Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

A macrophage uptaking near-infrared chemical probe CDnir7 for *in vivo* imaging of inflammation

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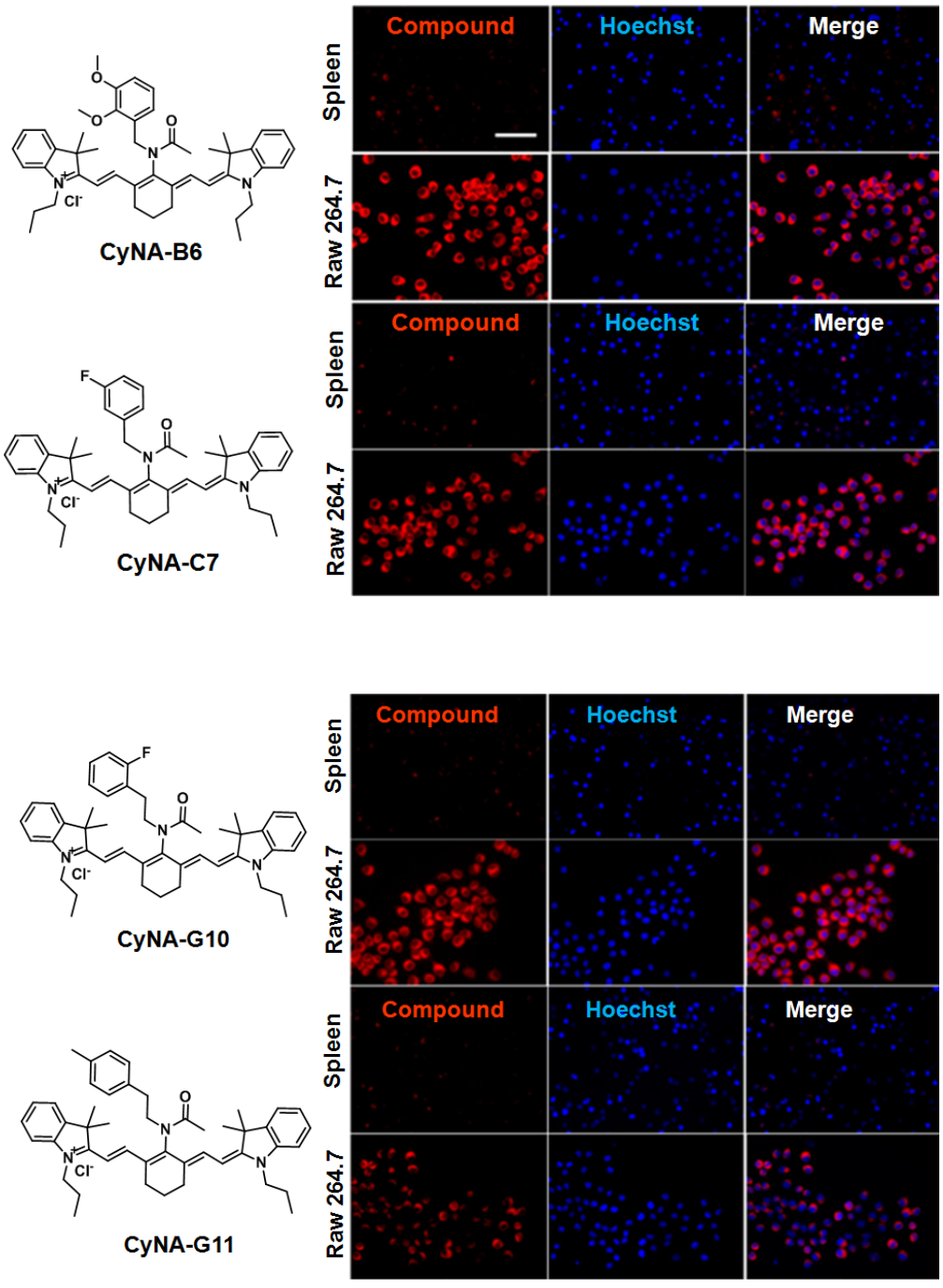


Figure S1. **Macrophage selective hit compounds**. NIR fluorescence images of mouse splenocytes and macrophage Raw264.7 cells. Through cell based screening, we identified five macrophage selective probes including **CDnir7** against mouse splenocytes. Cells incubated with 1μM concentration of libraries for 1hr. The probes selectively stained for live mouse macrophages. The fluorescence images were taken by Eclipse Ti Inverted Microscope (Nikon) installed with NIR light source. and NIR for macrophages images taken by NIR filter (em 700/800). Nucleus images taken by DAPI filter. *Scale bar, 50 μm*

