Supplementary Materials

A Two-Step Pretargeted Nanotherapy for CD20 Crosslinking May Achieve Superior Anti-Lymphoma Efficacy to Rituximab

Te-Wei Chu[1,†], Rui Zhang[1,†], Jiyuan Yang[1], Mark P. Chao[2], Paul J. Shami[1,3], and Jindřich Kopeček[1,4,★]

[1]Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84112, USA. [2]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA 94305, USA. [3]Division of Hematology and Hematologic Malignancies and Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112, USA. [4]Department of Bioengineering, University of Utah, Salt Lake City, UT 84112, USA.

[†]These authors contributed equally to this work.

★Corresponding Author (J. Kopeček):

University of Utah, Center for Controlled Chemical Delivery, 2030 East 20 South, Biopolymers Research Building, Room 205B, Salt Lake City, Utah 84112-9452, USA.

Phone: +1 (801) 581-7211; *Fax*: +1 (801) 581-7848

E-mail: jindrich.kopecek@utah.edu

Supporting Methods

Synthesis and characterization of Fab'-MORF1

The 1F5 mAb (IgG2a) was prepared from a mouse hybridoma cell subclone 1F5 (ATCC) in a CellMax® bioreactor (Spectrum Laboratories) and purified on a Protein G Sepharose 4 Fast Flow column (GE Healthcare). Preparation of Fab' followed a previously reported procedure [1]. Briefly, antibodies were digested into $F(ab')_2$ with 10% (w/w) pepsin (Sigma) in citric buffer (pH 4.0). Immediately before conjugation, $F(ab')_2$ was reduced to Fab'-SH by 10 mM tris(2-carboxyethyl)phosphine (Thermo Scientific). The Fab'-MORF1 conjugate was synthesized as previously described [2]. A typical procedure was as follows: first, 200 nmol $MORF1-NH_2$ (Gene Tools) was reacted with 0.67 mg (2 μmol) succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) (Soltec Ventures) in 170 μL DMSO to produce the MORF1-mal (containing a 3'-maleimide). The reaction was performed at room temperature (RT) for 24 h. The product was isolated by precipitation into 1.5 mL acetone and dried under vacuum; SMCC was removed by dissolution-precipitation in deionized water-acetone twice. Second, 200 nmol MORF1-mal was dissolved in 200 μL 10 mM PBS (pH 6.5), and then the solution was mixed with 200 nmol (~10 mg) freshly reduced Fab'-SH in 2 mL PBS (pH 6.5). The reaction was performed at 4 °C for 24 h. Finally, the Fab'-MORF1 conjugate was purified using size exclusion chromatography (SEC) to remove free, unconjugated Fab' and MORF1. An ÄKTA FPLC system equipped with Sephacryl S-100 HR16/60 column (GE Healthcare) eluted with PBS (pH 7.2) was used. Optionally, Fab'-MORF1 was labeled with 5-10 molar excess Rhodamine Red™-X succinimidyl ester (R6010) (Molecular Probes®) for imaging studies. The product was purified using a PD-10 desalting column (GE Healthcare). To determine Fab' equivalent concentration of the Fab'-MORF1 conjugate, a bicinchoninic acid (BCA) protein assay (Pierce) was used. UV-visible spectroscopy (Varian Cary 400, Agilent Technologies) was used for quantification of the MORF1 equivalent concentration;

molar absorptivity = 278,000 $M^{-1} cm^{-1}$ (at 265 nm, in 0.1 N HCl). The comparison between Fab' and MORF1 equivalent concentrations confirmed a 1:1 stoichiometry of the coupling reaction.

Synthesis and characterization of P-MORF2

The polymer backbone was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization, using the following monomers: *N*-(2-hydroxypropyl)methacrylamide (HPMA), *N*-methacryloylglycylglycine thiazolidine-2-thione (MA-GG-TT), and optionally *N*-methacryloylaminopropyl fluorescein thiourea (MA-FITC). Monomers HPMA [3], MA-GG-TT [4] and MA-FITC [5] were synthesized as previously described. MORF2 containing a 3'-primary amine (Gene Tools) was attached to the side chain of the polymer *via* amide linkage. P-MORF2 was purified using SEC. The valence and the MORF2 equivalent concentration of the P-MORF2 conjugate were determined by UV-Vis spectroscopy.

RAFT polymerization. 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044; Wako Chemicals) was used as the initiator, and 4-cyanopentanoic acid dithiobenzoate (CPDB) as the chain transfer agent. CPDB was synthesized as previously described [6]. The polymerization was carried out in a mixture of methanol and DMSO containing 0.3% (v/v) acetic acid; abbreviated as MeOH/H$^+$ and DMSO/H$^+$. A typical procedure was as follows: HPMA (117 mg, 0.82 mmol) and MA-GG-TT (55 mg, 0.18 mmol) were added into an ampoule attached to a Schlenk-line. After three vaccum-nitrogen cycles to remove oxygen, 0.45 mL degassed MeOH/H$^+$ and 0.15 mL degassed DMSO/H$^+$ were added to dissolve the monomers, followed by addition of CPDB solution (0.11 mg in 50 μL MeOH/H$^+$; [monomer]/[CPDB]=2500; total amount of monomers was 1 mmol) and VA-044 solution (0.07 mg in 50 μL MeOH/H$^+$; [CPDB]/[VA-044]=1.8) *via* syringes. The mixture was bubbled with nitrogen for 15 min before sealing the ampoule; the copolymerization was performed at 40 °C for 34 h. The copolymer was isolated by precipitation into acetone and purified by dissolution-precipitation in methanol-acetone twice and dried under vacuum. The number average molecular weight (Mn) and molecular weight distribution (polydispersity, Pd) of the polymer precursor (P-TT)

were determined by SEC, using an ÄKTA FPLC equipped with miniDAWN and OptilabREX detectors (GE Healthcare). Superose 6 HR10/30 column (GE Healthcare) was used, with sodium acetate buffer (pH 6.5) and 30% acetonitrile (v/v) as mobile phase. To remove the terminal (active) dithiobenzoate groups, the P-TT copolymer was reacted with 2,2'-azobis(2,4-dimethyl valeronitrile) (V-65) (Wako Chemicals). P-TT (34.5 mg, Mn=136 kDa) and V-65 (40× molar excess, 2.6 mg) were added into an ampoule. After three vacuum-nitrogen cycles to remove oxygen, 0.35 mL MeOH/H$^+$ was added. The solution was bubbled with nitrogen for 15 min, sealed and reacted at 50 °C for 2 h. The end-modified copolymer was purified by precipitation into acetone twice and then dried under vacuum (yield 33.5 mg; 97%). The content of TT groups in the copolymer was determined by UV absorbance at 305 nm (molar absorptivity = 10,900 M^{-1} cm^{-1}; in methanol) [4].

