Supporting information

A Tumor-targeting Probe Based on Mitophagy Process for Live Imaging

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This Supplementary Information file replaces that originally published on 4th August 2018, in which incorrect sugar structures were shown for CyT and CyTH. This error did not affect any of the results or conclusions in the article.

Experimental section

Reagent and Instruments

Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The silica column chromatography used the silica gel (100–200 mesh). LysoTracker Blue and MitoTracker Green were obtained from KeyGen BioTech (Jiangsu, China). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphe-nyltetrazolium bromide (MTT), Dulbecco’s modified Eagle’s medium (DMEM), trypsin-EDTA, and fetal bovine serum (FBS), were purchased from the Institute of Biochemistry and Cell
Biology, SIBS, CAS (China). Phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4, 4.3 mM Na2HPO4, pH 7.4) solution was obtained from Invitrogen. All other chemicals were obtained from by J&K Scientific Ltd. (Beijing, China) or Sinopharm chemical reagent Co. Ltd. (Shanghai, China) and utilized without further purification. Ultrapure water from a Milli-Q reference system (Millipore) was applied in all experiments.

**Synthesis of CyO**

To an anhydrous DMF (20 mL) solution of CyCl (0.80 g, 1.33 mmol, 1 equiv) was added sodium acetate (0.436 g, 5.32 mmol, 4 equiv). The mixture was reacted at 90°C for 6 h under the protection of nitrogen atmosphere and then cooled to room temperature. After that, the mixture was diluted with dichloromethane (100 mL) and washed with water (60 mL × 3) and brine (60mL × 3). After being extraction, the mixture was dried with anhydrous sodium sulfate and then filtered. After the reaction, the solvent was dried by vacuum rotary evaporator and obtained crude product which was purified by the column chromatography (silica gel, eluted with CH2Cl2/MeOH= 25:1, v/v) to acquire the desired purple solid powder (340 mg, 44 % yield). 1H NMR (500 MHz, DMSO -d6), δ(ppm): 12.43 (s, 2H), 7.93 (d, J = 10 Hz, 2H), 7.33 (t, J = 5 Hz, 3H), 7.19 (t, J = 10 Hz, 3H), 6.92 (d, J = 5 Hz, 2H), 5.77 (s, 1H), 5.56 (s, 1H), 3.90 (t, J = 15 Hz, 4H), 3.34 (t, J = 15 Hz, 4H), 2.57(t, J = 15 Hz, 4H), 1.56 (s, 12H), 1.26 (m, J = 13.5 Hz, 2H). 13C NMR (500 MHz, DMSO-d6), δ (ppm): 184.66, 172.61, 168.63, 161.05, 143.35, 138.7, 131.69, 127.70, 126.26, 121.67, 120.45, 107.28, 92.42, 45.75, 37.95, 30.89, 28.93, 28.13, 25.26. MS (ESI): m/z, calculated for C36H40N2O5: 579.7321[M-H]-, found 579.3.

**Synthesis of CyT**

The mixture of DIPEA (0.09 g, 0.72 mmol, 4 equiv) and HATU (0.27 g, 0.72 mmol, 4 equiv) was added to a solution of CyO (0.10 g, 0.18 mmol, 1equiv) in anhydrous DMF (8 mL) in under N2 atmosphere. After stirring for 10 minutes, D-glucosamine hydrochloride (0.13 g, 0.72 mmol, 4 equiv) was added, and the mixture was stirred at room temperature for 12 h in the dark under N2 atmosphere, and then poured into
ether (50 mL). The precipitation was collected by filtration and was purified by the column chromatography (silica gel, eluted with CH$_2$Cl$_2$/MeOH= 9:1, v/v ) to obtain the title compound as a purple solid (0.11 g, 73%).$^1$H NMR (500 MHz, DMSO-$d_6$), δ/ppm: 8.69 (s, 1H), 8.48 (s, 1H), 7.95 (t, J=5 Hz, 2H), 7.80 (d, J=5 Hz, 1H), 7.47 (d, J=10 Hz, 1H), 7.34(t, J=10 HZ, 1H), 7.22(t, J=7.5 Hz, 1H), 6.96(m, J=10 Hz, 4H), 6.45(t, J=7.5 Hz,1H), 6.31(s, 1H), 5.56 (d, J=15 Hz, 2H), 5.12(m, J=15 Hz, 2H), 4.83(m, J=17.5 Hz, 2H), 4.51(m, J=15 Hz, 4H), 3.98(s, 2H), 3.75(m, J=17.5 Hz, 10H), 3.50(m, J=15 Hz, 2H), 2.96(d, J=20 Hz, 6H), 2.71(s, 2H), 2.56(s, 2H)1.58(m, J=20 Hz, 14H), $^{13}$C NMR (500 MHz, DMSO-$d_6$), δ (ppm): 185.19, 170.80, 161.77, 144.04, 139.27, 128.25, 126.63, 122.19, 120.96, 107.90, 96.39, 92.85, 91.49, 70.90, 68.60, 67.79, 61.03, 50.87, 46.33, 40.47, 39.47, 32.69, 28.69, 22.67. HRMS (ESI): m/z, calculated for C$_{48}$H$_{62}$N$_4$O$_{13}$: 903.0452[M+H]$^+$, found 903.4391.

