Hyaluronic acid-functionalized nanoparticles for ulcerative colitis-targeted therapy: A comparative study of oral administration and intravenous injection

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1.1. Materials

PLGA (lactide:glycolide = 50:50, Mw = 38–54 kg/mol), CS, PVA (86-89% hydrolyzed, low molecular weight), sodium nitrite, CUR, EDC, NHS, MES, and LPS from Salmonella enteric serotype typhimurium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular weight of chitosan was tailored by depolymerization using sodium nitrite following a reported method [19]. Viscosity-average molecular weights of the original chitosan and resulting chitosan were determined as respective 246 kDa and 18 kDa using a reported method [20]. The depolymerized chitosan was used in the NPs fabrication process. HA (Mw = 20 kDa) was obtained from Lifecore Biomedical, LLC (Chaska, MN, USA). Paraformaldehyde stock solution

(16%) was from Electron Microscopy Science (Hatfield, PA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), DAPI, and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) were supplied from Invitrogen (Eugene, OR, USA). Dextran sulphate sodium (DSS, 36–50 kDa) was obtained from MP Biomedicals (Solon, OH, USA). Buffered formalin (10%) was supplied from EMD Millipore (Billerica, MA, USA). Hematoxylin and eosin were from Richard-Allan Scientific (Kalamazoo, MI, USA). All commercial products were used without further purification.

1.2. Physicochemical characterization of NPs

Particle sizes (nm), polydispersity index (PDI), and zeta potential (mV) of NPs were measured by dynamic light scattering (DLS) using 90 Plus/BI-MAS (Multi-angle particle sizing) or DLS after applying an electric field using a ZetaPlus (Zeta potential analyzer, Brookhaven Instruments Corporation). The diameters (nm), PDI, or zeta potential (mV) of NPs were measured using 3 runs. Each run is an average of 10 measurements. The average values were based on the measurement on repeated NPs.

The morphology of NPs was observed with a transmission electron microscopy (TEM, LEO 906E, Zeiss, Germany) at an 80 kV accelerating voltage. A drop of diluted NP suspension was mounted onto 400-mesh carbon-coated copper grids and dried before analysis.

The entrapped CUR in NPs was determined by measuring intrinsic fluorescence of CUR on a Perkine Elmer EnSpire multimode plate reader (Perkin Elmer, Boston, MA, USA). In a typical example, NPs (3 mg) were dissolved in dimethyl sulfoxide (DMSO). Then the solution (100 μL) was transferred to a black 96-well plate. The fluorescence intensity of CUR was measured at 528 nm emission wavelength (485 nm excitation wavelength).

XRD spectra were examined using a Cu Kα-ray with tube conditions of 40 kV and 30 mA

ranging from 10° to 50° (XRD-7000, Shimadzu, Japan).

The amount of HA on the surface of HA-CUR-NPs was quantified using the cetyltrimethylammonium bromide turbidimetric method (CTM) as reported previously. Briefly, NPs (5 mg) were dissolved in DCM at room temperature for 15 min. The free HA or HA conjugates were extracted from the organic phase by adding 0.8 mL sodium acetate solution (0.2 M). And the resultant mixture was vigorously vortexed for 5 min and then centrifuged at 12,000 rpm for 5 min at 4 ^{o}C. The HA content in the supernatant was further analyzed. Fifty microliters of HA standard solution (0.05–2 mg/mL) or HA sample was added to a 96-well plate. The solutions were incubated with 50 μL of sodium acetate solution (0.2 M) for 10 min at 37 ^{o}C. Then, 100 μL of 10 mM cetyltrimethylammonium bromide solution was added to the mixture for another 10 min of co-incubation, and the absorbance of the precipitated complex was read against the blank at 570 nm using a microplate reader. The amount of HA on HA-CUR-NPs was then calculated by standard curve of native HA.

1.3. Release profiles of CUR from NPs

The release behaviors of CUR from CUR-NPs and HA-CUR-NPs were conducted by the dialysis method. Briefly, NPs were dispersed in PBS to form a suspension (equal to 250 μg of drug). The suspension was transferred into a regenerated cellulose dialysis tube (molecular weight cut-off = 10,000 Da) and the sample-filled tube was closed tightly at both ends to keep each tube surface area equivalent. The closed bag was subsequently put into a centrifuge tube, and immersed in 20 mL PBS release medium containing 0.5% Tween-80. Tween-80 was employed in PBS to maintain the solubility of drugs in aqueous phase. The tube was put in a water bath with a shaking at 100 rpm at 37 ^{o}C. At appropriate time points, outer solution was taken for measurement and equal amount of fresh release medium was added. The amount of CUR in the

outer solution was measured according to the method described in Section 2.3. All the operations were carried out in triplicate.