Preparation of P-MORF2. The P-TT polymer precursor described above was reacted with MORF2-NH$_2$ to produce the multivalent P-MORF2 conjugate. Preparation of P-MORF2 followed a previously reported procedure [2]. A typical reaction was as follows: 4.2 mg P-TT (136 kDa; containing 2.5 µmol TT groups) was mixed with 4.2 mg (0.5 µmol) MORF2-NH$_2$ in 200 µL deionized water and 70 µL DMF. The solution mixture in an ampoule was stirred at RT for 24 h; then 5 µL 1-amino-2-propanol (Sigma-Aldrich) was added and stirred for another 5 to 10 min to aminolyze unreacted TT groups on the polymer chains. After reaction, the solution was filtered through a 0.22 µm syringe filter, and the conjugate was purified by SEC using ÄKTA FPLC with Superose 6 HR16/60 column (GE Healthcare) eluted with PBS (pH 7.2). P-MORF2 was characterized by UV absorbance at 265 nm after removal of unconjugated MORF2. To quantify the content of MORF2 and determine the valence (number of MORF2 per polymer chain), the fractionated P-MORF2 conjugate was freeze-dried and dissolved in 0.1 N HCl prior to UV-Vis spectroscopy analysis. A molar absorptivity of 252,000 (M^{-1} cm^{-1}) was used for quantification of MORF2. The valence of the P-MORF2 conjugate was calculated based on the resulting MORF2 content and the Mn of the polymer backbone (136 kDa, determined by SEC).

Confocal fluorescence microscopy

Human Burkitt's B-cell non-Hodgkin lymphoma Raji cell line (ATCC) was cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (HyClone) at 37 °C in a humidified atmosphere with 5% CO_2 (v/v). Experiments were performed using cells in exponential growth phase. Cells at a density of 10^5 per well were incubated with 0.5 mL rhodamine-labeled Fab'-MORF1 (1 µM) in culture medium at 37 °C for 1 h; then the cells were washed twice with PBS prior to incubation with 0.5 mL of FITC-labeled P-MORF2 (1 µM, MORF2 equivalent) or FITC-labeled P-scMORF2 (1 or 5 µM, MORF eqv.) for another 1 h. Conjugates P-MORF2 and P-scMORF2 were prepared from the same polymer backbone and had a similar valence (~5 oligos/chain). After the incubation, cells were washed twice with PBS, and plated onto sterile 35-mm glass bottom dishes with 14-mm microwells (MatTek Corporation) for imaging. An Olympus laser scanning confocal microscope (FV 1000) was used. Please, note that all other experiments (except confocal microscopy) were performed with P-MORF2 containing 9 oligonucleotides/chain.

Radiolabeling of Fab'-MORF1

Iodine-125 labeling of the Fab'-MORF1 conjugate was performed immediately before use. Na^{125}I (PerkinElmer) was reacted with Fab'-MORF1 in 10 mM PBS (pH 7.4) in a pre-coated iodination tube (Thermo Scientific). The reaction mixture was gently stirred at room temperature for 10 min, followed by purification with a Sephadex PD-10 column (GE Healthcare) and then a Millipore® ultrafiltration tube (30 kDa cut-off). The specific radioactivity of the hot samples was in the range of 1.6 – 2.2 mCi/mg.

Bioluminescence imaging

Female C.B-17 SCID mice (6- to 8-week-old; 18–20 g; Charles River Laboratories) were intravenously engrafted with 4×10^6 Raji-luc cells *via* the tail vein and underwent different treatments as above-mentioned. *In vivo* imaging was performed twice per week on weeks 1–4 and

once per week on weeks 5–7. Mice were anesthetized with 2% (v/v) isoflurane gas (IsoFlo[®], Abbott Laboratories) in oxygen from a precision vaporizer and intraperitoneally injected with 3 mg firefly D-luciferin (Biosynth). At 15 min post-injection of luciferin (a predetermined time interval, with maximal luciferase signal intensity), mice were scanned at the prone position. Xenogen IVIS[®] Spectrum (Perkin Elmer) was used, with 1 min exposure time, medium binning, and 1 f/stop. Images were acquired and analyzed under the Living Image[®] (Perkin Elmer) software environment. Region of interest (ROI) was selected by drawing contours to include the whole mice. Luciferase light unit was quantified in average radiance (photons/sec/cm^2/sr). For *ex vivo* analysis, mice were injected with 3 mg luciferin 12 min prior to being sacrificed. Various organs and tissues (heart, liver, spleen, kidney, lung, intestine, stomach, muscle, brain, spinal cord, femur, tibia, and mesenteric and inguinal lymph nodes) were harvested for imaging. Acquisition parameters were as follows: 1 min exposure time, small binning, and 1 f/stop.

Microcomputed tomography (microCT) imaging

In vivo microCT imaging of mice was performed on week 10 after tumor implantation. Mice were anesthetized with 2% (v/v) isoflurane gas in oxygen and positioned left lateral on the scanner bed. The hind limbs of mice were scanned using a high-resolution microCT Quantum FX system (Perkin Elmer) at 90 keV and 160 mA, with a field of vision (FOV) of 24 mm. The acquisition time was 4.5 min. Three-dimensional (3D) reconstruction of bone architecture was performed under a Quantum FX viewer 1.3.0 software environment (Perkin Elmer).

Pathological and histopathological examination

Immediately after mice were sacrificed, the following organs and tissues were harvested for pathological evaluation: brain, heart, lung, liver, spleen, kidney, spinal cord, and lymph nodes. These organs/tissues were fixed in 10% formalin overnight at room temperature, and then transferred and preserved in 70% ethanol. Histopathological examination was performed by a

blinded veterinary pathologist at Animal Reference Pathology (Salt Lake City, UT). Tissue sections (2–6 per organ) were cut at 4-μm thickness, mounted on glass slides, and stained by hematoxylin and eosin (H&E).