**Spectral Measurement**

Absorption and fluorescence spectra of CyO, CyT and glucosamine in PBS buffer: The measured concentration of CyO, CyT was finally fixed at 10 μM, and the concentration of glucosamine was fixed at 20 μM. The absorption and fluorescence spectra were measured at room temperature.

Absorption and fluorescence spectra of CyT in different solvents: The measured concentration of CyT was finally fixed at 10 μM. Preparation of CyT stock solution concentration: 1mM. CyT stock solution was dissolved in different solvents to a final volume of 3ml, and the absorption and fluorescence spectra were measured immediately at room temperature.

Absorption and fluorescence spectra of CyT at different pH: 30 μL of stock solution of CyT (1 mM) was mixed with 10 mM SDS in different pH PBS buffer to a final volume of 3ml with a final concentration of 10μM. The absorption and fluorescence spectra were recorded at room temperature.

**DFT calculations**

The changes in absorption and emission spectra of CyT and CyTH was evaluated by the time-dependent density functional theory (TD-DFT) with B3LYP functional
analysis based on the 6-31G* level of theory. Calculations were implemented using the commercial Gaussian 09W software package. HOMO and LUMO orbital energies were determined from the molecular geometries optimized at the B3LYP 6-31G(d) level. Molecular orbitals were visualized using the Gauss View 5.0 software package.

Cell culture

MCF-7 (human breast adenocarcinoma cell line), A549 (adenocarcinomic human alveolar basal epithelial cell), and L02 cell (normal human hepatic cell) provided from American Type Culture Collection (ATCC, USA). The cell lines were propagated in Dulbecco’s Modified Eagle’s Medium (DMEM). Unless otherwise stated, the basic media were supplemented with 10% fetal bovine serum (FBS) and 1 % (v/v) penicillin-streptomycin. All cells were cultured in a humidified incubator at 37 °C and 5% CO₂.

Cytotoxicity Assay

The in vitro cytotoxicity was examined by an MTT assay in L02 and A549 cell lines. The cells were transferred to 96-well cell culture plate (1 × 10⁴/well) and subsequently incubated for 24 h in CO₂ culture box. The cells were further maintained at 37 °C for 24 h under 5 % CO₂ after treatment of CyT (100 μL/well) at a wide concentration range from 0 to 150 μM. Before adding MTT solution (15 μL, 5.0 mg mL⁻¹), each well was washed three times with PBS (pH =7.4) and the cells were incubated for another 4 h. The medium containing MTT was carefully discarded and then 150 μL of DMSO was added into each well. Before measuring the absorbance, the plates were gently shaken for 15 min at room temperature. The viability of cell growth was calculated by the following formula: viability (%) = (mean absorbance of test wells - mean absorbance of medium control wells) / (mean absorbance of untreated wells - mean absorbance of medium control well) × 100%.

Confocal fluorescence imaging of Cells Stained by probes

MCF-7, A549 and L02 cells were seeded onto laser scanning confocal microscope (LSCM) culture dishes with a concentration of 5×10⁵ cells/well, subsequently cultured in an incubator (37 °C, 5% CO₂ and 20% O₂). After the 70%-80% space of
culture dishes was occupied by cells, 100 μM of CyT solutions were added to culture dishes, and then incubated under the same conditions for 0.5 h, 1h and 2h. For mitochondria or LysoTracker Blue staining, the cells were stained with Mito-Tracker Green (2.0 μM) for 30 min or LysoTracker Blue (6.0 μM) for 1h and then slightly rinsed with PBS buffer three times to remove the free dye. A549 and MCF-7 Cells were incubated with CyT (100 μM) and CyO (100 μM) at 37 °C for 2 h. The blocking experiments were implemented which the cells incubated with saturated glucosamine (200μM) for 1 h prior to treat with CyT. Fluorescence images were detected on an FV1000 confocal fluorescence microscope (Olympus, Japan). The fluorescence signal was recorded at the MitoTracker Green fluorescence channel (500-600 nm, λex = 488 nm) or LysoTracker Blue fluorescence channel (425-475 nm, λex = 405 nm), CyT fluorescence channel (570-610 nm, λex = 559 nm; 660-740 nm, λex = 647 nm) respectively.

**Western blot Assay**

The expression levels of LC3-I and LC3-II protein on different culture conditions detected using Western blotting analysis. MCF-7 cells were lysed after cultured for 2 hours in normal medium and serum-free medium. Protein concentrations were measured by the BCA protein assay. The equal amounts of protein were separated by 15% SDS-PAGE before being transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). They were incubated with anti-LC3 (Proteintech Group, Inc) and anti-β-tubulin (Boster) antibodies overnight at 4 °C. Blots were then incubated once again with horseradish peroxidase conjugated secondary antibodies for 1 h, coupled with enhanced chemiluminescence (ECL).

