Nanoparticles containing a combination of a drug and an antibody for the treatment of breast cancer brain metastases

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Supporting Information

Materials and Methods

^{1}H NMR spectra were acquired on a Varian 600 MHz spectrometer (Inova). Electrospray ionization (ESI) masses of small molecules were acquired on a Finnigan LCQ ion trap mass spectrometer. Matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectra for polymers were acquired on an Applied Biosystems Voyager DE-PRO.

Synthesis of MAP-CPT Conjugate.
Synthesis of MAP-CPT Conjugate. MAP was prepared and characterized as previously described.[1] Anhydrous dimethyl sulfoxide (5 mL) was added under argon to dissolve MAP (100 mg, 1 equiv) in a 25 mL round-bottomed flask. To this was added EDC (42 mg, 4 equiv) and NHS (16 mg, 3 equiv) dissolved in anhydrous dimethyl sulfoxide (1.5 mL), followed by 20-O-Glycincamptothecin trifluoroacetic acid salt (CPT-gly.TFA, 85 mg, 3 equiv) dissolved in dimethyl sulfoxide (1.5 mL) and anhydrous N,N-diisopropylethylamine (28 μL) dried over molecular sieves. The reaction was stirred under argon at room temperature overnight. The solution was dialyzed against dimethyl sulfoxide 3 times and nanopure water 2 times using a 10 kDa MWCO Spectra/Por 7 membrane (Spectrum). Precipitate was removed by centrifugation at 3220 g for 15 min, and the supernatant was filtered through a 0.2 μm Supor membrane Acrodisc syringe filter (Pall) to yield MAP-CPT conjugate as self-assembled nanoparticles in solution. A portion of this clear yellow solution was lyophilized to determine percent CPT conjugation. The remaining product was formulated into 0.9% (w/v) saline and stored at -20 ºC.
Determination of CPT Content in MAP-CPT. Lyophilized MAP-CPT was dissolved in dimethyl sulfoxide at 10 mg/mL, diluted to 0.1 mg/mL with 1 N NaOH, and incubated overnight. Fluorescence was measured at 370/440 nm (ex/em) using a Safire 2 multi-mode plate reader (Tecan). A calibration curve of known concentrations of CPT was prepared and used to determine the CPT concentration in the mixture.

Synthesis of MAP-AF568 Conjugate. Anhydrous dimethyl sulfoxide (3 mL) was added under argon to dissolve MAP (30 mg, 1 equiv) in a 10 mL round-bottomed flask. To this was added EDC (13 mg, 4 equiv) and NHS (5 mg, 3 equiv) dissolved in anhydrous dimethyl sulfoxide (1 mL), followed by Alexa Fluor 568 hydrazide, sodium salt (AF568, 12 mg, 1 equiv) dissolved in dimethyl sulfoxide (1 mL). The reaction was stirred under argon at room temperature overnight. The solution was dialyzed against dimethyl sulfoxide 3 times and nanopure water 4 times using a

10 kDa MWCO Spectra/Por 7 membrane (Spectrum). The retentate was filtered through a 0.2 μm Supor membrane Acrodisc syringe filter (Pall) to yield MAP-AF568 conjugate as self-assembled nanoparticles in solution. The product was then formulated into 0.9% (w/v) saline and stored at -20 ºC.

Synthesis of CO_2H-$PEG_{3.5k}$-nitroPBA and CO_2H-PEG_{5k}-nitroPBA.

Synthesis of 3-acyl chloride-5-nitrophenyl boronic acid. 3-carboxy-5-nitrophenyl boronic acid (nitroPBA, 100 mg, 1 equiv, Alfa Aesar) was added to an oven-dried 10 mL round-bottomed flask. The reaction flask was sealed and vented with argon. Anhydrous tetrahydrofuran with BHT inhibitor (4 mL) was added to dissolve the boronic acid, followed by anhydrous dimethylformamide (7 μL, 0.2 equiv). The flask was cooled to 0 ºC in an ice bath, and oxalyl chloride (98 μL, 2.4 equiv) was added dropwise. After addition of oxalyl chloride, the ice bath was removed and the reaction was stirred under argon for 2 hrs. Solvent was evaporated under vacuum to yield 3-acyl chloride-5-nitrophenyl boronic acid (101 mg) as a yellow solid.

Synthesis of CO_2H-$PEG_{3.5k}$-nitroPBA. 3-acyl chloride-5-nitrophenyl boronic acid (46 mg, 2 equiv) was added to an oven-dried 25 mL round-bottomed flask. The reaction flask was sealed, vented with argon, and cooled to 0 ºC in an ice bath. Anhydrous DCM (5 mL) was added to dissolve the boronic acid. Acetic acid-$PEG_{3.5k}$-amine (3.5 kDa, 350 mg, 1 equiv, JenKem) was added to a separate oven-dried 10 mL round-bottomed flask. The flask was sealed, and vented with argon. To this was added anhydrous N,N-diisopropylethylamine (35 μL, 2 equiv) dried over molecular sieves, and anhydrous DCM (5 mL) to dissolve the PEG. The PEG solution was added dropwise to the boronic acid solution. The reaction flask was left in the ice bath to slowly warm to room temperature, and stirred under argon overnight protected from light. Solvent was removed under vacuum, and the solid reconstituted in 0.5 N HCl (4 mL) and stirred for 15 min. The solution was filtered through a 0.2 μm Supor membrane Acrodisc syringe filter (Pall) and dialyzed against nanopure water until constant pH using a 1 kDa MWCO Spectra/Por 7 membrane (Spectrum). The retentate was filtered through a 0.2 μm Durapore PVDF membrane Millex syringe filter (Millipore), and lyophilized to yield CO_2H-$PEG_{3.5k}$-nitroPBA (238 mg) as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6): 12.52 (s - COOH, 1H), 8.90 (t, 1H), 8.72 (m, 1H), 8.69 (m, 1H), 8.64 (m, 1H), 8.61 (s, 2H), 4.01 (s, 2H), 3.53–3.46 (s - PEG). MALDI: 3978.4.

