Supporting Information

Amplification of CD20 Crosslinking in Rituximab Resistant B-Lymphoma Cells Enhances Apoptosis Induction by Drug-Free Macromolecular Therapeutics

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1. Synthesis and Characterization of Fab'-MORF1 and Fab'-MORF1-Cy5

Monoclonal antibody Rituximab (Genentech) was first digested into $F(ab')_2$ with 10% (w/w) pepsin (Sigma-Aldrich) in 100 mM citric buffer (pH 4.0) at 37 ^oC. Then, $F(ab')_2$ was reduced to Fab'-SH using 20 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich) in 100 mM citric buffer (pH 5.5) at 37 ^oC. Freshly reduced Fab'-SH was used to conjugate with 3'-maleimide-derived MORF1 (see below). Each step was monitored using size-exclusion chromatography (SEC) to ensure the reaction was completed.

To conjugate MORF1 to Fab'-SH, the end amino group of MORF1 (3'-amine-derivatized 25-mer phosphorodiamidate morpholino oligonucleotide, customized and purchased from Gene Tools, Philomath, OR) was converted to maleimide group by reaction with 40 x excess of succinimidyl-(*N*-maleimidopropionamido)-diethyleneglycol]ester (SM(PEG)$_2$,Thermo Fisher Scientific). The reaction was performed at room temperature (RT) for 2 h. Excess SM(PEG)$_2$ was removed by ultracentrafiltration (Ultracel® 3,000 Da MWCO, Millipore) with DI H_2O washing four times. The MORF1-mal was obtained as white powder after lyophilization. Maleimide content was measured using modified Ellman's assay, and >90% conversion was achieved.