**Fluorescence imaging in tumor-bearing mouse models**

All animal experiments were implemented in accordance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (Document NO. 55, 2001) and the guidelines for the Care and Use of Laboratory Animals of China Pharmaceutical University. For construction of the tumor models, 2×10^6 of MCF-7, A549 and EAC cells were injected subcutaneously into the axillary
fossa of the mice. After three weeks, when the size of tumors reached 0.1 cm in diameter, the mice were immobilized for in vivo NIR fluorescence imaging. **CyT** (0.1 mmol) or **CyO** (0.1 mmol) dissolved in 0.2 mL of PBS was injected into the tail vein of MCF-7 and A549 tumor bearing nude mice. For the blocking experiment in vivo investigation, glucosamine was intravenously injected to EAC tumor-bearing mice 0.5 h before the injection of **CyT**. NIR fluorescence images were obtained at different time points (0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 8 h, 12 h and 24 h, which were collected by a CCD camera (750 ± 15 nm) with a laser excitation at 660 nm. The fluorescence images of the organs (intestine, kidney, heart, lung, liver, spleen and tumor) at 4 h post injection were collected to investigate the bio-distribution of **CyT** in organs and tumor *in vitro*.

![Scheme S1](image)

**Scheme S1** The synthesis route for **CyT**.
**Fig. S1** The MS spectrum of CyO (m/z = 579.3 was correspond to [M-H]).

**Fig. S2** $^1$H NMR spectrum of CyO in DMSO-d$_6$. 
Fig. S3 $^{13}$C NMR spectrum of CyO in DMSO-d6.
Fig. S4 HRMS spectrum of CyT (m/z = 903.4391 was correspond to [M+H]+).
Fig. S5 ¹H NMR spectrum of CyO in DMSO-d6.

Fig. S6 ¹³C NMR spectrum of CyT in DMSO-d6.
Fig. S7  The frontier molecular orbitals (MOs) of (A) CyT and (B) CyTH involved in the vertical excitation (the left columns) and emission (right columns) shown in solid line. The vertical excitation and emission related calculations were performed computationally using the B3LYP 6-31g(d) level of theory. The electron distribution at HOMO and LUMO is shown in red and green color. The dashed line represents the internal conversion.
**Fig. S8** Cytotoxicity of MCF-7 (a), A549 (b) and L02 (c) cells incubated with different concentration of CyO and CyT.

**Fig. S9** Fluorescence imaging of MCF-7 cells stained with different concentrations CyT. Cells were incubated with CyT at 37 °C for 2 h. Scale bar: 20 μm.
Fig. S10 Fluorescence imaging of MCF-7 cells stained with CyT (100 μM, λ<sub>ex</sub> = 568
nm or 647 nm) and MitoTracker Green (A) and LysoTracker Blue (B). Intensity profile and localization of CyT and Mito-Tracker Green and LysoTracker Blue in MCF-7 cells were in 2 hours group (C, D). Scale bar: 20 μm (A); 10 μm (B).

**Fig. S11** (A) The expression levels of LC3-I and LC3-II protein in MCF-7 cells on different culture conditions by Western blot analysis. (B) Semi-quantitative analysis. (Normal: MCF-7 cells were cultured for 2 hours in normal medium. Starvation: MCF-7 cells were cultured for 2 hours in serum-free medium.)
Fig. S12 Fluorescence images of MCF-7 cells incubated with CyT (50 μM, λ<sub>ex</sub> =568 nm or 647 nm) and LysoTracker Blue in serum-free medium for different times. Scale bar: 20 μm.
Fig. S13 Fluorescence imaging of MCF-7 cells co-stained with CyT (100 μM, λ<sub>ex</sub> = 568 nm or 647 nm) and CyO (100 μM). Cells were incubated with probes at 37°C for 2 h. The blocking experiments were performed by adding glucosamine into the cells before CyT incubation. Scale bar: 20 μm.

Fig. S14 Fluorescence imaging of L02 cells co-stained with CyT (100 μM, λ<sub>ex</sub> = 568 nm or 647 nm) and MitoTracker Green (A) and LysoTracker Blue (B). Scale bar: 10 μm (A); 5 μm (B).
Fig. S15 (A) In vivo fluorescence images of CyT and CyO in EAC tumor-bearing mice model within 24 h. (B) The fluorescence images of isolated organs (intestine, kidney, heart, lung, liver, spleen and tumor) at 4 h post injection of CyT from the tumor-bearing mice. (C) Relative Fluorescence intensity of tumor in different group at a different post-injection time. The blocking experiment was carried out by intravenously injecting glucosamine to EAC tumor-bearing mice 0.5 h before the injection of CyT.