1.4. Cell culture

Colon-26 cells and Raw 264.7 macrophages were maintained in respective RPMI 1640 medium, DMEM medium, and DMEM medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. The cells were grown at 37 °C under 5% CO_2, the growth medium was replaced every 48 h, and the cells were sub-cultured 1:10 twice a week. Colon-26 cells were detached from the culture flasks by incubating the cells for around 3 min at 37 °C with trypsin. Raw 264.7 macrophages were gently detached with a sterile cell scraper.

1.5. In vitro biocompatibility of NPs

For MTT assay, Colon-26 cells and Raw 264.7 macrophages were seeded at a respective density of 2×10^4 and 8×10^3 cells/well in 96-well plates and incubated overnight. After 24 or 48 h of exposure to NP suspensions, the cells were then incubated with 100 μL MTT working solution at 37 °C for 4 h. This solution was prepared in serum-free medium with the MTT concentration of 0.5 mg/mL. Thereafter, the media were discarded and 50 μL DMSO was added to each well prior to spectrophotometric measurements at 570 nm. Untreated cells were used as a negative reference, whereas cells treated with 0.5% Triton X-100 were the positive control.

1.6. In vitro cellular uptake visualization of NPs

Raw 264.7 macrophages were cultured in eight-chamber tissue culture glass slide (BD Falcon, Bedford, USA) at a density of 2×10^4 cells/well and incubated for 12 h. After 3 h of co-incubation with HA-CUR-NPs (CUR, 50 μM), the cells were rinsed with phosphate-buffered saline (PBS) supplemented with calcium and magnesium to remove surplus NPs and fixed with a 4% paraformaldehyde solution for 30 min. Subsequently, 4',6-diamidino-2-phenyl-indole

dihydrochloride (DAPI) was diluted 10,000 times and added to each well for 5 min to stain nucleus. Images were acquired using the fluorescein isothiocyanate (FITC) channel and DAPI channel on an Olympus fluorescence microscopy (ORCA-03G, Hamamatsu Photonics, Hamamatsu, Japan).

1.7. Quantification of cellular uptake using flow cytometry (FCM)

Raw 264.7 macrophages were seeded in 12-well plates at a density of 1×10^5 cells/well and incubated overnight. The medium was exchanged to serum-free medium containing CUL-CUR-NPs or HA-CUR-NPs (equal to 16 μM CUR). Cells treated with blank NPs were used as negative controls. After incubation for different time periods (0, 1, 3, and 5 h), the cells were thoroughly rinsed with PBS to eliminate non-uptake NPs. Subsequently, the treated cells were harvested using trypsin, transferred to centrifuge tubes, and centrifuged at 1,500 *g* for 5 min. Upon removal of the supernatant, the cells were re-suspended in FCM buffer, transferred to round-bottom polystyrene test tubes (BD Falcon, 12 × 75 mm), and kept at 4 ^{o}C until further analysis. Analytical FCM was performed using the FITC channel on the FCM Canto™ (BD Biosciences). A total of 5,000 ungated cells were analyzed.

1.8. In vitro anti-inflammation activity of NPs

Raw 264.7 macrophages were seeded in 6-well plates at a concentration of 2×10^5 cells/well for a 12-h period. Subsequent to a 5-h period of exposure to HA-CUR-NPs and CUL-CUR-NPs, the cells were incubated in medium containing 10% FBS for 19, 43, 67, and 91 h, respectively. Following this, macrophages were activated with lipopolysaccharide (LPS, 1 μg/mL) for 3 h. Total RNA was extracted using a RNeasy Plus Mini Kit (Qiagen), and complementary DNA (cDNA) was generated from the extracted RNA *via* a Maxima first strand cDNA synthesis kit (Fermentas). The expression levels of TNF-*α* mRNA were quantified by quantitative reverse

transcriptase polymerase chain reaction (qRT-PCR) utilizing Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas). The Sequences for all utilized primers are outlined in Table S1.