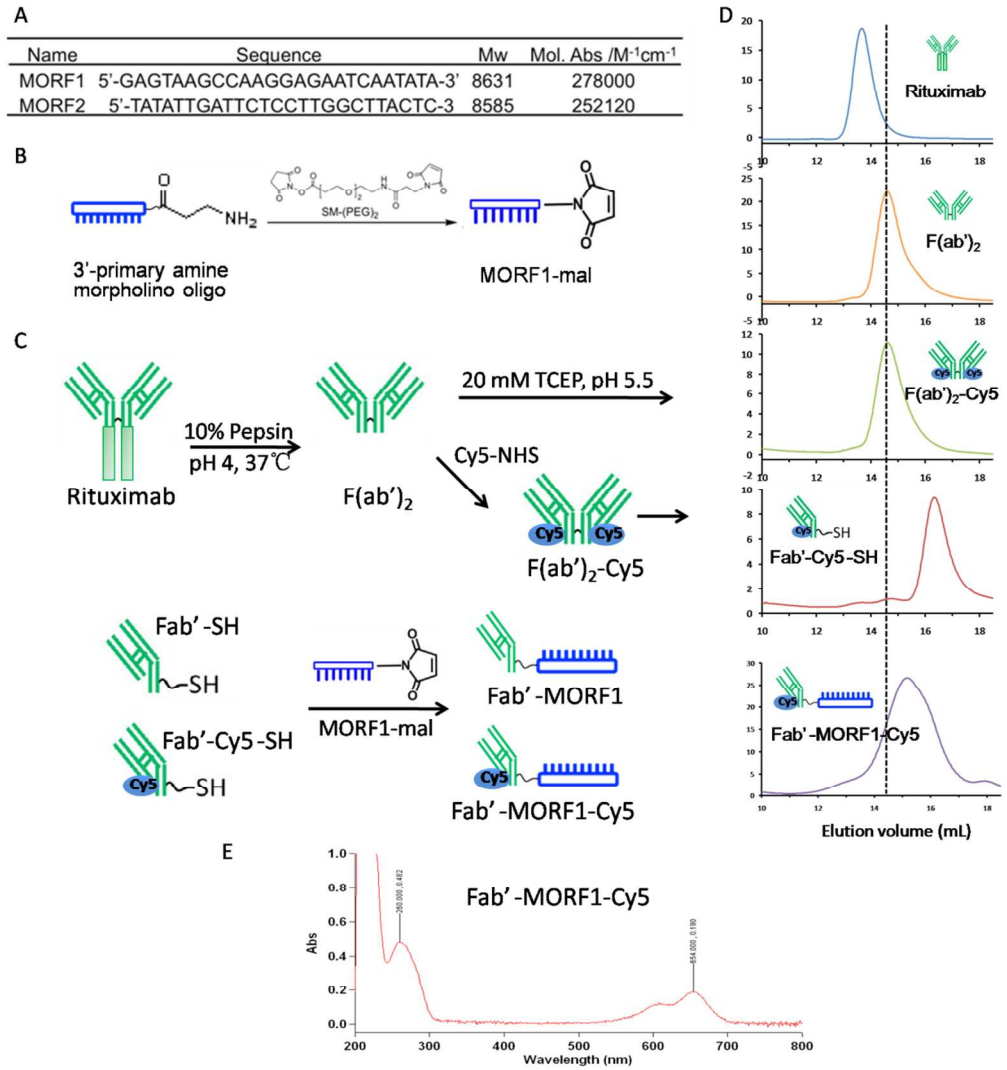


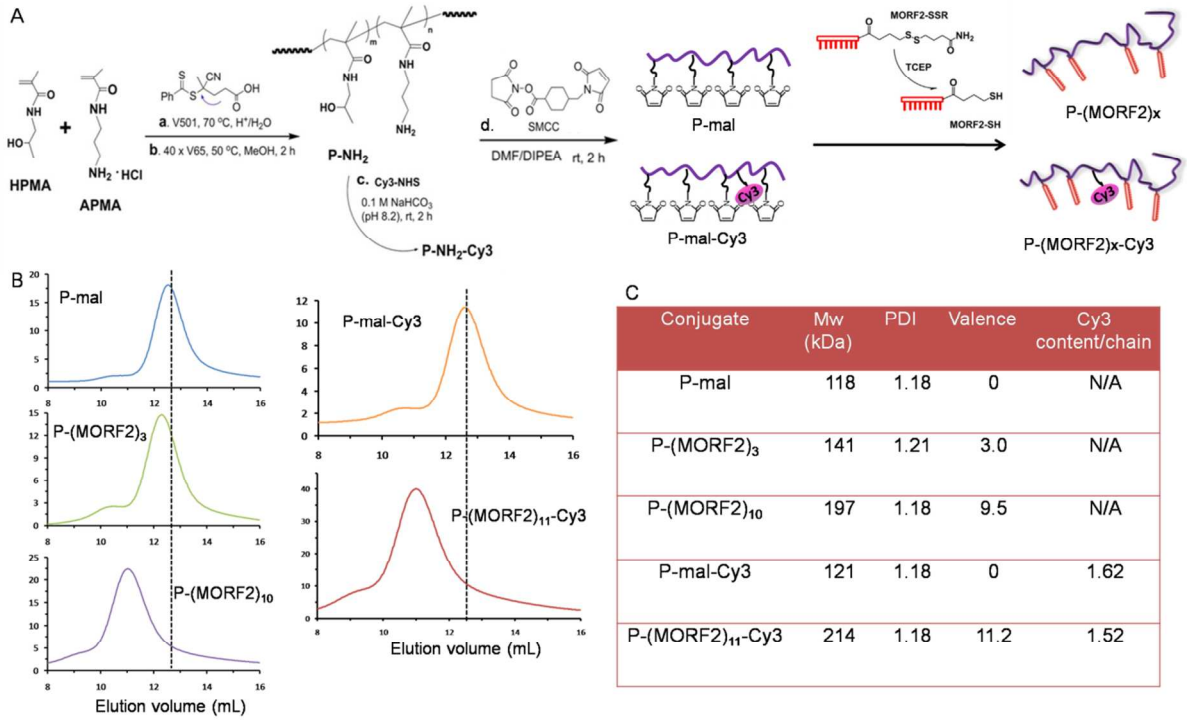
Figure S1. Synthesis and characterization of Fab'-MORF1 and Fab'-MORF1-Cy5. (A) Structures of complementary MORF1 and MORF2. Schematic illustration of the preparation of (B) MORF1-mal and (C) Fab'-MORF1 and Fab'-MORF1-Cy5. (D) Size-exclusion chromatography and (E) UV-Vis spectrophotometry (in 0.1 HCl) of Fab'-MORF1-Cy5.

The conjugation of MORF1-mal to Fab'-SH was performed in 10 mM PBS (pH 6.5) at RT for 2 h with the ratio of 1.1:1. The Fab'-MORF1 conjugate was purified using 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. Fab' equivalent concentration and MORF1 content in Fab'-MORF1 conjugate were determined using bicinchoninic acid (BCA) protein assay (Pierce) and UV-vis spectroscopy (solution in 0.1 N HCl at 260 nm, molar absorption coefficient 278000 $M^{-1}cm^{-1}$) on a Varian Cary 400 Bio UV-visible spectrophotometer, respectively.

To prepare Fab'-MORF1-Cy5, $F(ab')_2$ was labeled with cyanine 5 monosuccinimidyl ester (Cy5-NHS, Lumiprobe, Hunt Valley, MD) by 2 h reaction with lysine side-chains at RT. $F(ab')_2$-Cy5 was purified using a PD 10 column to remove the unreacted dye, then worked up as described above.

2. Synthesis and Characterization of P-$(MORF2)_x$ and P-$(MORF2)_x$-Cy3

HPMA copolymer grafted with multiple copies of MORF2 was prepared via thiol-ene reaction in three steps: HPMA copolymer containing pendent amino groups (P-NH_2) was first synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization of HPMA with *N*-(3-aminopropyl)methacrylamide (APMA); Polymer precursor (P-mal) was then obtained by reaction of P-NH_2 with a heterobifunctional agent succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate, SMCC). Conjugation was achieved by attaching freshly reduced MORF2 (3'-primary terminated with thiol group) to P-mal.



Conjugate	Mw (kDa)	PDI	Valence	Cy3 content/chain
P-mal	118	1.18	0	N/A
P-$(MORF2)_3$	141	1.21	3.0	N/A
P-$(MORF2)_{10}$	197	1.18	9.5	N/A
P-mal-Cy3	121	1.18	0	1.62
P-$(MORF2)_{11}$-Cy3	214	1.18	11.2	1.52

Figure S2. Synthesis and characterization of P-$(MORF2)_x$ and P-$(MORF2)_x$-Cy3. (A) Schematic illustration of the synthesis of P-$(MORF2)_x$ and P-$(MORF2)_x$-Cy3. (B) Size-exclusion chromatography of P-$(MORF2)_x$ and P-$(MORF2)_x$-Cy3 with different MORF2 valences. (C) Characterization (molecular weight, molecular weight distribution, MORF2 valence, Cy3 content) of P-$(MORF2)_x$ and P-$(MORF2)_x$-Cy3.

HPMA (1 g, 7 mmol) and APMA (66 mg, 0.37 mmol) were dissolved with 5 mL DI H_2O containing 0.1% (v/v) acetic acid.4-cyanopentanoic acid dithiobenzoate as RAFT agent (100 μL with conc. 27 mg/mL in methanol) and 4,4′-azobiscyanovaleric acid (V501) as initiator (100 μL

with conc. 9 mg/mL in methanol) were added and the reaction was bubbled with N_2 in ice bath for 30 min then sealed and put into 70°C oil bath for 20 h. The copolymer was precipitated twice into acetone to remove unreacted monomers. The dithiobenzoate end group was removed by radical-induced end-modification using excess of V-65 in methanol at 55 ^oC for 2 h. After precipitation into acetone and filtration, white powder was obtained. The polymer was further purified by dialysis (MWCO 6,000-8,000) against water over 16 h followed by lyophilization. The average molecular weight and the polydispersity of the conjugates were determined by SEC on an AKTA FPLC system equipped with a UV detector (GE Healthcare), miniDAWN TREOS and OptilabrEX (refractive index, RI) detector (Wyatt Technology) using a Superose 6 HR10/30 column with sodium acetate buffer containing 30% acetonitrile (pH 6.5) as mobile phase. The number average molecular weight was 8.72 $\times 10^4$ Da (Mw/Mn = 1.14). The amine content in the copolymer P-NH_2 was 366 nmol/mg (32 NH_2 groups per chain) as determined by ninhydrin test.