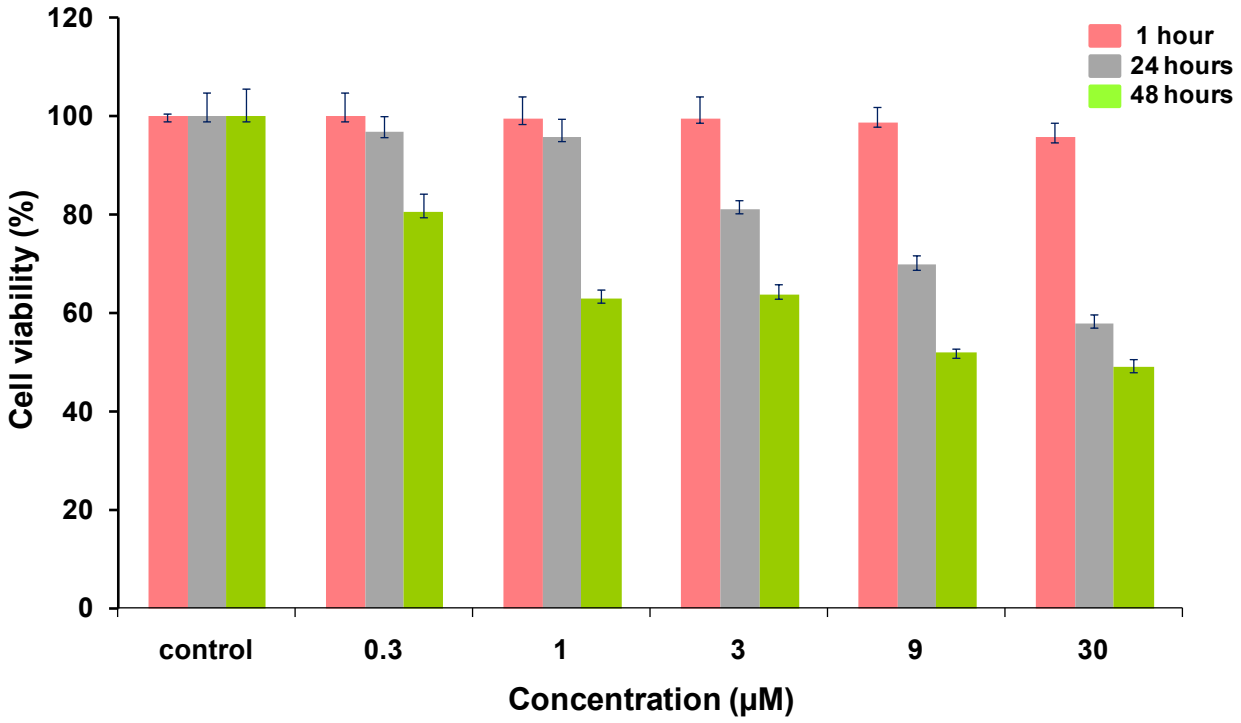


Figure S2. **Cell viability assay of CDnir7 in Raw 264.7 cells**. To estimate cell viability effect of **CDnir7** , different concentration of **CDnir7** was treated to Raw264.7cells and incubated them for 1 hour, 24 hours and 48hours. A Cell Proliferation Assay (CellTiter 96®AQueous One Solution, Promega) was used for measuring the cell proliferation. The concentrations of **CDnir7** from at 0.3 μM to 30 μM were not affected to cell viability for 1 hour incubation. The changes of cell viability was observed after 48 hours incubation of **CDnir7** at 1μM concentration. The assay was performed in triplicate.

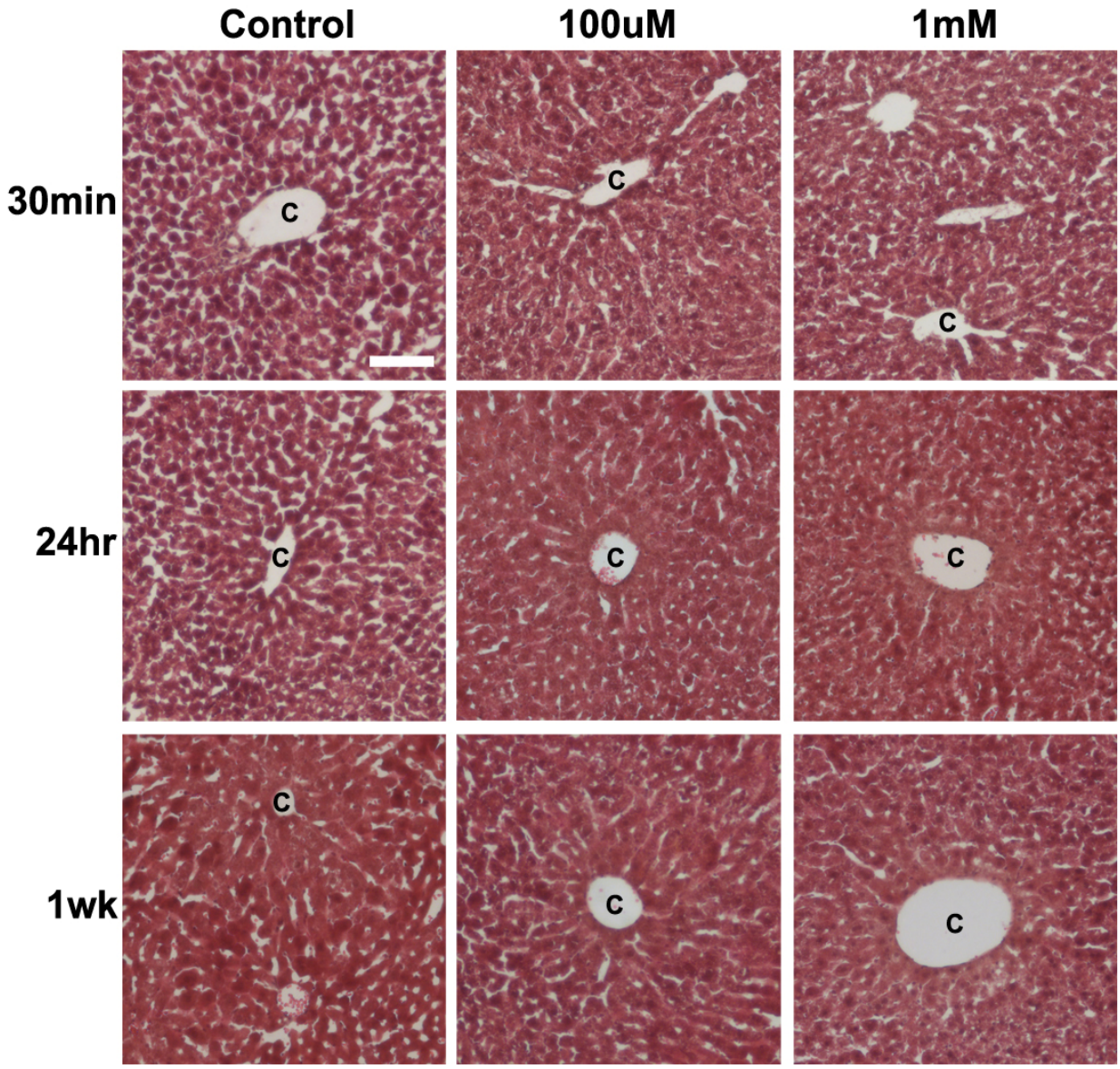


Figure S3. **Toxicity testing of CDnir7 in Liver**. To investigate toxicity of **CDnir7** in vivo, dye was administrated to mouse tail vein with two different concentrations like 100μM and 1 mM and leave them until 1 week. To recognize the change of various tissues by **CDnir7,** we performed Haematoxylin and Eosin (H&E) staining. The liver toxicity was not observed in the long term period (1 week) and the high concentration (1mM). **C**: central vein. *Scale bar: 100μm.*