1.9. Ex vivo imaging

To track the HA-functionalized NPs throughout the mice body after oral administration or *i.v.* injection, the near infrared dye, DiR, was employed as a fluorescence probe. The fabrication process of HA-DiR-NPs was as the same as that of HA-CUR-NPs, except that CUR was replaced by DiR. The amount of DiR in HA-DiR-NPs was examined by a UV-Vis spectra method. Colitis was induced in FVB male mice (8 weeks of age, The Jackson Laboratory) by replacing their drinking water with a 3.5% (wt/vol) DSS (36-50 kDa). Mice receiving NPs were treated with 0.5 mg DiR/kg per mouse after 6 days of DSS treatment. After oral administration for respective 4, 8, or 24 h, the mice were sacrificed to obtain GIT and the major organs (heart, liver, spleen, lung, and kidney). The images were captured using the IVIS spectrum imaging system (PerkinElmer/Caliper LifeSciences, Hopkinton, MA).

1.10. Quantification of in vivo bio-distribution of NPs

Mice with DSS (3.5%, w/v)-induced UC were orally or intravenously administered with CUL-CUR-NP or HA-CUR-NP suspensions at a CUR dose of 5 mg/kg body weight. After sacrificing the mice at the desired time point, major organs (heart, liver, spleen, lung, and kidney), different sections of GIT (stomach, duodenum, jejunum, ileum, cecum, and colon) and plasma of mice were collected, and further homogenized in DMSO. Consequently, the mixtures were centrifuged, and the supernatants were collected to quantify the CUR amounts at 425 nm excitation wavelength and 530 nm emission wavelength using a fluorescence spectrophotometer (RF−5301 PC, Shimadzu, Japan).

1.11. MPO activity

Neutrophil infiltration into the colon was quantified by measuring MPO activity. Briefly, a portion of colon was homogenized in 1:20 (w/v) of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma) on ice using a homogenizer (Polytron, Luzern, Switzerland). The homogenate was then sonicated for 10 s, freeze-thawed three times, and centrifuged at 16,000 *g* for 15 min. The supernatant was then added to 1 mg/mL *O*-dianisidine hydrochloride (Sigma) and 0.0005% hydrogen peroxide, and the change in absorbance at 460 nm was measured. MPO activity was expressed as units per g of colonic tissue, where one unit was defined as the amount that degrades 1 μmol of hydrogen peroxide per min at 25 °C.

1.12. H&E staining

Colon samples were evaluated for mucosal architecture change, cellular infiltration, inflammation, goblet cell depletion, surface epithelial cell hyperplasia, and signs of epithelial regeneration using light microscopy of H&E staining. These values were used to assess the degrees of mucosal damage and repair in various groups. The major organs (heart, liver, spleen, lung, and kidney) from healthy control group and *i.v.* injection mice group were also conducted H&E staining to evaluate the systemic toxicity. Tissues were fixed in 10% buffered formalin (Fisher) and embedded in paraffin. Tissue sections with a thickness of 5 μm were stained with hematoxylin and eosin followed by imaging using bright-field microscopy.

1.13. In vivo TNF-α mRNA expression level

Total RNA was extracted from the tissue samples using RNeasy Plus Mini Kit (Qiagen). The cDNA was generated from the total RNAs isolated above using the Maxima first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instruction. The mRNA expression levels of TNF-*α* were quantified by real-time RT-PCR using Maxima® SYBR Green/ROX qPCR

Master Mix (Fermentas).