To conjugate 3'-thio-modified MORF2 to polymer backbone, the pendant amino group was further converted to maleimide group by reaction with SMCC in DMF in the presence of a tertiary amine (DIPEA) at RT for 2 h (molar ratio of [NH_2]:[SMCC]:[DIPEA]= 1:1.5:3). The maleimide content of the precursor was 294 nmol/mg (26 maleimide groups per chain) as measured by modified Ellman's assay.

To fluorescently label copolymer conjugate, 20 μL Cy3-NHS stock solution in DMSO was added into copolymer P-NH_2 in 0.1 M $NaHCO_3$ (pH 8.2) with molar ratio of [Cy3]: [NH_2] = 1:16) and stirred in dark at RT for 2 h. After reaction, the sample was first precipitated into acetone and then purified using PD-10 Sephadex G25 column (GE Healthcare) to remove free dye. To quantify Cy3 substitution, P-NH_2-Cy3 was dissolved in PBS and the absorbance at 547 nm was determined on a UV-Vis spectrophotometer (Agilent Cary Bio 400). The amount of Cy3 per chain was calculated based on a Cy3 standard curve. The rest of amino groups at the side chains were converted to maleimide groups as described above to yield P-mal-Cy3.

3'-disulfide amide derivatized MORF2 was reduced with TCEP (100 mM, pH 7.0) in 10 mM PBS (pH 5.1) at 37 ^oC for 30 min to generate a free thiol end group. Then TCEP was removed by ultrafiltration (3000 Da cut-off) and washed three times with 10 mM NaH_2PO_4 (pH 5.1). The freshly prepared MORF2-SH was added into P-mal/P-mal-Cy3 solution in 10 mM PBS and kept stirring at RT for 3 h. At the end, unreacted MORF2-SH was removed by ultrafitration (30,000 Da cut-off) with 4 times DI water wash and freeze-dried. Graft copolymers with different valences of MORF2 were obtained by varying the feed ratio of thiol to maleimide groups. The content of MORF2 in the conjugates P-$(MORF2)_X$ or P-$(MORF)_X$-Cy3 was determined by UV-visible spectrophotometry at 260 nm in 0.1 N HCl, and calculated based on the molar absorptivity of MORF2 (252120 $M^{-1}cm^{-1}$, provided by the manufacturer Gene Tools).

3. Synthesis and Characterization of 2P-GEM.

a) Synthesis of polymerizable drug derivative *N*-methacryloylglycylphenylalanylleucylglycyl gemcitabine (MA-GFLG-GEM)

3-(N-methacryloylglycylphenylalanylleucylglycyl) thiazolidine-2-thione (MA-GFLG-TT, 15.0 g, 26.6 mmol), gemcitabine hydrochloride (7.0 g, 23.4 mmol), and small amount of free-radical inhibitor t-octylpyrocatechine (~70 mg) were added into a 500 mL round-bottom flask with a magnetic stir bar. After addition of 150 mL pyridine, the flask was sealed with a rubber septum, then bubbled with nitrogen for 30 min before placing into 50 ^oC oil bath for reaction. After 20 h, the solvent was removed by rotary-evaporator at 40-50 ^oC. The residue was purified by silica gel chromatography (~200 g) with gradient elution: the column was first eluted with ethyl acetate (3 column volumes), then ethyl acetate/acetone (3:1; 3 column volumes), and finally eluted with ethyl acetate/acetone 1:3. MA-GFLG-GEM was obtained after removal of the solvent by rotary-evaporation below 30 ^oC, and further dried under vacuum at room temperature. Yield 13.2 g (80.1 %).