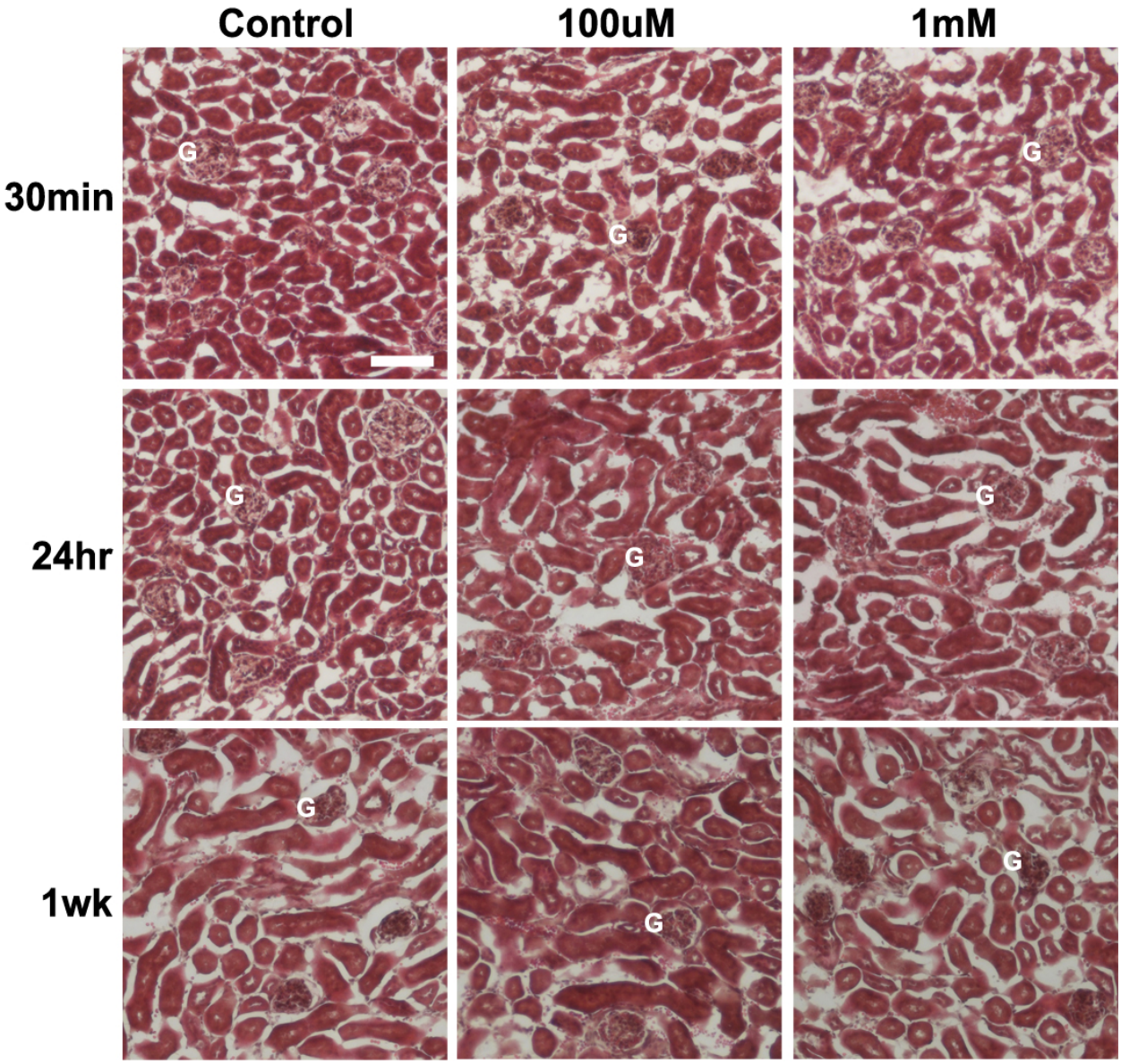


Figure S4. **Toxicity testing of CDnir7 in Kidney**. To investigate toxicity of **CDnir7** in vivo, dye was administrated to mouse tail vein with two different concentrations like 100μM and 1 mM and leave them until 1 week The kidney toxicity was not observed in the long term period (1 week) and the high concentration (1mM) **G** : glomerulus in cortex. *Scale bar: 100μm.*