Patient samples analysis and apoptosis assay

Primary mantle cell lymphoma (MCL) cells were isolated from 4 patients. Two isolates were from lymph node biopsies (patients 1 and 2) and two were from the peripheral blood (patients 3 and 4). Cells (2×10^5) were suspended in 0.4 mL RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Hyclone) and treated consecutively (1 h or 5 h interval) with Fab'-MORF1 and P-MORF2 (at equimolar MORF1/MORF2 concentrations). Untreated cells in the culture medium were used as negative controls. For positive controls, cells were treated consecutively (identical time intervals) with anti-CD20 mAbs (1F5 or rituximab) and a goat anti-mouse secondary antibody (KPL) (molar ratio mAb:2°Ab = 2:1). Incubation was carried out for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. All experiments were conducted in duplicate or triplicate wells. After treatments, cells were washed twice with PBS prior to staining by propidium iodide (PI) and FITC-labeled annexin V. Staining was performed following the RAPID™ protocol provided by the manufacturer (Oncogene Research Products). Percentage of total apoptotic cells (annexin V^+ PI^+ + annexin V^+ PI^-) was quantified by flow cytometry where data of 10^4 cells were recorded.

Supporting Discussion

Comparison with other CD20 crosslinking platforms

Several other platforms for CD20 crosslinking and apoptosis induction of B-cells have been developed [7–15]. One strategy is to use multivalent polymer-mAb or polymer-Fab' conjugates. For instance, multimeric rituximab bound to activated dextran [7] or lipid nanoparticles [8] have been produced. Our laboratory synthesized multivalent HPMA copolymer – anti-CD20 Fab' conjugates,

which successfully induced apoptosis of malignant B-cells *in vitro* [9–11]. The advantage of such approaches [7–13] is the more straightforward one-step treatment, likely resulting to better patient compliance. However, due to the large size of the antibodies or their fragments, it is difficult to synthesize such constructs with high valency (due to steric hindrance). This undesirable feature may severely limit the therapeutic efficiency. Previously we attempted to increase the valency by synthesizing branched HPMA copolymer backbones [9]. A small amount of tetraethyleneglycol dimethacrylate was used as the crosslinker, and copolymers with high polydispersity were produced. Consequently, fractionation was required to prepare narrowly dispersed copolymers. Other researchers used biological polymers such as DNA [12] or polypeptides [13] as scaffolds to attach antibodies or the smaller size single-chain variable fragments (scFv). These polyvalent constructs indeed achieved better efficacies than their monovalent counterparts. However, one fundamental difference between these single-treatment designs and our "binary system" reported here is that the binary systems have the advantage of performing pretargeting. Previously our laboratory has designed and developed a pilot "binary" anti-CD20 drug-free macromolecular therapeutic system using a pair of pentaheptad peptides (CCE and CCK) that formed antiparallel coiled-coil heterodimers as the biorecognition moieties [14, 15]. When compared to this previous design using peptides, the MORF oligos clearly demonstrated faster binding kinetics as well as more efficient self-assembly (equimolar MORF1/MORF2 reached binding saturation *in vitro*), therefore resulting in superior apoptosis induction and *in vivo* anti-lymphoma efficacy [2]. This is because the individual peptide sequences do not have a pronounced secondary structure at the physiological pH [16]. Fab'-CCE and P-CCK interact first *via* hydrophobic and electrostatic interactions, and then the oligopeptides fold into a strong antiparallel coiled-coil heterodimer. Therefore, we aimed to identify a biorecognition pair that would bind efficiently at the 1:1 molar ratio. Morpholino oligonucleotides were selected due to their fast hybridization, excellent binding affinity and stability in plasma, as well as water-solubility [17].