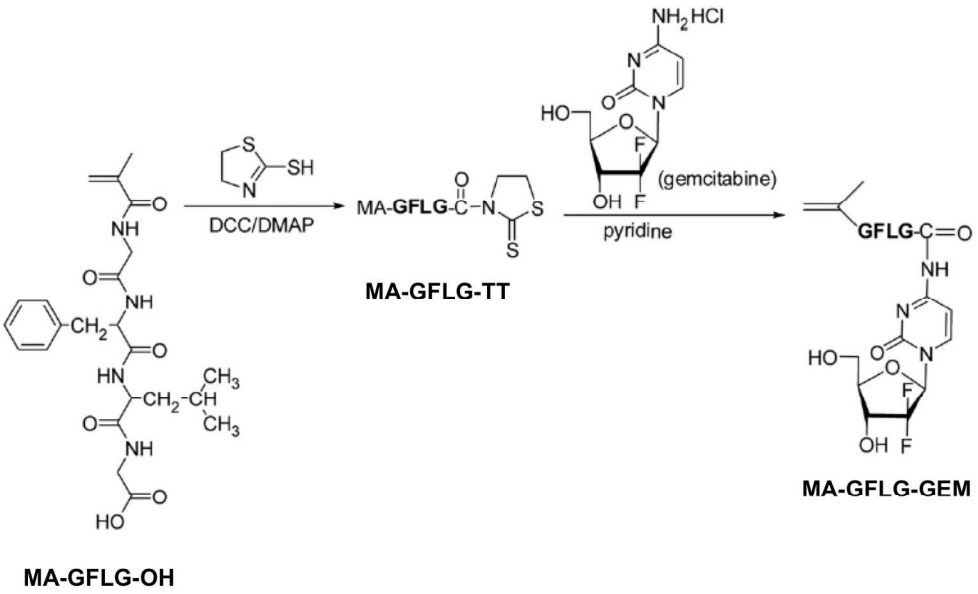


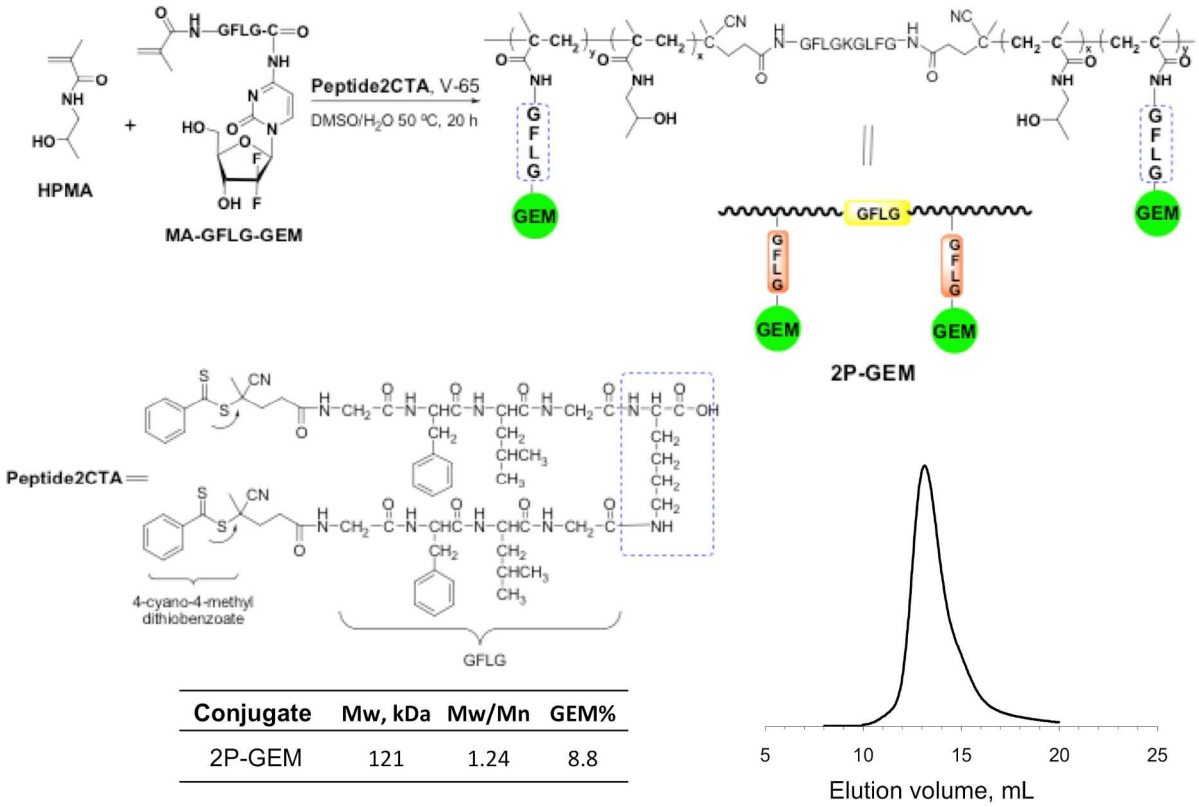
Figure S3. Schematic illustration of MA-GFLG-GEM synthesis.

b) Synthesis of diblock degradable HPMA copolymer-gemcitabine conjugate (2P-GEM)

Polymerizable gemcitabine derivative MA-GFLG-GEM (66 mg, 0.093 mmol) and HPMA (134 mg, 0.94 mmol) were added into a 2 mL-ampoule with a stir bar, followed by adding 0.75 mL degassed DMSO/H_2O(1:1) containing 0.2%(v/v) acetic acid. The ampoule was bubbled with N_2 for 30 min, then 100 µL stock solution of peptide2CTA and 50 µL stock solution of initiator V-65 were added via syringe. The ampoule was sealed after another 5 min N_2 bubbling and kept stirring at 50 ^{o}C oil bath for 20 h. The polymer was isolated by precipitation into acetone and purified by re-dissolving in methanol and precipitating into acetone two more times. The conjugate (2P-GEM) was obtained as a light pink powder with yield of 130 mg (65%).

The molecular weight and the polydispersity index (PDI) of 2P-GEM were determined using size-exclusion chromatography (SEC) on an ÄKTA FPLC system (GE Healthcare) equipped with Superose 6 HR10/300 column, UV (280 nm, GE Healthcare), miniDAWN TREOS and OptilabEX detectors (Wyatt Technology, Santa Barbara, CA). Sodium acetate buffer containing 30% acetonitrile (pH 6.5) served as mobile phase and flow rate 0.4 mL/min. HPMA homopolymer fractions were used as molecular weight standards.

The conjugate was post-polymerization end-modified with excess of V-65 to remove dithiobenzoate end-groups. Gemcitabine content in the conjugate was estimated by UV spectrophotometry in methanol (ε_{300} = 5710 L mol^{-1} cm^{-1}).



Conjugate	Mw, kDa	Mw/Mn	GEM%
2P-GEM	121	1.24	8.8

Figure S4. RAFT polymerization for synthesis of the 2nd generation backbone degradable HPMA copolymer-gemcitabine conjugate 2P-GEM; the structure of RAFT agent Peptide2CTA, the Mw and drug content of 2P-GEM, and its FPLC profile on Superose 6 HR10/300 column eluted with sodium acetate buffer containing 30% acetonitrile (pH 6.5, flow rate 0.4 mL/min).