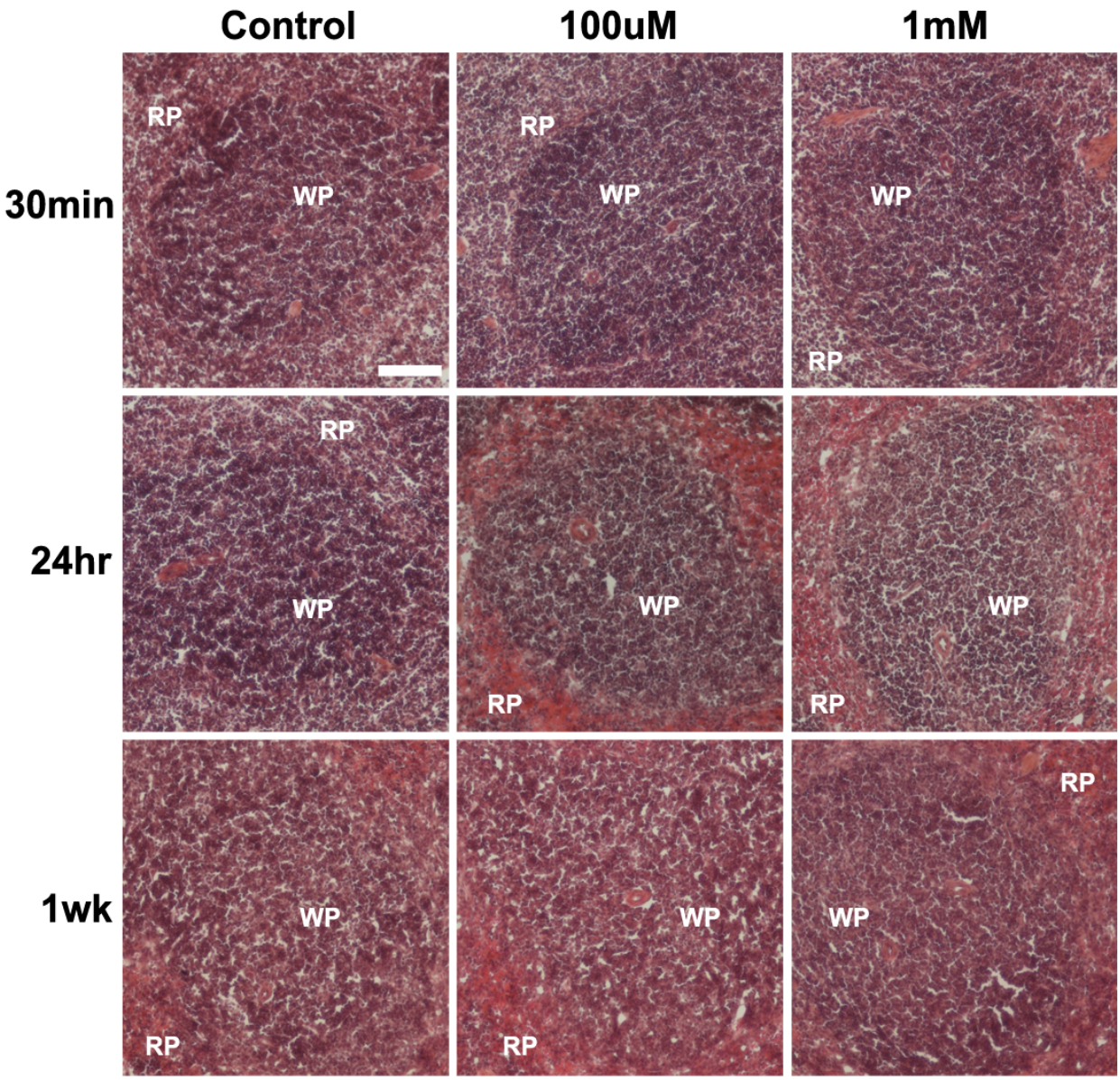
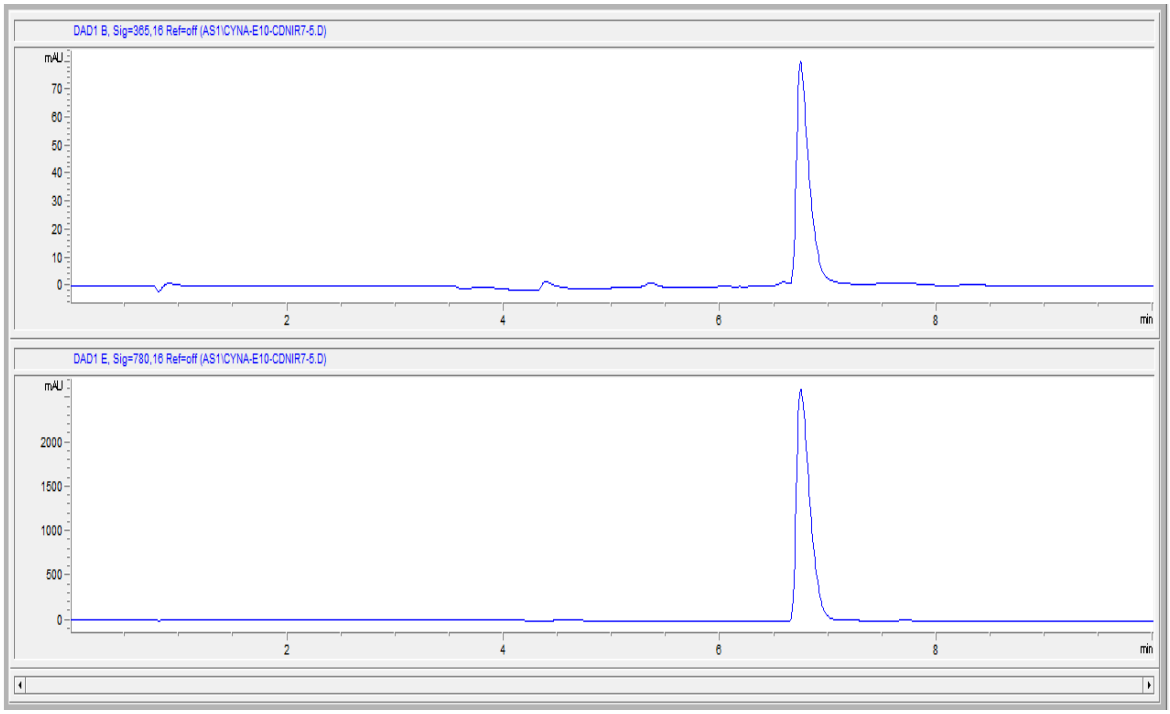
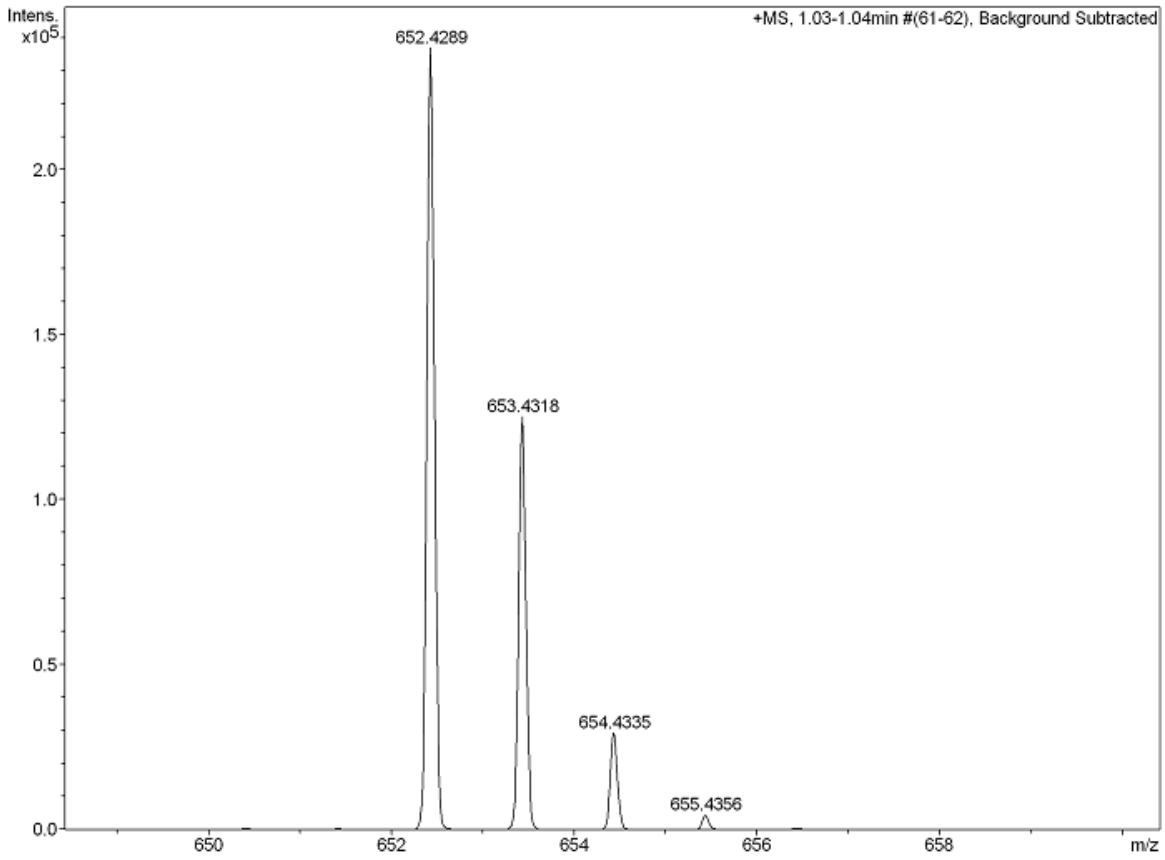


Figure S5. **Toxicity testing of CDnir7 in Spleen**. To investigate toxicity of **CDnir7** in vivo, dye was administrated to mouse tail vein with two different concentrations like 100μM and 1 mM and leave them until 1 week The spleen toxicity was not observed in the long term period (1 week) and the high concentration (1mM). **RP** : red pulp, **WP** : white pulp. *Scale bar: 100μm.*

HPLC of **CDnir7**:



HRMS of **CDnir7**:



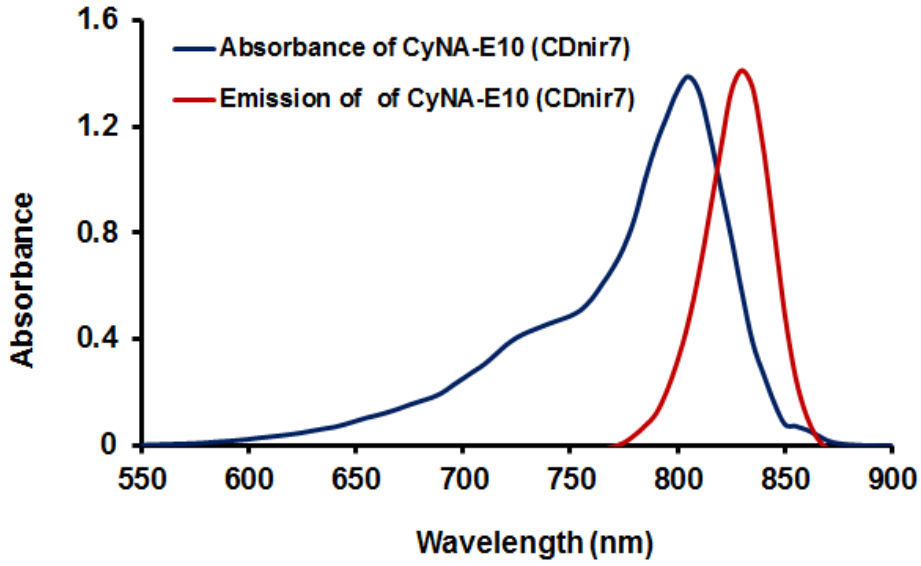


Figure S6. Analytical characterization was performed on a HPLC-MS (Agilent-1200 series) with a DAD detector and a single quadrupole mass spectrometer (6130 series) with an ESI probe. Analytical method, unless indicated: eluents: A: H_2O (0.1% HCOOH), B: ACN (0.1% HCOOH), gradient from 5 to 95%B in 6 min; C_{18}(2) Luna column (4.6 x 50mm^2, 5 µm particle size). HRMS of **CDnir7** ($C_{45}H_{54}N_3O^+$) calc: 652.4261; found: 652.4289. High resolution mass spectrometry (HRMS) data was recorded on a Micromass VG 7035 (Mass Spectrometry Laboratory at National University of Singapore (NUS)).Absorbance and emission spectra of **CDnir7** in DMSO (Final concentration of dye in DMSO; 10 µM).

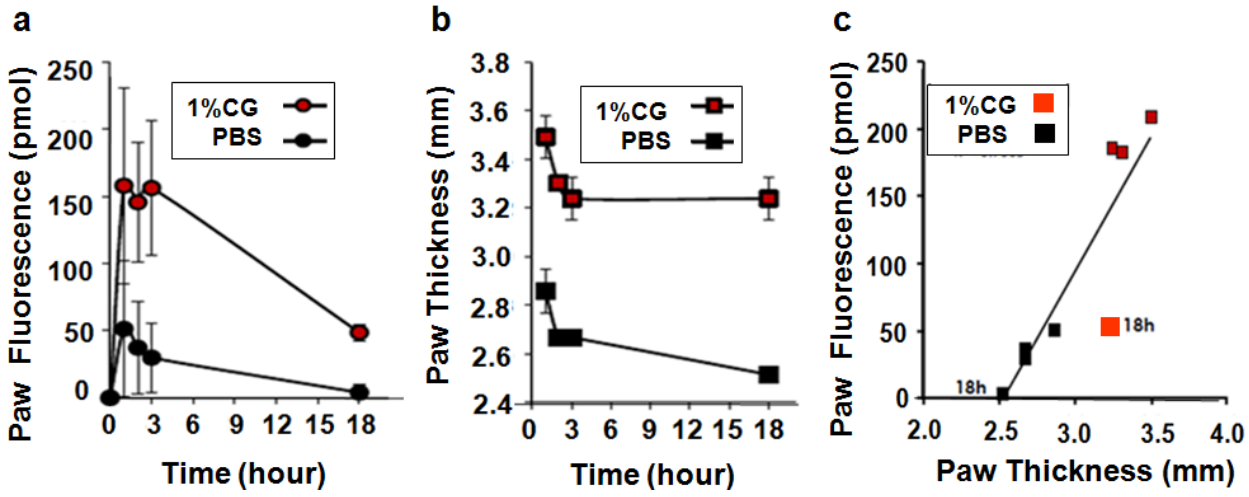


Figure S7. **CDnir7 fluorescence measurement of CG-induced inflammation area a**. fluorescence concentrations in region of carrageenan-induced inflammation remained stable from 1-3hr then declined by 66% by 18hr. **b.** Paw thickness according to fluorescence intensity. **c.** Correlation of paw thickness and fluorescence intensity. There is an excellent linearity between paw thickness (1-3hr) and **CDnir7** imaging. Each experimental has been performed in 2 mice separately. R2= 0.7665. n=2.