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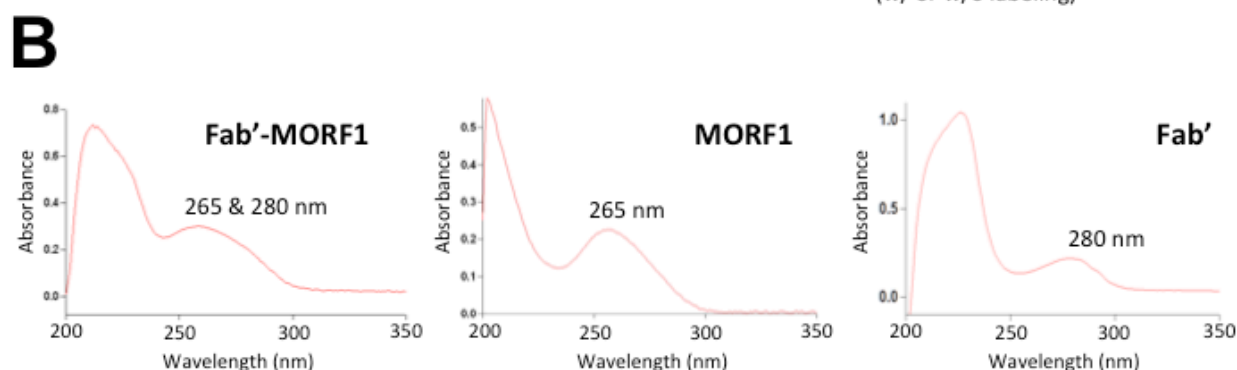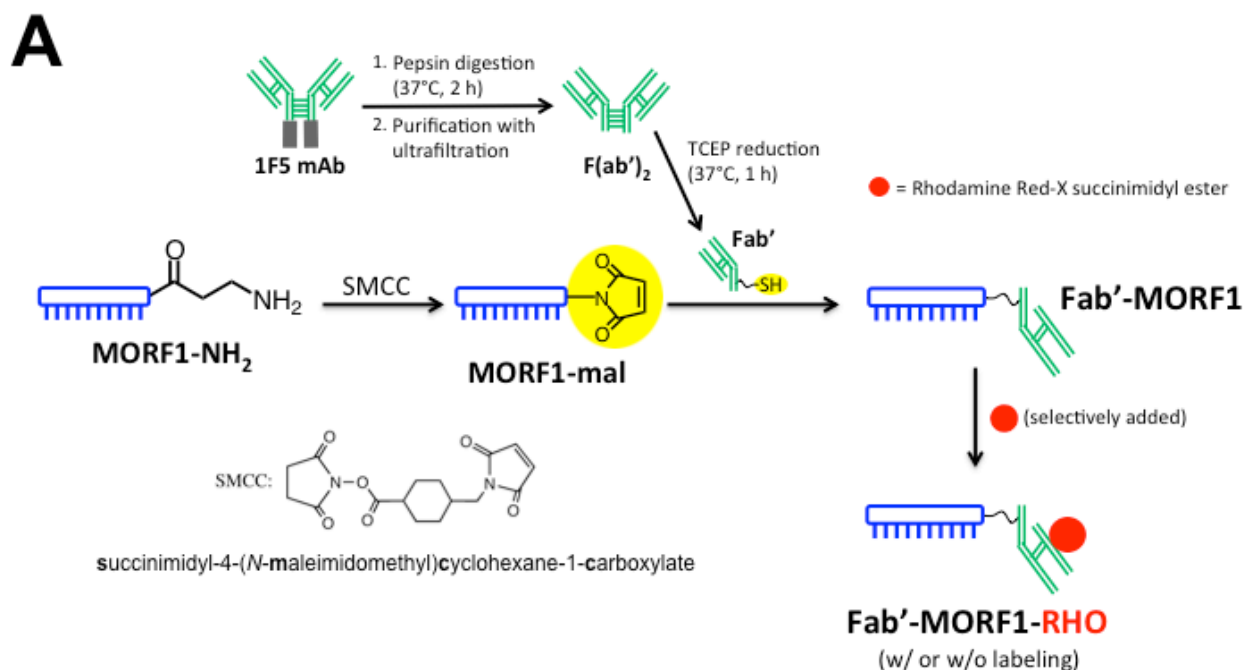
Figure S1. Synthesis and characterization of Fab'-MORF1. (**A**) The synthetic route of the Fab'-MORF1 conjugate. MORF1: 5'-GAGTAAGCCAAGGAGAATCAATATA-3' (8630.5 Da). TCEP, tris(2-carboxyethyl)phosphine; mal, maleimide. (**B**) UV-visible spectra of the purified Fab'-MORF1 (1 μM), unconjugated MORF1 (1 μM), and Fab' fragment (2.5 μM). The Fab'-MORF1 conjugate had a main absorbance at 265 nm (contributed by MORF1) and an enhanced absorbance at 280 nm (contributed by Fab') when compared to unconjugated MORF1. The Fab' equivalent concentration of Fab'-MORF1 was further determined by BCA protein assay. The obtained values were compared to the MORF1 equivalent concentrations obtained from UV-visible spectroscopy.
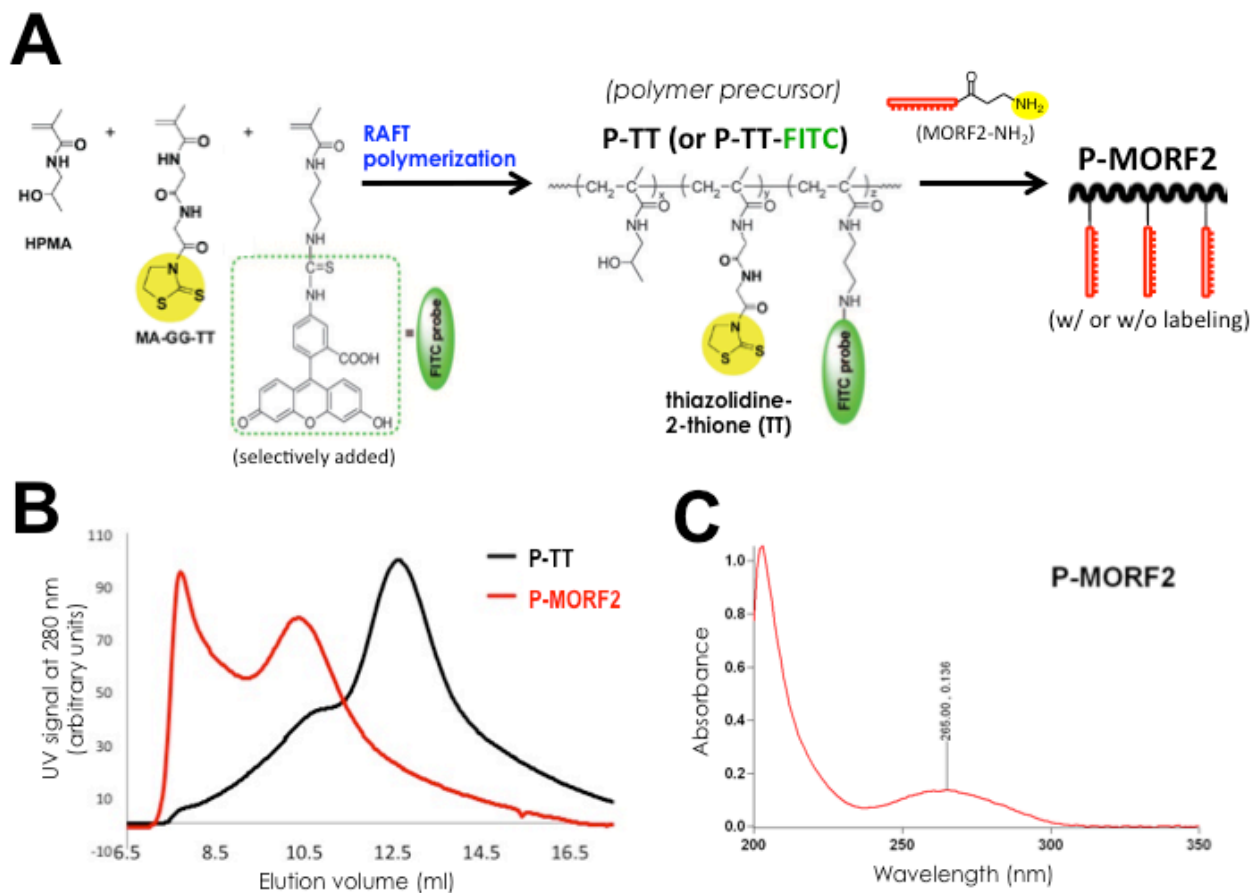
Figure S2. Synthesis and characterization of P-MORF2. (**A**) The synthetic route of the multivalent P-MORF2 conjugate. MORF2: 5'-TATATTGATTCTCCTTGGCTTACTC-3' (8438.5 Da). MA-GG-TT, *N*-methacryloylglycylglycine thiazolidine-2-thione. (**B**) Size exclusion chromatography (SEC) analysis of P-MORF2 and its P-TT polymer