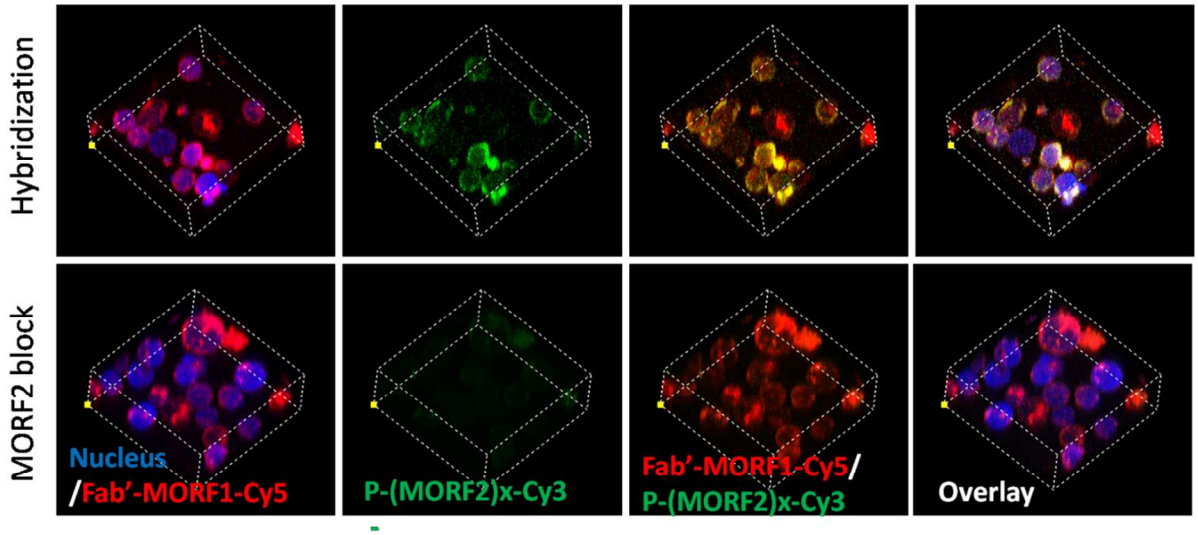


Figure S5. Confocal imaging of hybridization of Fab'-MORF1-Cy5 and P-$(MORF2)_{11}$-Cy3 in the absence and presence of excessive MORF2 block. Blue: nuclei; Red: Cy5; Green: Cy3.

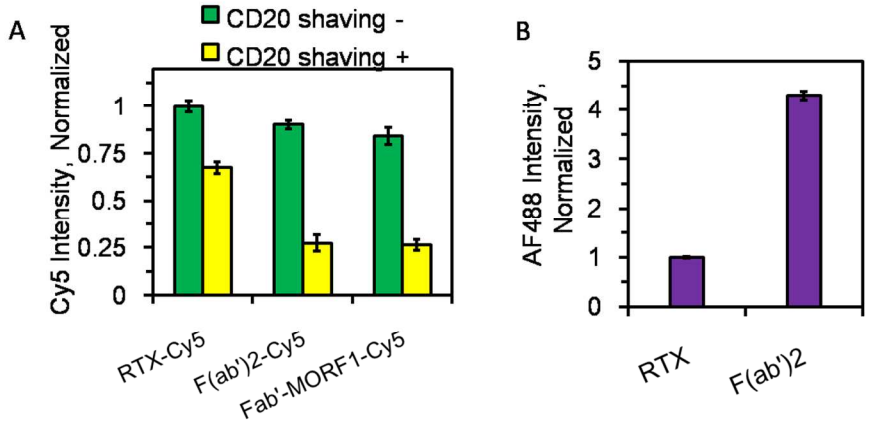


Figure S6. U-2932 4RH cells were treated with RTX-Cy5, F(ab')$_2$-Cy5 and Fab'-MORF1-Cy5 for 1 h and further incubated in cell culture medium for 3 h. The surface accessible RTX and F(ab')$_2$ were detected by AF488 labeled-GAH IgG(H+L) staining at 4 ^{o}C for 20 min. (A) The overall uptake (before surface CD20 shaving) and intracellular uptake (after surface CD20 shaving) of RTX-Cy5, F(ab')$_2$-Cy5 and Fab'-MORF1-Cy5. (B) The intensity of AF488 labeled-GAH IgG(H+L) binding to RTX-Cy5 and F(ab')$_2$-Cy5 on cell surface.

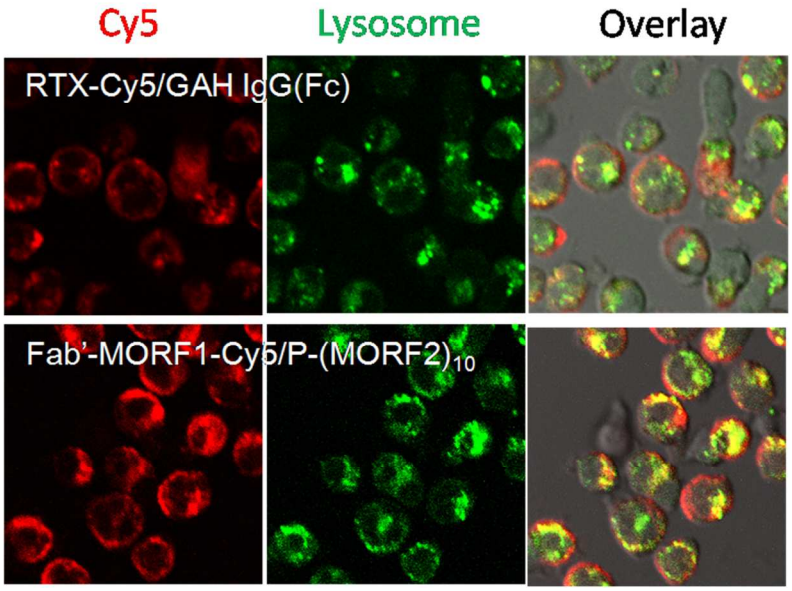


Figure S7. U-2932 4RH cells were first treated with RTX-Cy5 or Fab'-MORF1-Cy5 for 1 h, then GAH IgG(Fc) or P-(MORF2)$_x$ was added and incubated for 3 h. The lysosomes within cells were stained with Lysosome-tracker Green, prior to confocal imaging. Red: Cy5; Green: lysosome.