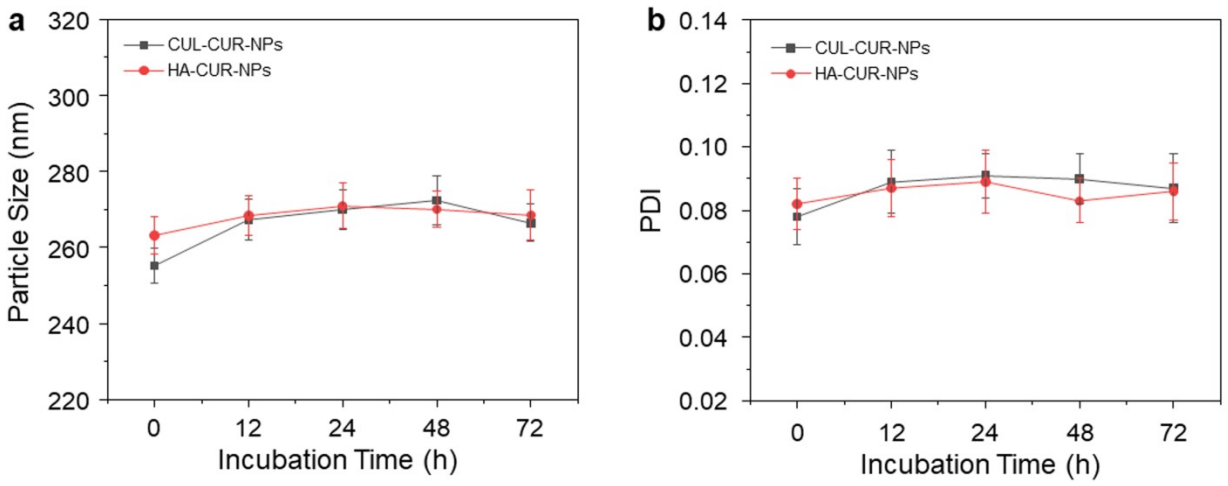

Fig. S1. Variations of average particle sizes and PDI values of CUL-CUR-NPs and HA-CUR-NPs during co-incubation in FBS (10%)-contained medium for 72 h. Data are presented as means ± S.E.M. (n = 3).