precursor by ÄKTA FPLC; Superose 6 HR10/30 column (acetate buffer pH 6.5 + 30% acetonitrile v/v). The retention limit of this column is about 7 mL. The number average molecular weight (Mn) of P-TT was 136 kDa; polydispersity (Pd) = 1.15. (**C**) UV-Vis spectrum of the SEC-purified P-MORF2 conjugate (13 µg/mL). P-MORF2 was characterized by UV absorbance at 265 nm (contributed by MORF2). The valence (number of oligos per chain) of this P-MORF2 conjugate was 8.9.
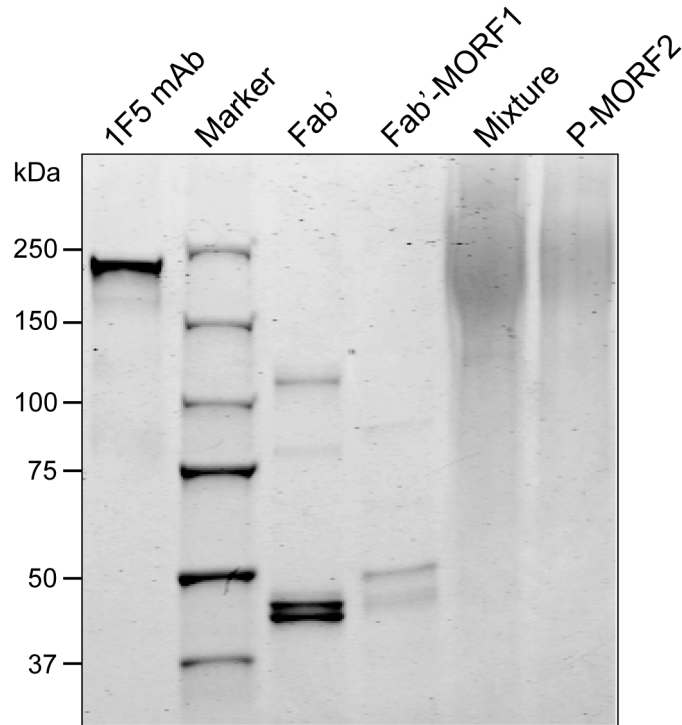
Figure S3. SDS-PAGE analysis of the two conjugates. Lane 1, 1F5 mAb (1.5 µg). Lane 2, protein ladder. Lane 3, Fab' fragment of 1F5 (1.5 µg). Lane 4, Fab'-MORF1 conjugate (0.3 µg, 5 pmol). Lane 5, mixture of Fab'-MORF1 (5 pmol) and P-MORF2 (5 pmol MORF2 equivalent). Lane 6, P-MORF2 conjugate (5 pmol MORF2 equivalent). Samples were incubated with 1× Laemmli buffer without reducing reagents at 37 °C for 30 min prior to loading; 8-16% polyacrylamide gel. Fab'-MORF1 and P-MORF2 were mixed in PBS (pH 7.4) at room temperature for 1 h. When mixed, the Fab'-MORF1 band completely disappeared from the gel, indicating an efficient binding between the two conjugates.
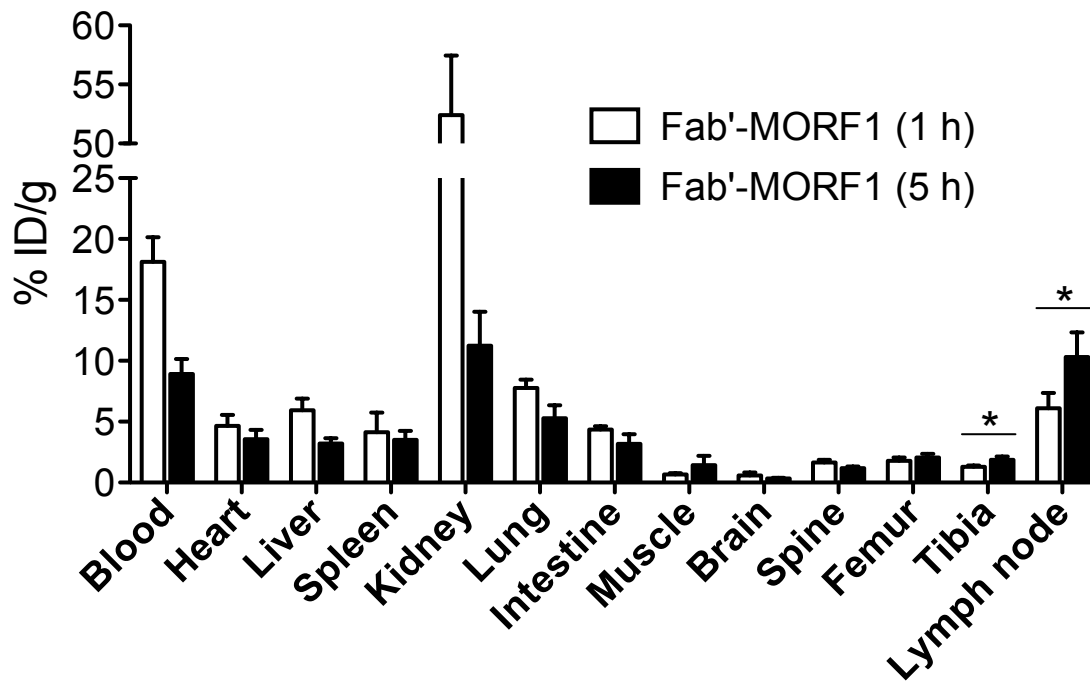