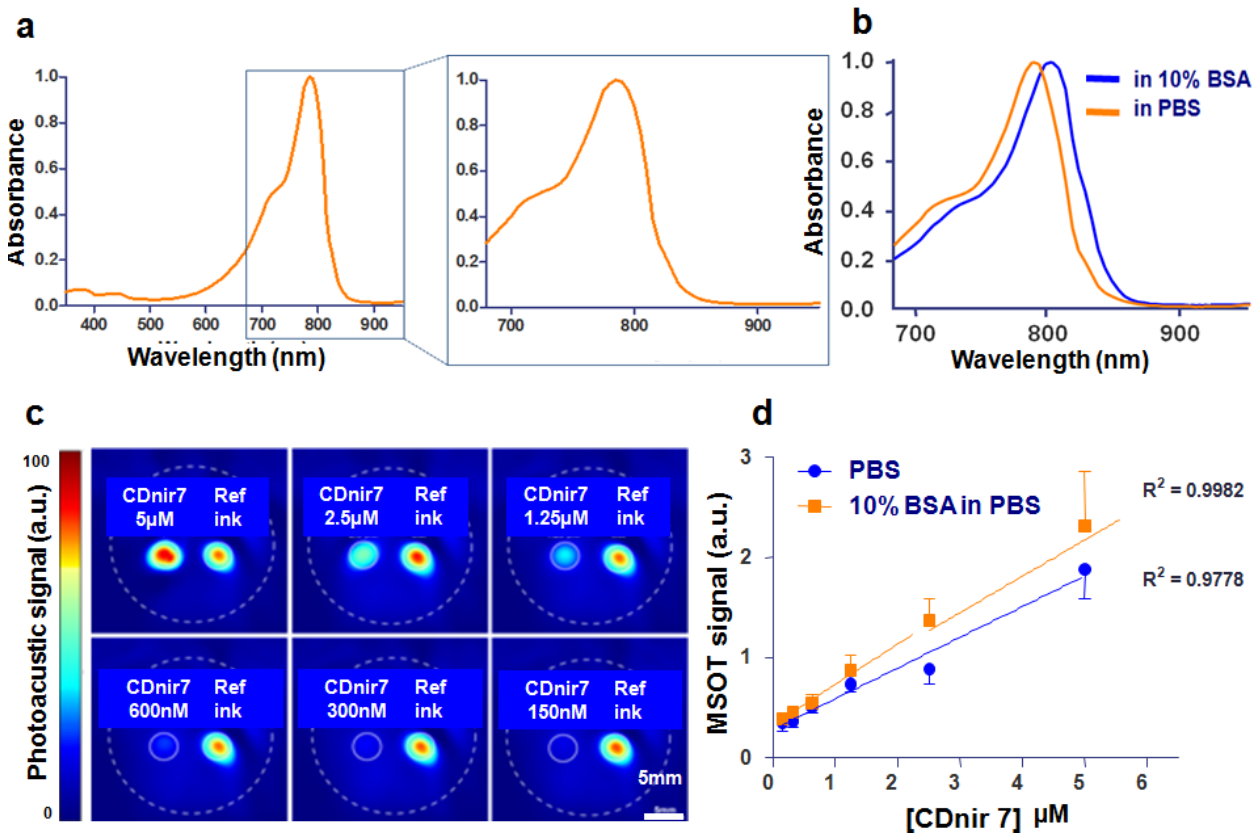


Figure S8. **Phantom measurement and sensitivity of CDnir7 by MSOT** **a.** Absorbance spectrum data of **CDnir7.** It has excellent spectral properties with a prominent spectral signature in the MSOT imaging range (680-980nm). **b.** Absorbance spectrum of **CDnir7** in PBS and PBS containing 10% bovine serum albumin **c.** In phantoms **CDnir7** can be detected ranging from 5μM-150nM. **d. CDnir7** displayed strong photoacoustic signal and excellent linearity in the presence of serum components.

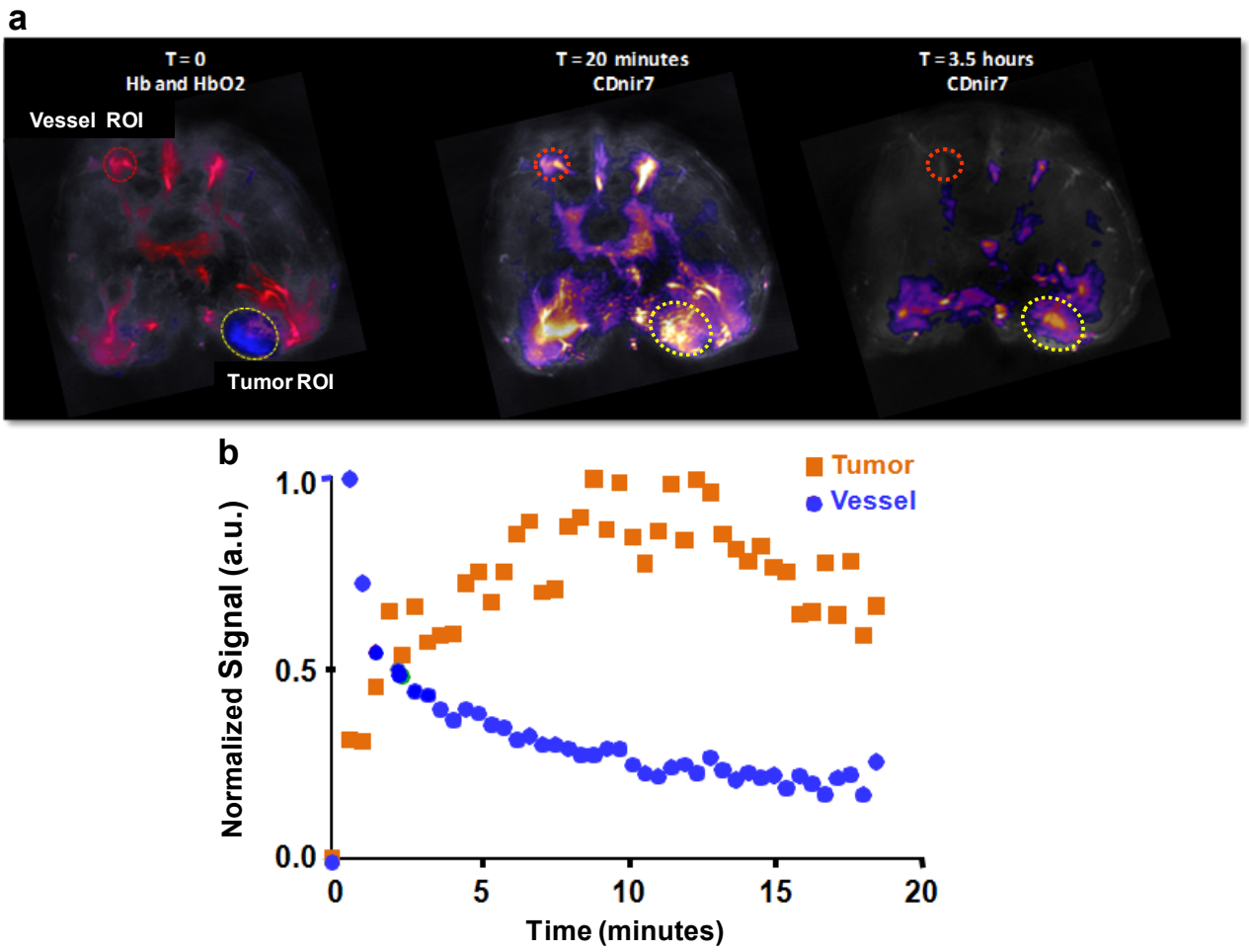


Figure S9. **Time lapse imaging of CDnir7 in hypoxic areas within 4T1 tumors. a.** Accumulation of **CDnir7** in tumors occurs quickly (<10 mins) and remains apparent up to 3.5hrs. **b.** Time-lapse quantification of **CDnir7** signal in the vessel and orthotopic breast tumor.

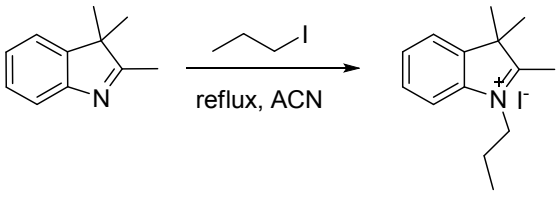
Scheme S1

1. Synthesis of 1a

1a

To a chilled solution of dimethylformamide (20 mL, 273 mmol, 5.4 eq.) in 20 mL CH_2Cl_2 under N_2 atmosphere, 20 mL of $POCl_3$ (17.5 ml, 115 mmol, 2.3 eq.) in DCM were added dropwise under an ice bath. After 30 min, cyclohexanone was added (5 g, 50mmol, 1 eq.), and the resulting mixture was refluxed with vigorous stirring for 3 h at 80°C, poured into ice-cold water, and kept it overnight to obtain **1a** as a yellow solid (8.0 g, 92%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 1.57 (m, 2H), 2.35 (t, 4H, *J*=6.3 Hz), 2.5 (s, 1H), 10.10 (s, 1H). tR: 4.30 min, ESI *m/z* ($C_8H_9ClO_2$): calc: 172.0; found: 173.1.