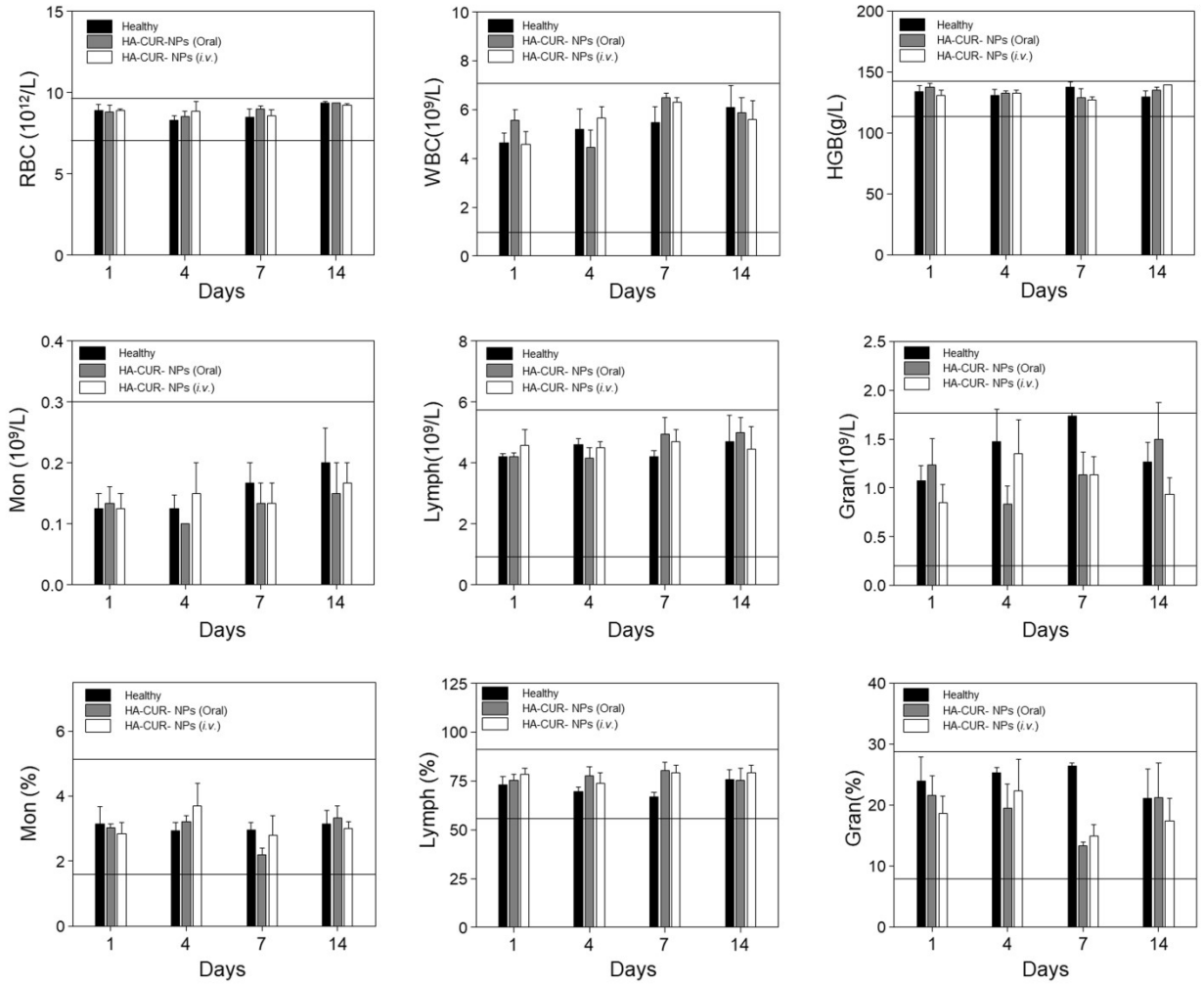

Fig. S2. Hematological parameters of various mouse groups at different time points (1, 4, 7, and 14 d). RBC: red blood cell; WBC: white blood cell; HGB: hemoglobin concentration; Mon: mononuclear cell; Lymph: lymphocyte; Gran: neutrophilic granulocyte; Mon (%): percentage of mononuclear cell; Lymph (%): percentage of lymphocyte; Gran (%): percentage of neutrophilic granulocyte (n = 3).

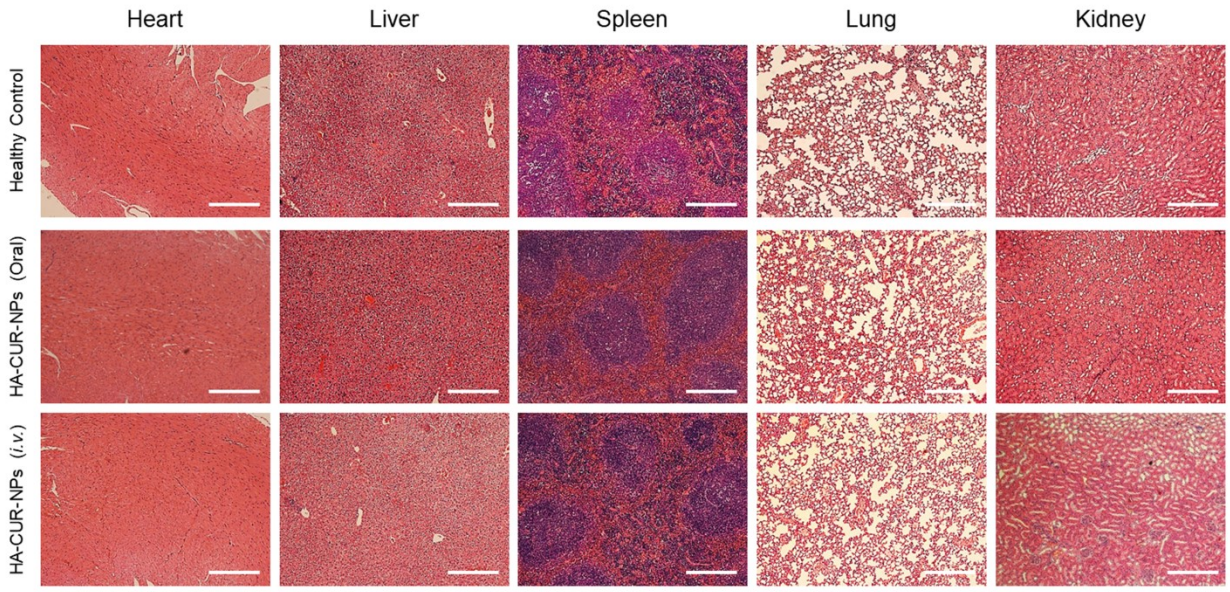

Fig. S3. H&E staining images of the five principal organs from various mouse groups in the biosafety evaluation experiment of HA-CUR-NPs with oral administration or intravenous injection. Scale bar = 200 μm.