Figure S4. Biodistribution of Fab'-MORF1 in mice at day 1 post-tumor inoculation. Four million Raji B-cells were injected from the tail vein of SCID mice (n = 4) 1 day prior to the study. Mice were sacrificed at 1 h or 5 h after i.v. injection of ^{125}I-labeled Fab'-MORF1. Uptake of the conjugates was calculated as the percentage of the injected dose per gram of organs (% ID/g). Data are presented as mean + SD. Statistics was performed by student's t test (*: $p < 0.05$).
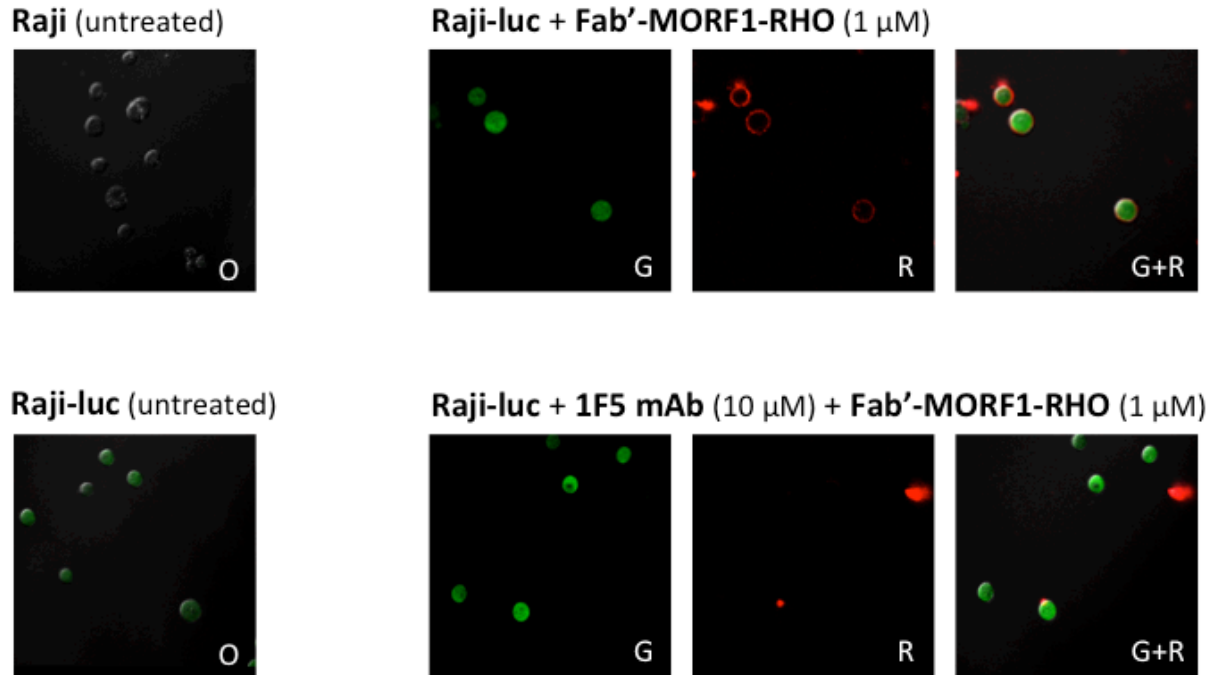
Figure S5. Characterization of the Raji-luc cell line by confocal fluorescence microscopy. Raji-luc cells harbor a dual reporter expressing both luciferase and green fluorescence protein (GFP). The Fab'-MORF1 conjugate was labeled with rhodamine (RHO; red). G: green channel, R: red channel, O: overlay of G and R and under transmitted light. Fab'-MORF1 bound to the surfaces of Raji-luc cells, which confirmed the CD20 expression. Pretreatment with an anti-CD20 1F5 mAb blocked the CD20 binding sites at cell surface, resulting in no red signal decoration after the Fab'-MORF1 treatment.
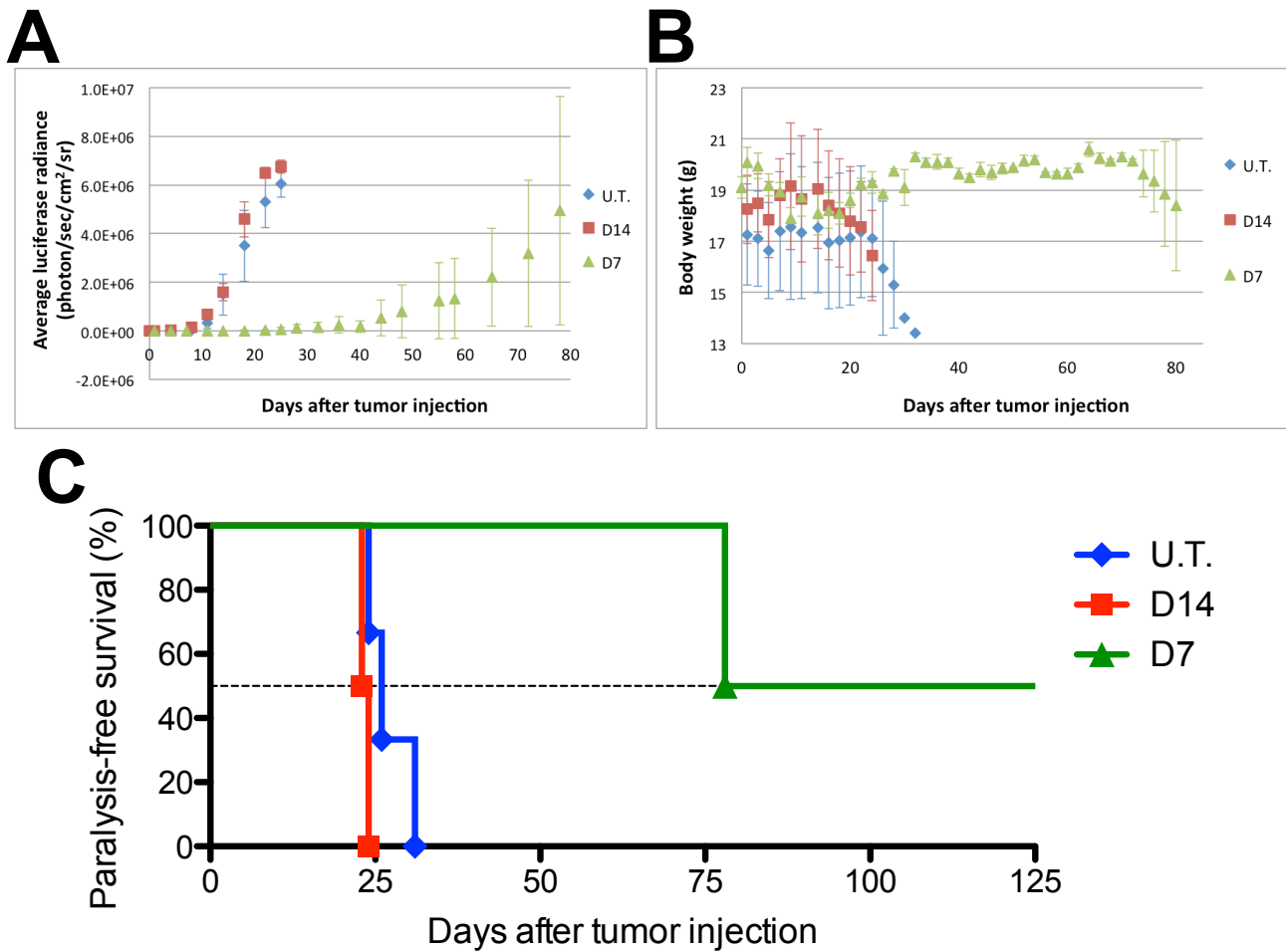