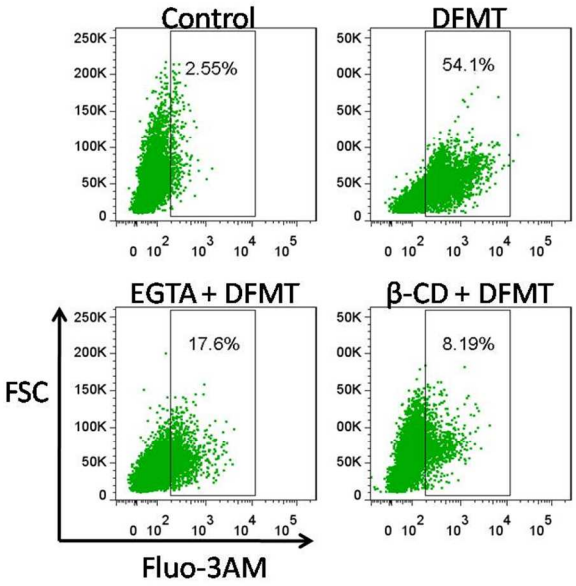


Figure S8. Calcium influx was significantly inhibited by lipid raft inhibitor β-CD (inhibiting CD20 crosslinking) or Ca^{2+} chelating agent EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid).

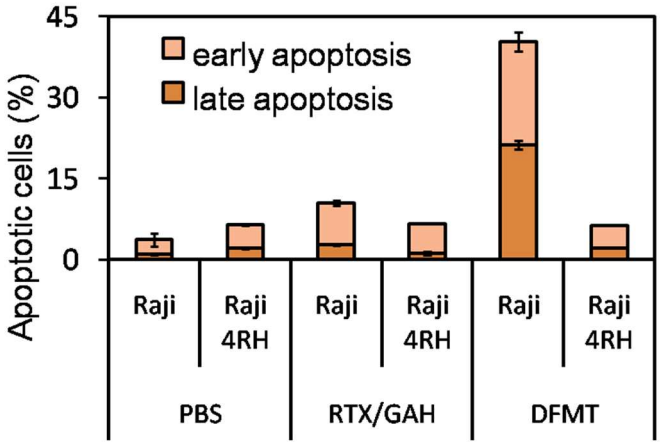


Figure S9. Apoptosis induction in Raji and Raji 4RH cells after treatments with PBS, RTX (1 μM, 1 h)/GAH (1 μM, 24 h) and DFMT, Fab'-MORF1 (1 μM, 1 h) /P-$(MORF2)_{10}$ (1 μM MORF2, 24 h).

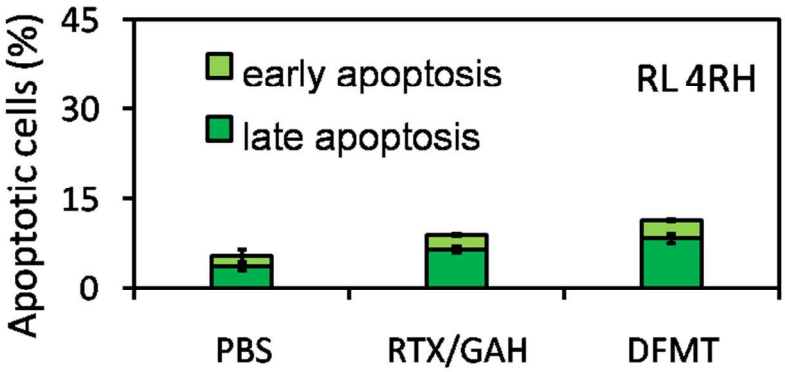


Figure S10. Apoptosis induction in Raji and Raji 4RH cells after treatments with PBS, RTX (1 μM, 1 h)/GAH (1 μM, 24 h) and DFMT, Fab'-MORF1 (1 μM, 1 h) /P-$(MORF2)_{10}$ (1 μM MORF2, 24 h).

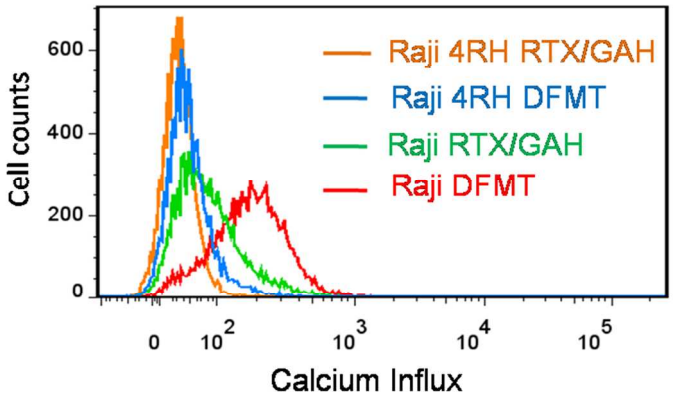


Figure S11. Calcium influx in Raji and Raji 4RH cells triggered by the treatments of RTX (1 μM, 1 h)/GAH (1 μM, 24 h) and DFMT, Fab'-MORF1 (1 μM, 1 h) /P-$(MORF2)_{10}$ (1 μM MORF2, 24 h).

