After reaction, the solvent was evaporated under reduced pressure to obtain a blue solid, which was directly used for subsequent synthesis without purification. The crude intermediate was added to 20% piperidine in DMF (1.25 mL) and stirred for 1 h at room temperature. Then, excess petroleum ether was added to remove solvent, and the crude product was purified by semi-preparative HPLC to afford hCy7-CA-LAP as a blue solid (3 mg, 9%). ¹H NMR (400 MHz, DMSO) δ 10.88 (s, 1H), 8.54 (d, J = 14.9 Hz, 1H), 8.15 (s, 1H), 7.84 (d, J = 7.3 Hz, 1H), 7.76 (dd, J = 21.0, 8.2 Hz, 4H), 7.58 – 7.46 (m, 4H), 7.39 (s, 1H), 7.31 (s, 1H), 6.60 (d, J = 15.0 Hz, 1H), 5.36 (s, 2H), 4.45 (s, 2H), 4.34 (s, 1H), 4.09 (s, 1H), 3.99 (s, 1H), 3.73 (s, 1H), 3.53 (s, 1H), 3.21 (d, J = 7.4 Hz, 1H), 3.19 – 3.10 (m, 3H), 2.78 – 2.58 (m, 4H), 2.17 – 2.08 (m, 2H), 2.01 – 1.90 (m, 4H), 1.79 – 1.74 (m, 6H), 1.72 – 1.65 (m, 4H), 1.64 – 1.58 (m, 5H), 1.45 – 1.38 (m, 4H), 1.28 – 1.17 (m,12H), 0.91 – 0.84 (m, 9H), 0.74 (s, 3H), 0.50 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 177.91, 174.31, 172.86, 159.64, 155.96, 152.24, 144.94, 142.26, 141.31, 138.70, 131.19, 130.88, 129.63, 128.90, 128.70, 128.24, 127.78, 127.44, 122.93, 119.52, 118.83, 115.84, 114.28, 113.37, 105.33, 102.00, 70.97, 70.40, 66.21, 50.63, 46.13, 45.69, 41.50, 41.33, 35.81, 35.26, 35.07, 34.86, 34.33, 32.47, 31.63, 31.27, 30.37, 29.02, 28.69, 28.54, 27.70, 27.44, 27.27,
26.58, 26.19, 25.10, 23.92, 23.64, 22.74, 22.54, 22.07, 19.81, 17.12, 13.94, 12.29. HRMS \( m/z \) (C\(_{65}\)H\(_{86}\)ClN\(_4\)O\(_7\)) calculated (M): 1069.6180, found (M): 1069.6185.

**Table S1** Comparison of LAP-activated imaging probes (hCy-CA-LAP and hCy-LAP) with corresponding products.

|             | \( \lambda_{abs} \) (nm) | \( \lambda_{em} \) (nm) | Max Fl. Time (min) | F/F\(_0\) | Detection limit (U/mL) | \( \Phi \) |
|-------------|--------------------------|--------------------------|-------------------|--------|------------------------|--------|
| hCy-CA-LAP  | 615                      | -                        | -                 | -      | -                      | <0.01  |
| hCy-LAP     | 603                      | -                        | -                 | -      | -                      | <0.01  |
| hCy-CA-LAP +LAP | 680                  | 710                      | 60                | 27     | 0.0067                 | 0.20   |
| hCy-LAP +LAP | 680                  | 705                      | 40                | 78     | 0.002                  | 0.27   |

**Figure S1** Photostability of hCy-CA-LAP and hCy-LAP (10 \( \mu \)M) before and after the addition of LAP monitored at 710 nm at 37°C. \( \lambda_{ex} = 680 \) nm, once per min, 180 times, source: xenon lamp (150 W).
**Figure S2** *In vitro* response of hCy-LAP toward LAP. (A) The sensing kinetics of hCy-LAP ($F_{705\text{ nm}}$) and (B) fluorescence responses of hCy-LAP (10 $\mu$M) to different concentrations of LAP (0-0.02 U·mL$^{-1}$). (C) Fluorescence intensity of hCy-LAP (10 $\mu$M) at 705 nm with different concentrations of LAP (0-0.2 U·mL$^{-1}$). (D) Linear correlation between fluorescence intensity at 705 nm and low concentrations of LAP (0-0.02 U·mL$^{-1}$), $\lambda_{ex}=680$ nm. All spectra measurements were recorded in PBS solution (pH = 7.4) at 37°C.
Figure S3 HRMS spectrum of hCy-CA-LAP.

Figure S4 HRMS spectrum of the reaction solution of hCy-LAP-CA with LAP.
Figure S5 HRMS spectrum of hCy-CA.

Figure S6 HRMS spectrum of the reaction solution of hCy-LAP with LAP.
Figure S7 Chromatograms of hCy-LAP, hCy-LAP reacting with LAP and hCy. $\lambda_{abs} = 600$ nm.

Figure S8 Fluorescence response of hCy-LAP (10 μM) to different analytes (analytes: 1 mM, enzyme: 0.1 U·mL$^{-1}$) in PBS buffer (pH 7.4) at 37°C, $\lambda_{ex} = 680$ nm, $\lambda_{em} = 705$ nm.
Figure S9 Effects of pH on the fluorescence of (A) hCy-CA-LAP and (B) hCy-LAP (10 μM) in the absence (black) and presence (red) of LAP (0.2 U·mL⁻¹). All spectra measurements were recorded in PBS solution (pH = 7.4) at 37°C.

Figure S10 Cytotoxicity of hCy-CA-LAP and hCy-LAP to LO2 and HepG2 cells. Cells were incubated with the probe at corresponding concentrations for 24 h. The results are reported as percentage relative to untreated cells and the results are the mean standard deviation of three separate measurements.
**Figure S11** Organs dissected from mice 3 h after intravenous injection of hCy-CA-LAP (50 μM, 100 μL). $\lambda_{ex} = 640$ nm, $\lambda_{em} = 740$ nm.

**Figure S12** Time-dependent *in vivo* fluorescence imaging. (A) Balb/c female mice were pretreated with APAP (300 mg/Kg, 200 μL) or PBS (200 μL) for 1 h, and then intravenous injected with hCy-LAP (50 μM, 100 μL). (B) Organs dissected from mice
3 h after intravenous injection of **hCy-LAP** (50 µM, 100 µL). (C) Quantification of the fluorescence signal in (B) *: P < 0.05, and **: P < 0.01, compared with the control group (PBS-treated group). $\lambda_{ex} = 640$ nm, $\lambda_{em} = 740$ nm.

**Figure S13** Quantification of the fluorescence signal at the imaging site of APAP-treated mice after intravenous injection of **hCy-CA-LAP** and **hCy-LAP** (50 µM, 100 µL).

**Figure S14** Organs dissected from mice 60 min after intravenous injection of **hCy-CA-LAP** (50 µM, 100 µL). $\lambda_{ex} = 640$ nm, $\lambda_{em} = 740$ nm.
NMR spectra

Figure S15 $^1$H NMR spectrum of hCy-LAP-Fmoc.

Figure S16 $^{13}$C NMR spectrum of hCy-LAP-Fmoc.
Figure S17 $^1$H NMR spectrum of hCy-LAP.

Figure S18 $^{13}$C NMR spectrum of hCy-LAP.
Figure S19 $^1$H NMR spectrum of hCy-CA.

Figure S20 $^{13}$C NMR spectrum of hCy-CA.
Figure S21 $^1$H NMR spectrum of hCy-CA-LAP.

Figure S22 $^{13}$C NMR spectrum of hCy-CA-LAP.
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