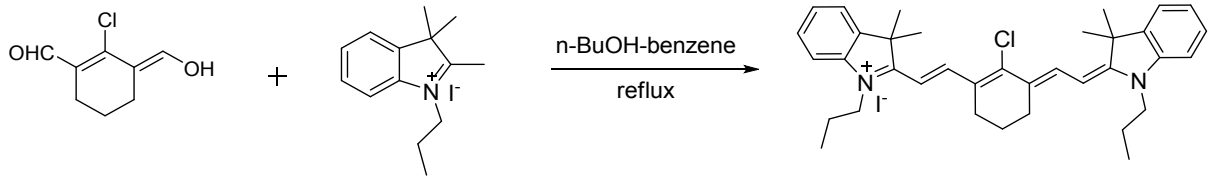
Synthesis of 1b



1b

To a solution of 2,3,3-trimethyl-3*H*-indole (2 g, 12.5 mmol, 1 eq.) in ACN, 1-iodopropane (10.6 mL, 62 mmol, 5 eq.) was added, and refluxed with continuous stirring for 15 h. The mixture was dried in high vacuum and washed by Et_2O. The resulting solid was recrystallized in acetone to obtain **1b** a white solid (3.9 g, 95%). ^{1}HNMR (300 MHz, DMSO-d_6): δ =1.04 (t, 3H, J=7.2), 1.64 (s, 6H), 2.67 (s, 3H), 1.34 (m, 2H), 4.17 (t, 2H, *J*=7.8 Hz), 7.63 (d, 2H), 7.82 (m, 2H). tR: 2.46 min, ESI *m/z* ($C_{14}H_{20}N^+$) calc: 202.4; found: 202.1.

Synthesis of 1

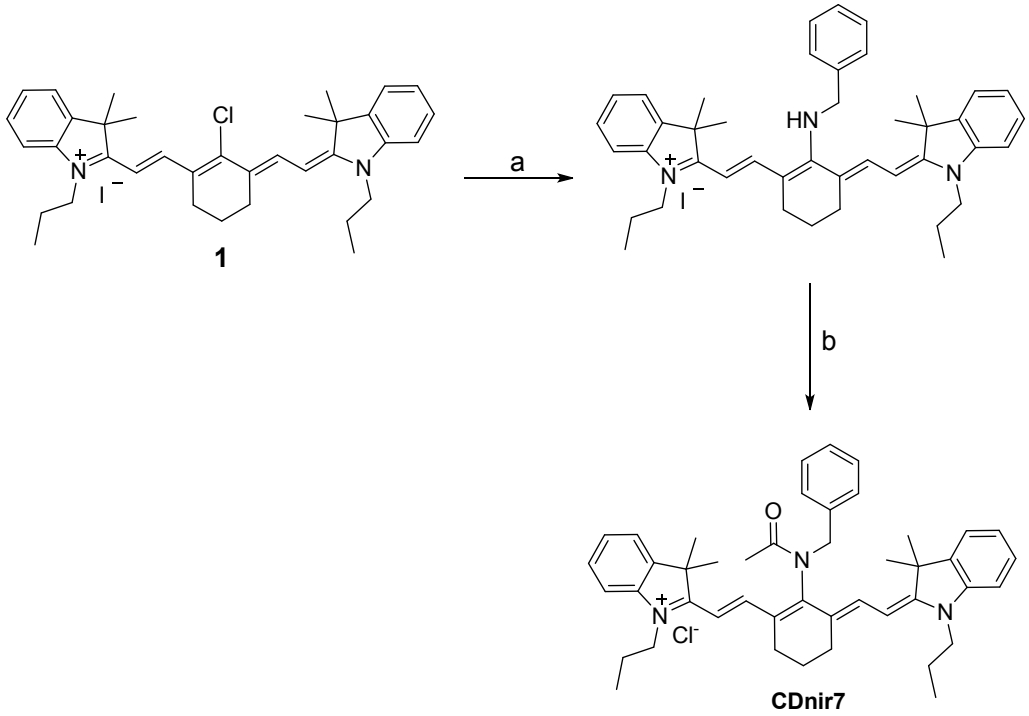


1a **1b** **1**

1a (500 mg, 2.9 mmol, 1eq.) and **1b (**1.91g, 5.81 mmol, 2 eq.) were dissolved in BuOH-benzene (7:3) under N_2 atmosphere, and refluxed at 160 °C for 10 h with a Dean-Stark condenser. Afterwards, the solvent was evaporated, and the resulting green solid mixture was washed with Et_2O and purified by flash chromatography (DCM-MeOH, 50:1) to obtain **1** as a green solid (1.8 g, 96%). ^{1}HNMR (300 MHz, $CDCl_3$) δ=1.06 (t, 6H, *J*=7.5 Hz), 1.31 (m, 4H), 1.64 (s, 12H), 1.95 (m, 2H), 2.73 (m, 4H), 4.15 (t, 4H, *J*=6.9Hz), 6.23 (d, 2H, *J*=14.2 Hz), 7.15-7.72 (m, 8H), 8.19 (d, 2H, *J*=13.8 Hz). tR: 5.64 min, ESI *m/z* ($C_{36}H_{44}ClN_2^+$), calc: 539.4; found: 539.1. This compound **1** is also commercially available (CAS Number: 207399-07-3)

For synthesis of **CDnir7, 1** (20 mg, 30 μmol, 1 eq.) and the benzyl amine (CAS: 100-46-9, Sigma Aldrich, Alfa Aesar) (15 μL, 120 μmol, 4 eq.) were dissolved in ACN, and *N,N*-diisopropylethylamine (DIEA) (7.7 μL, 60 μmol, 2 eq.) was added. The reaction mixture was heated at 80 °C for 30 min-45 min for the completion of reaction. The reaction mixture was monitored by TLC (even can be monitored in HPLC-MS). The resulting blue color crude mixtures were neutralized with 0.1 N HCl, and concentrated under vacuum. Resulting crudes were dissolved in DCM under N2 atmosphere, and treated with excess DIEA (96.2 μL, 750 μmol, 25 eq.) and acetyl chloride (11.7 μL, 150 μmol, 5 eq.) at 0 °C for 5 min. The final green products were washed with 0.1 N HCl to remove the excess of DIEA, concentrated under vacuum, and purified by a normal-phase silica short column using hexane-DCM and DCM-MeOH (ranging from 10:90 to 0:100 and 100:0 to 97:3) as the eluting solvent. Overall yield: 4 mg, 20 %. The characterization of the **CDnir7** was performed by HPLC, HRMS, and ^{1}H-NMR.

CDnir7: ^{1}H-NMR (300 MHz, $CDCl_3$): δ=1.06 (t, 6H, *J*=7.5Hz), 1.54 (t, 3H, *J*=7.0 Hz), 1.64 (s, 12H), 1.82-1.84 (m, 2H), 1.85-1.89 (m, 4H), 1.94 (s, 3H), 2.59 (t, 4H, *J*=6.3Hz), 3.79 (t, 4H, *J*=6.9Hz), 3.96 (m, 2H), 6.04 (d, 2H, *J*=14.02 Hz), 6.80-7. 28 (m, 13H, aromatic region), 8.12 (d, 2H, *J*=14.1 Hz), HRMS of **CDnir7** ($C_{45}H_{54}N_3O^+$) calc: 652.4261; found: 652.4289.