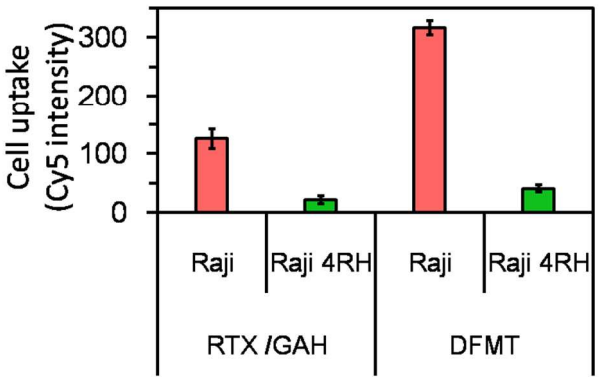


Figure S12. Intracellular internalization of Cy5-labeled RTX/GAH and DFMT (Fab'-MORF1-Cy5/P-$(MORF2)_{10}$) in Raji and Raji 4RH cells after surface CD20 shaving.

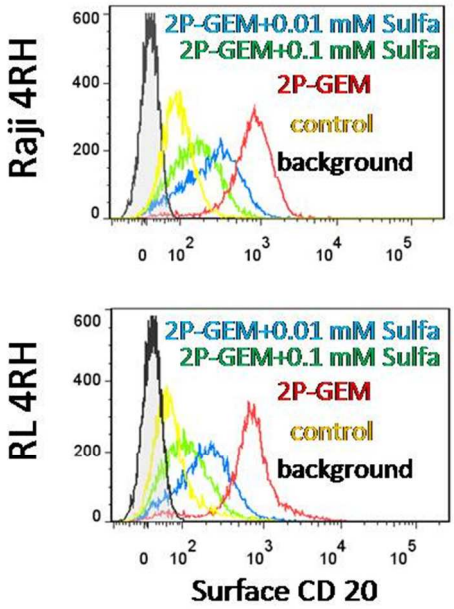


Figure S13. 2P-GEM (100 ng/mL GEM equivalent, 48 h) increases surface CD20 expression in Raji 4RH and RL 4RH cells via NF-kB signaling. Sulfasalazine (Sulfa) is the inhibitor of NF-kB pathway.

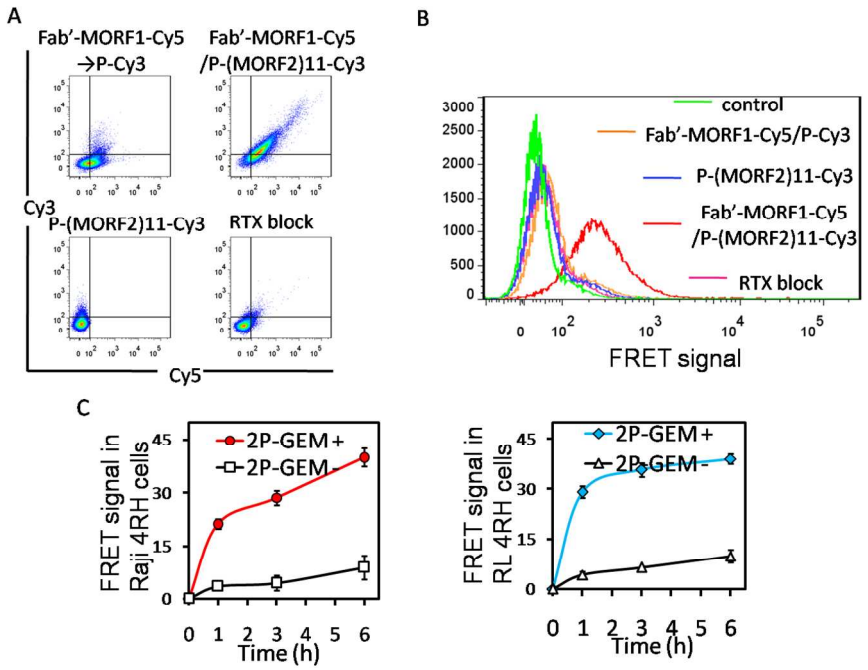


Figure S14. (A) Flow cytometric analysis and (B) Cellular Förster Resonance Energy Transfer (FRET) signals after cells were treated with Fab'-MORF1-Cy5 (1 μM, 1 h)/P-Cy3 (no MORF2 grafts, 3 h), Fab'-MORF1-Cy5 (1 μM, 1 h) /P-$(MORF2)_{11}$-Cy3 (MORF2 1 μM, 3 h), P-$(MORF2)_{11}$-Cy3 (MORF2 1 μM, 3 h) alone and excessive RTX (20 μM, 1 h) block + Fab'-MORF1-Cy5 (1 μM, 1 h) /P-$(MORF2)_{11}$-Cy3 (MORF2 1 μM, 3 h). After various treatments, the flow cytometry was applied with the laser excitation at 488 nm and emission at 675 nm to detect the FRET signal between Cy3 and Cy5. Only sequential exposure to Fab'-MORF1-Cy5 and P-$(MORF2)_{11}$-Cy3 resulted in the majority of cells double-stained with Cy5 and Cy3, and generated a FRET signal as compared with other groups, indicating the specific self-assembly of Fab'-MORF1 engager and P-$(MORF2)_x$ effector on cell surface. (C) FRET signal in Raji 4RH cells and RL 4RH cells after consecutive treatments with Fab'-MORF1-Cy5 (1 μM, 1 h) and P-$(MORF2)_{11}$-Cy3 (MORF2 1 μM, 0, 1, 3, 6 h) in the presence and absence of 2P-GEM pretreatment (100 ng/mL GEM equivalent, 48 h).