Figure S6. Preliminary *in vivo* therapy experiments. SCID mice were injected with luciferase-expressing Raji cells (4 × 10^6) *via* the tail vein on day 0. Fab'-MORF1 (57.5 μg/20 g; 1 nmol) and P-MORF2 (119 μg/20 g; 5 nmol MORF2) were i.v. administered consecutively, using a 1 h interval. *U.T.*: Untreated mice (*n* = 3). *D14*: Mice given 3 doses of treatments on days 14, 16, and 18 (*n* = 2). *D7*: Mice given 3 doses of treatments on days 7, 9, and 11 (*n* = 2). (**A**) Whole-body bioluminescence intensity of mice. Mice were i.p. injected with 3 mg firefly D-luciferin 15 min prior to imaging. Data are shown as mean ± SD. (**B**) Body weight charts. Data are shown as mean ± SD. (**C**) Kaplan-Meier analysis of animal survival. Incidence of hind-limb paralysis or survival of mice was monitored until day 125.
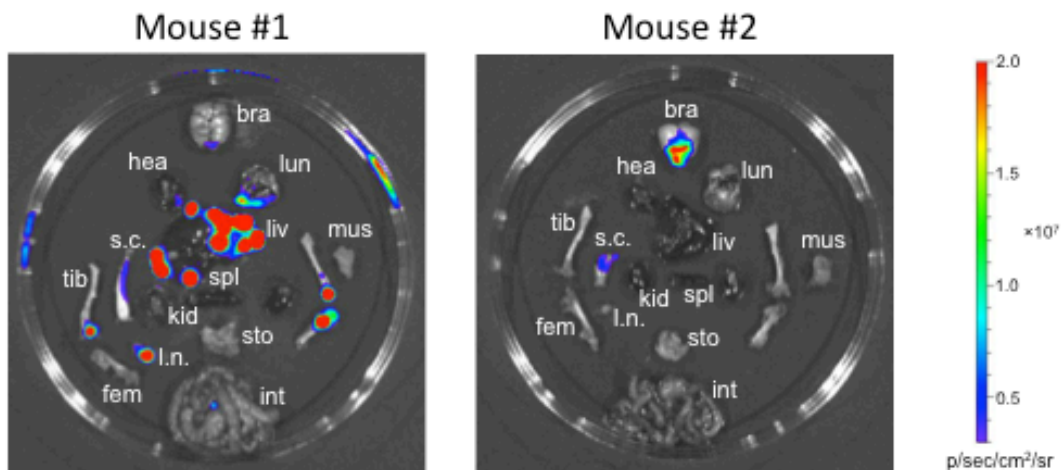
Figure S7. *Ex vivo* bioluminescent images of the PBS-treated mice. Both animals (#1 and #2) were implanted with Raji-luc lymphoma cells on day 0 and paralyzed on day 25. Mice were sacrificed 12 min after the injection (i.p.) of 3 mg firefly D-luciferin, and the organs and tissues were immediately harvested for imaging. *bra*: brain, *hea*: heart, *lun*: lung, *liv*: liver, *spl*: spleen, *kid*: kidney, *s.c.*: spinal cord, *l.n.*: lymph node, *tib*: tibia, *fem*: femur, *mus*: muscle, *sto*: stomach, *int*: intestine.