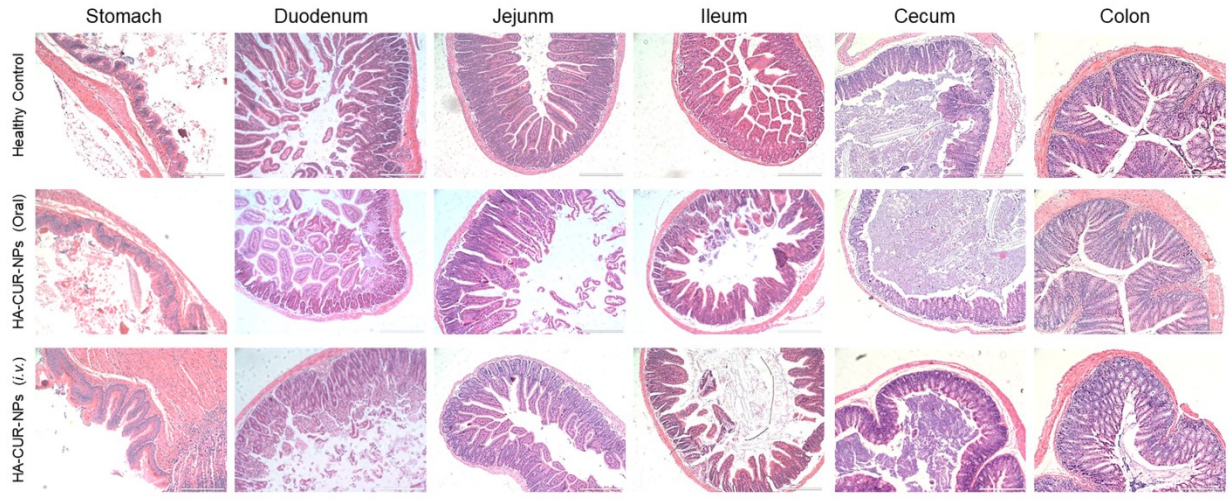

Fig. S4. H&E staining images of the stomachs, duodenums, jejunms, ileums, cecums and colons from various mouse groups in the biosafety evaluation experiment of HA-CUR-NPs with oral administration or intravenous injection. Scale bar = 100 μm.