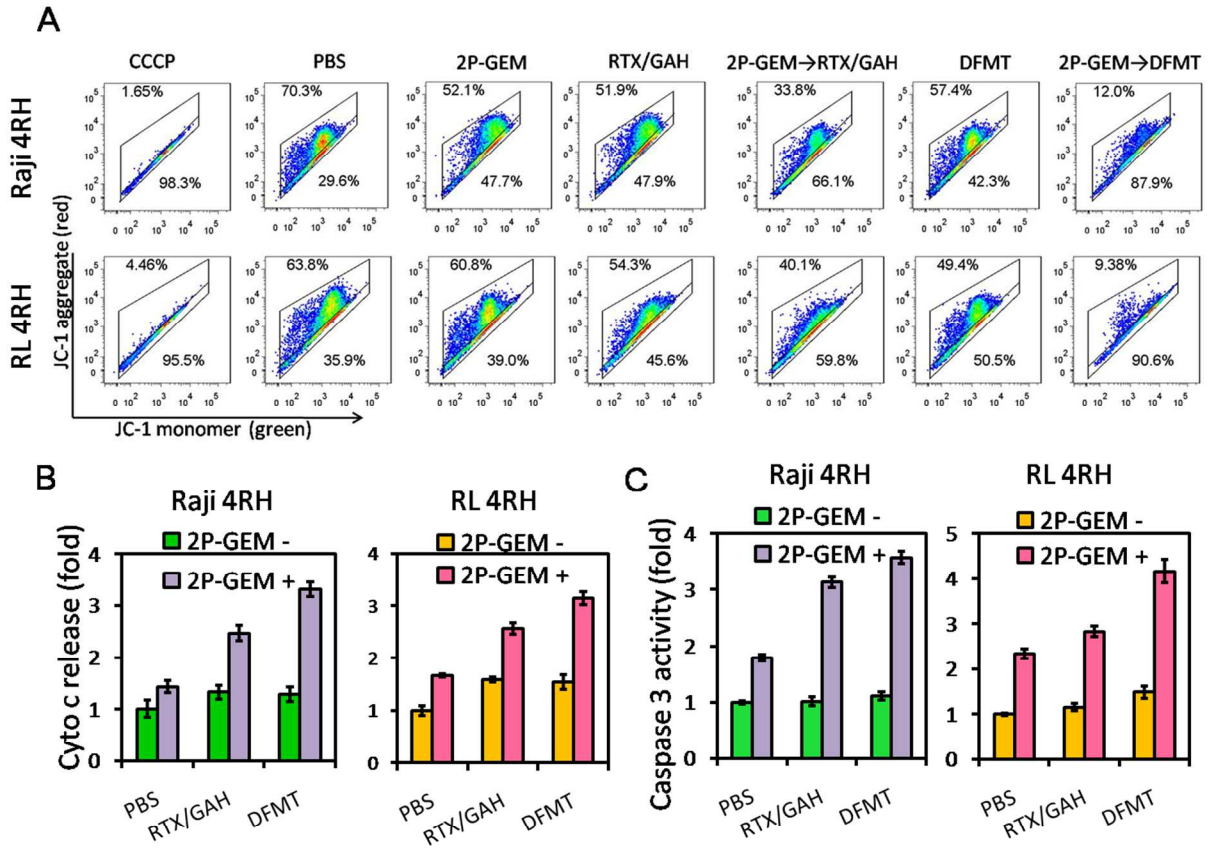


Figure S15. (A) Mitochondrial depolarization, (B) Cytochrome c release and (D) Caspase 3 activation in Raji 4RH and RL 4RH cells after treatments with sequential combination of 2P-GEM (100 ng/mL GEM equivalent, 48 h) and DFMT, Fab'-MORF1 (1 μM, 1 h) /P-$(MORF2)_{10}$ (1 μM MORF2, 24 h).

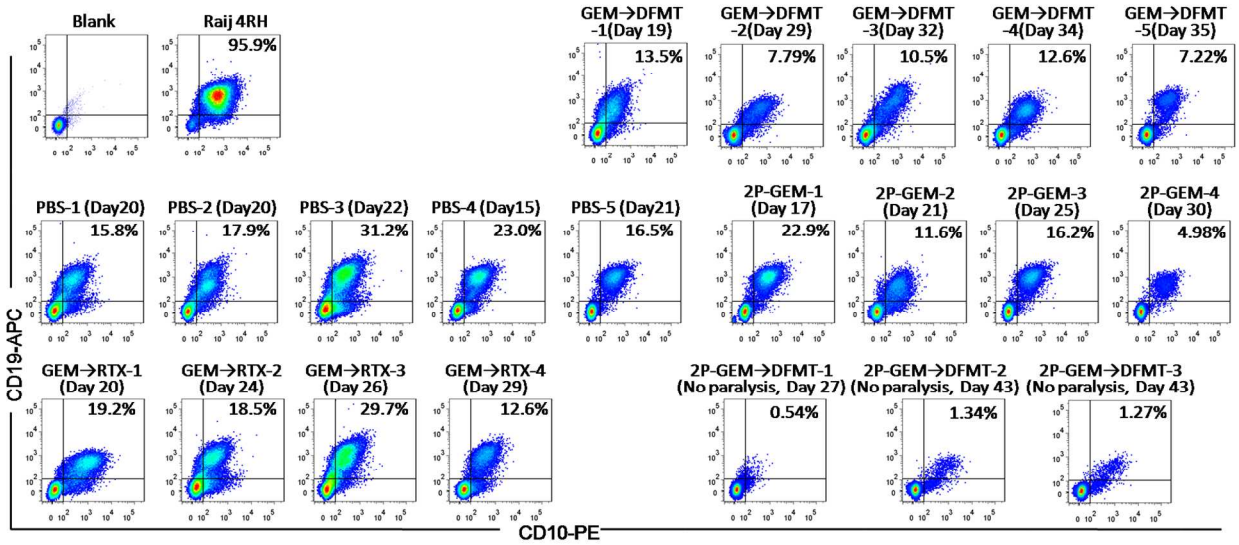


Figure S16. Flow cytometry analysis of residual Raji 4RH cells (human 10+19+) in the bone marrow (BM). BM cells were isolated from the femur of mice at endpoint (onset of paralysis), and Raji 4RH cells were dual stained with PE-labeled mouse anti-human CD10 and APC-labeled mouse anti-human CD19 antibodies.