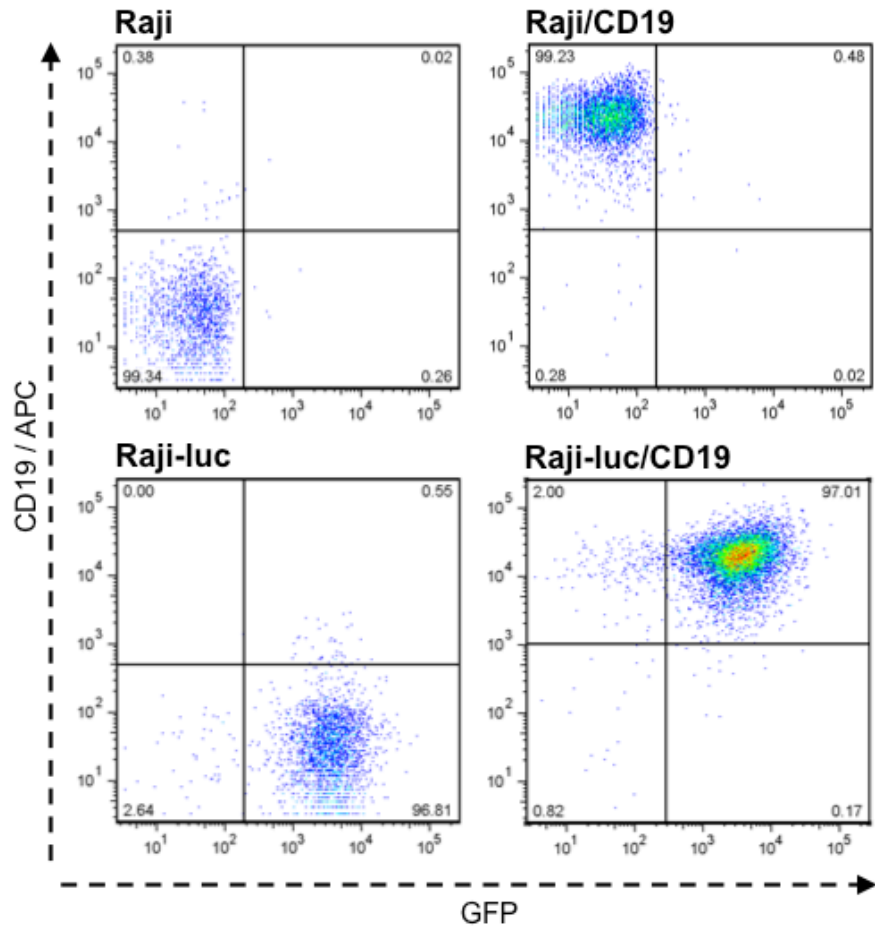
Figure S8. Flow cytometry analysis of Raji and Raji-luc cell lines. Raji-luc cells harbor a dual reporter expressing both luciferase and green fluorescence protein (GFP). Cells were stained with an anti-human CD19 antibody labeled with allophycocyanin (APC).
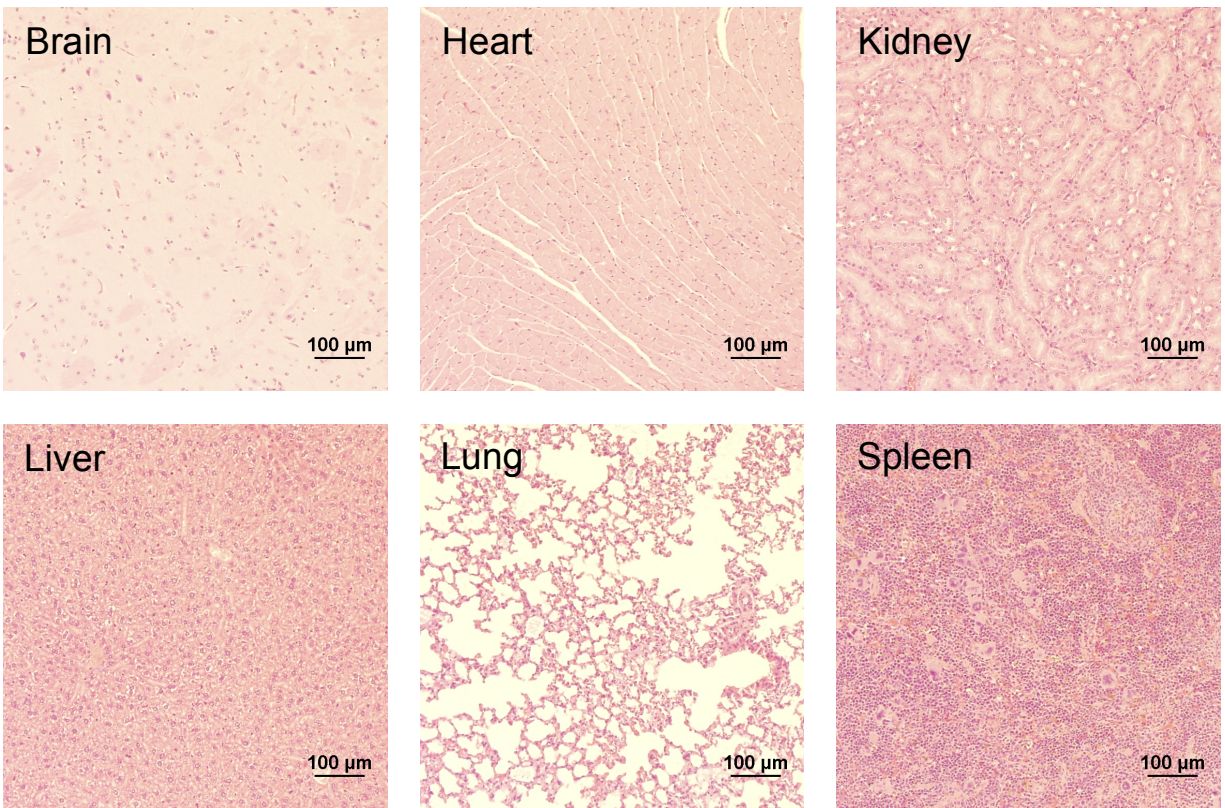
Figure S9. Histology of the surviving mice that were treated with the nanomedicine. Tissues were harvested from the long-term survivors that were treated with drug-free macromolecular therapeutics using a 5 h interval (Cons 5h). Three mice were examined, with 2-6 tissue sections obtained from each organ. H&E-stained specimens were examined, and no evidence of lymphoma invasion or metastatic tumors was found in any of the tissues. No toxicity of the treatment was suggested in all of the organs evaluated.
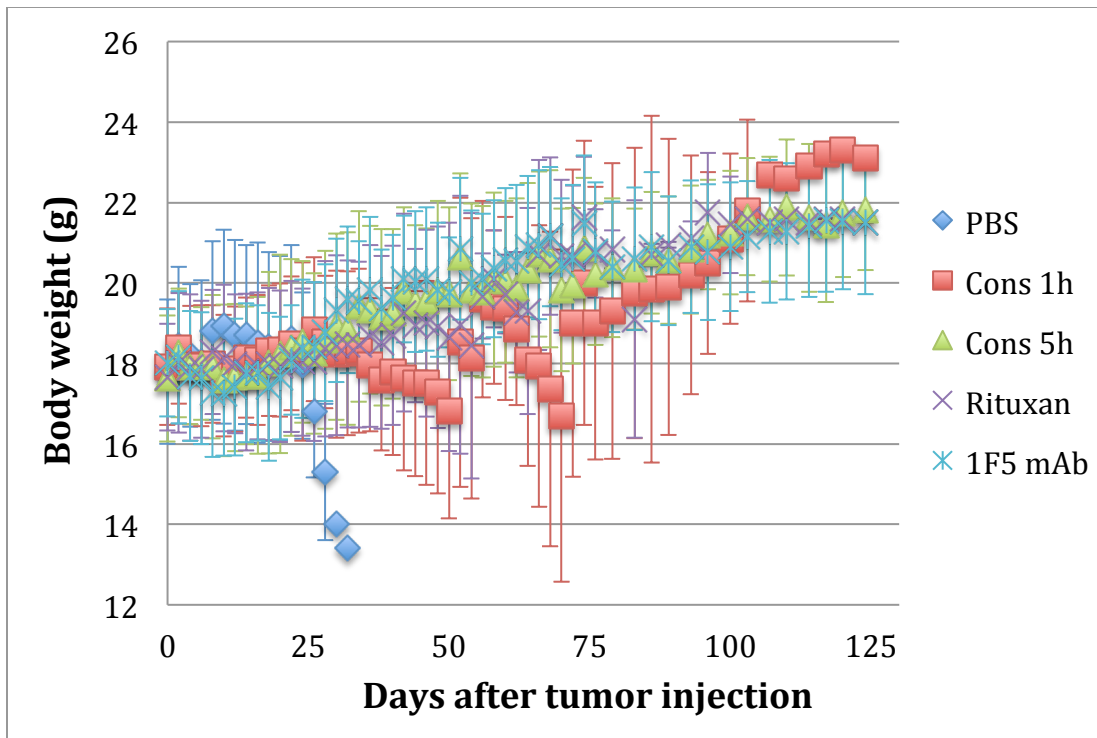
Figure S10. Body weight charts of mice. Mice were injected with Raji-luc cells *via* the tail vein on day 0 and exposed to different treatments (as indicated in Fig. 4 of the main article). Body weight is presented as mean ± SD.