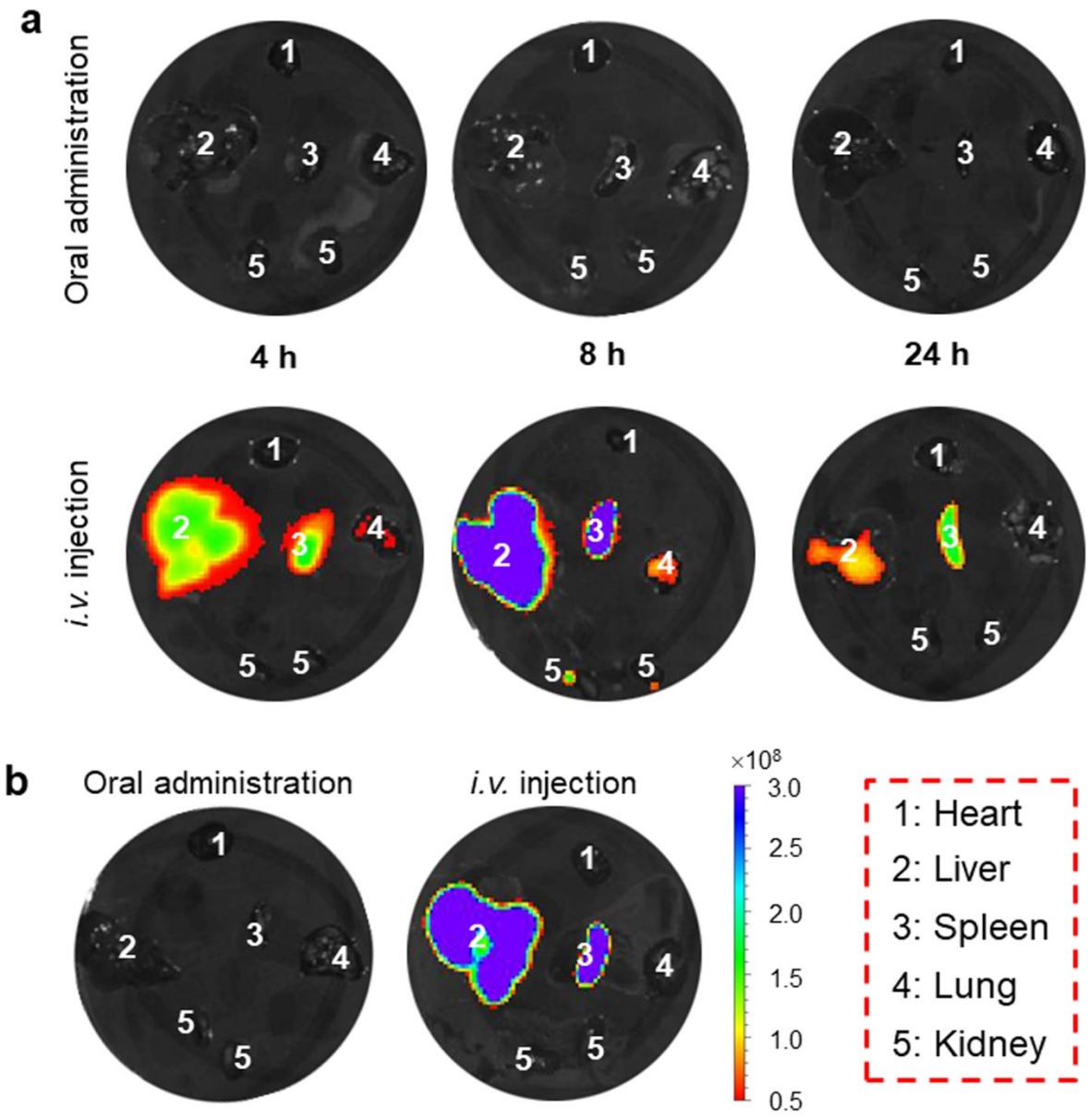

Fig. S5. Typical NIRF images of the five principal organs in mice with colitis after oral administered or intravenous injected HA-Cy7-NPs at different time points (4, 8, and 24 h). (b) Representative NIRF images of the five principal organs in healthy mice following oral administration or intravenous injection HA-Cy7-NPs at 12 h.

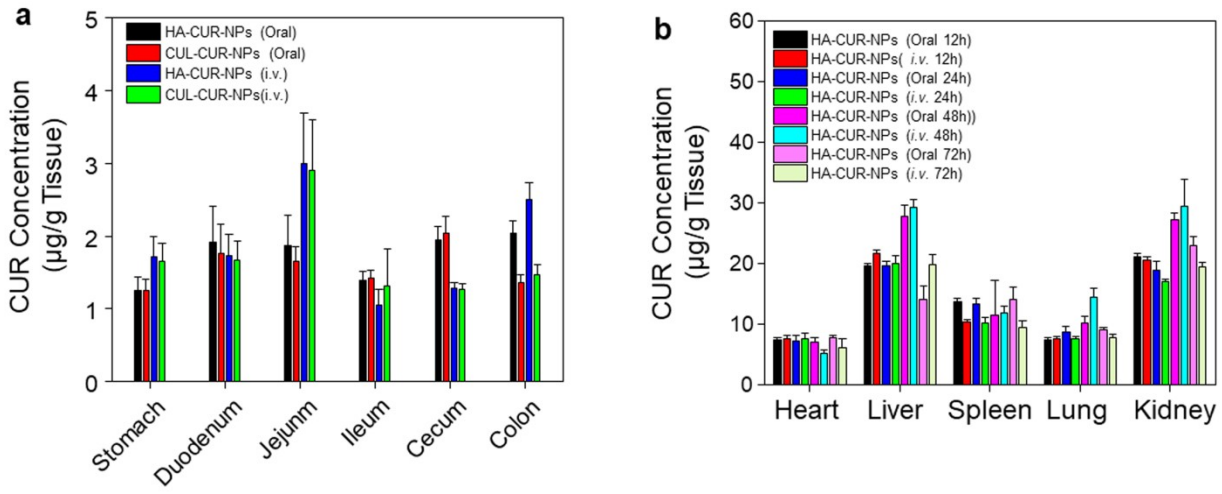

Fig. S6. (a) Concentration variations of CUR in the GIT after oral administered or intravenous injected NPs at 24 h. (b) Concentration variations of CUR in the main organs after oral administered or intravenous injected NPs at different time points. Data are presented as means ± S.E.M. (n = 3).