Reagents and conditions: a) Benzylamine, DIEA, CH_3CN, 80 °C, 30-45 min; b) CH_3COCl, DIEA, CH_2Cl_2, 0 °C, 5 min.

Experimental procedures

Cell culture/ NIR libraries screening

RAW 264.78 was grown in DMEM (BSF) supplemented with 10% FBS (PAA), 1% penicillin streptomycin glutamine (GIBCO) at 37ºC in a humidified incubator of 5% CO2. Spleen cells were prepared from the spleens of 6-8 week old C57BL6/J male mice via lysis buffer method. Briefly, the spleen was cut, washed with PBS and resuspended in 1mL of lysis buffer for 8 mins, and further resuspended in 1mL of lysis buffer for 4 mins. Cells were plated in 96-well plates at 4 x 10^3 cells/well for RAW 264.7, and 8 x 10^3 cells/well for spleen cells. After cells have settled, cells were incubated with 80 fluorescent NIR compounds at a concentration of 1uM. After 1 hour, Hoechst was added. NIR images were taken using an Eclipse Ti Inverted Microscope (Nikon) installed with NIR light source DAPI and bright-field images were also taken with the same microscope.

Cell viability assay using CellTiter 96 ®AQ_{ueous} Reagents

Raw264.7 cells were seeded into 96 well plate and cultured for 1day in a 100 µL volume. Next day, **CDnir7** was treated at 0.3, 1, 3, 9, 30 µM and incubated with cells from 1 hour to 48 hours. This assay is used CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corporation Cat.# G3580) and followed the protocol provided. The cell viability effect of **CDnir7** was measured on absorbance at 490 mm using an ELISA plate reader after incubation for 1–4 hours at 37°C in a humidified, 5% CO2.

H&E staining

For *in vivo* toxicity of **CDnir7**, dye was injected mouse tail vein at 0.1 mM and 1 mM and

dissect mouse after 1hour, 24 hours and 1week. The liver, kidney and spleen were harvested and prepared cryo-section to estimate toxicity using H&E staining. Staining times will vary based upon depth of stain required For slide-mounted immunohistochemistry, counterstain tissue for 30 seconds. For H&E staining, counterstain tissue for 5 minutes. In order to blue the stain, put slides through 4 changes of tap water, 5 minutes each.

Flow Cytometry analysis

Raw264.7 cells and primary spleen cells prepared as above were stained with 1 μM of CDnir7 for 1 hour. Remove stained media and change with fresh PBS three times. After harvesting cell, dissociated cells were centrifuged at 1500 rpm for 5min. Remove supernatant and wash with fresh PBS. After last washing, cell pellet was suspend with 500μ of PBS and bring flow cytometey. Analysis was done by Flowjo.

LPS-induced inflammation animal /Xenogen IVIS-Spectrum Optical In Vivo Imaging

6-8 week old C57BL6/J mice were injected with 100 μL of 1 mg/mL LPS (Sigma) on each right paws. After 2 days, 250 μL of 100 μM CDnir7 (mixed with 1% PEG and 0.1% Tween 20) was injected via tail-vein together with a control (non-LPS injected) mouse. At scheduled time points, the mice were imaged using an IVIS Imaging System (Caliper) under excitation 720 nm and emission 821 nm bandpass 30 wavelength filter.

The confirmation of CDnir7 stain pattern by CD11b

After injection **CDnir7** into the LPS induced inflammation models for 20min, Right (experimental part) and left (control) paws were prepared for cryosection. With 10um cryosection, each samples was observed by Eclipse Ti Inverted Microscope (Nikon) with NIR

channel for **CDnir7** signals. After the observation of tissue, all samples were fixed by 4% paraformaldehyde in PBS for 20min and treated by 1% bovine serum albumin in PBS for 1hr. For the confirmation of **CDnir7** stain pattern, rat anti mouse CD11b antibody (Abcam, dilution factor 1:300) was applied and was visualized by Alexa Flour® 488 conjugated goat anti rat secondary antibody (Life Technologies™, dilution factor 1:300). All images were taken by Eclipse Ti Inverted Microscope (Nikon).

Carrageenan Paw Edema Model in vivo imaging by FMT

CG injection into mouse paw generates a biphasic inflammatory response, characterized in the first 3-4 hours by neutrophil-driven edema and by decreased edema and increased macrophage infiltration from 3-24 hours. To induce paw inflammation, BALB/c mice were injected in the right hind footpad with 30 μL of a 1% CG solution prepared in PBS. The left hind footpad was injected with 30 μL PBS and served as a negative internal control. Immediately after CG challenges, 50μM of CDnir7 was injected intravenously. Mice were imaged by FMT at 1, 2, 3 and 18 h after CDnir7 injection.

FMT Reconstruction and Analysis

The collected fluorescence data was reconstructed by FMT 4000 system software (TrueQuant v3.0, PerkinElmer, Waltham, MA) for the quantification of three-dimensional fluorescence signal within the tumors and lungs. Three dimensional regions of interest (ROI) were drawn encompassing the relevant biology.

In vivo imaging by MSOT

MSOT in vivo animal imaging was performed with a previously described system[1] and with the inVision 256-TF small animal scanner (iThera Medical GmbH, Munich, Germany). For generating the orthotopic breast tumor model, 500,000 4T1 cells were injected into the mammary fat pad BALB/c nude mice. Naïve mice and the mice bearing tumors were anesthetized by isoflurane a venous catheter was placed into the tail vein. A thin layer of ultrasound gel was onto the skin and a plastic membrane was positioned around the body. MSOT imaging was performed at 715, 730, 760, 800, 850 and 900 nm. After baseline imaging, CDnir7 (100uM; 200ul) was injected via the planted venous catheter. Second whole body imaging at the same mouse was performed at the same excitations with time lapse and acquired images were reconstructed using the model-based algorithm. Spectral unmixing of Hb, HbO2 and CDnir7 signals was achieved by the pseudo-inverse algorithm. In naïve mice, CDnir7 signals in jugular vein area was measured by ROI and, in tumor model, CDnir7 in breast area was measured for image-based quantification.

Phantom measurement of CDnir7 by MSOT

Agar phantoms were made by the mixture of 1.2% agar and 1% intralipid and had the hole in the center. PBS or PBS containing 10% bovine serum albumin with **CDnir7** were injected into the hole in the center of agar phantom and absorbance spectrum of **CDnir7** was measured by from 680 to 900 nm excitation wavelength by the inVision 256-TF small animal scanner (iThera Medical GmbH, Munich, Germany).

Reference

1. A. Buehler, E. Herzog, A. Ale, B. D. Smith, V. Ntziachristos, D. Razansky, *Eu. J. Nucl. Med. Mol. Imaging Res.* 2012, **2**, 14; D. Razansky, A. Buehler, V. Ntziachristos, *Nat. Protoc.* 2011, **6**,

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