Synthesis of CO_2H-PEG_{5k}-nitroPBA.

A similar synthesis procedure was followed using acetic acid-PEG_{5k}-amine (5 kDa, 500 mg, 1 equiv, JenKem) to synthesize CO_2H-PEG_{5k}-nitroPBA. Solvent was removed under vacuum, and the solid reconstituted in 0.5 N HCl (5 mL) and stirred for 15 min. The solution was filtered through a 0.2 μm Supor membrane Acrodisc syringe filter (Pall) and dialyzed against nanopure water until constant pH using a 15 mL Amicon Ultra 3 kDa spin filter (Millipore). The retentate was filtered through a 0.2 μm Durapore PVDF membrane Millex syringe filter (Millipore), and lyophilized to yield CO_2H-PEG_{5k}-nitroPBA (452 mg) as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6): 12.52 (s - COOH, 1H), 8.90 (t, 1H), 8.73 (m, 1H), 8.68 (m, 1H), 8.65 (m, 1H), 8.62 (s, 2H), 4.00 (s, 2H), 3.53–3.46 (s - PEG). MALDI: 5476.7.

Synthesis of Herceptin-$PEG_{3.5k}$-nitroPBA and Tf-PEG_{5k}-nitroPBA.

Synthesis of Herceptin-$PEG_{3.5k}$-nitroPBA. CO_2H-$PEG_{3.5k}$-nitroPBA (11.2 mg, 25 equiv), EDC-HCl (6.1 mg, 250 equiv), and NHS (5.5 mg, 375 equiv) were dissolved in 0.1 M MES buffer, pH 6.0 (0.33 mL), and stirred for 15 min at room temperature. The reaction mixture was then added to a 0.5 mL Amicon Ultra 3 kDa spin filter (Millipore), and centrifuged to isolate the activated

nitroPBA-$PEG_{3.5k}$-NHS ester. The ester was added to Herceptin (20 mg, 1 equiv) dissolved in 0.1 M PBS, 0.15 M NaCl, pH 7.4 (1 mL). The reaction was lightly agitated for 2 h at room temperature, and then dialyzed against 0.1 M PBS, 0.15 M NaCl, pH 7.4 using 0.5 mL Amicon Ultra 50 kDa spin filters (EMD Millipore) to remove excess PEG. A portion of this solution was dialyzed into 10 mM PB, pH 7.4, and conjugation was verified by MALDI-TOF using a sinapinic acid matrix. MALDI: 153063.6. The remaining Herceptin-$PEG_{3.5k}$-nitroPBA was formulated into PBS, pH 7.4, and stored at 4 ºC.

Synthesis of Tf-PEG_{5k}-nitroPBA. A similar procedure was followed using CO_2H-PEG_{5k}-nitroPBA (16 mg, 25 equiv) and human holo-Tf (10 mg, 1 equiv, Sigma) to prepare Tf-PEG_{5k}-nitroPBA. Following dialysis against 0.1 M PBS, 0.15 M NaCl, pH 7.4 to remove excess PEG, the amount of iron loaded to the Tf after conjugation was verified by UV-VIS on a NanoDrop system (Thermo Scientific) using the ratio of A_{465}/A_{280}. This ratio was compared to that of the unreacted human holo-Tf, and a value ≥ 80% of the unreacted ratio confirmed adequate iron retention following synthesis steps. A portion of this solution was dialyzed into 10 mM PB, pH 7.4, and conjugation was verified by MALDI-TOF using a sinapinic acid matrix. MALDI: 85210.7. The remaining Tf-PEG_{5k}-nitroPBA was formulated into PBS, pH 7.4, and stored at 4 ºC.

Preparation of Nanoparticles. To prepare TfR-targeted CPT nanoparticles, Tf-PEG_{5k}-nitroPBA conjugates in PBS, pH 7.4 were added at 20x molar excess to MAP-CPT nanoparticles (20 Tf per particle), as previously described.[1] The solution was gently mixed by pipette and allowed to equilibrate for 10 min. To prepare Herceptin and combination CPT/Herceptin nanoparticles, Herceptin-$PEG_{3.5k}$-nitroPBA conjugates in PBS, pH 7.4 were added at an equal molar ratio to either MAP-AF568 or MAP-CPT nanoparticles (1 Herceptin per particle), respectively. The solution was gently mixed and allowed to equilibrate as above. Tf-PEG_{5k}-nitroPBA conjugates in PBS, pH 7.4 were then added at 20x molar excess to either Herceptin or combination CPT/Herceptin nanoparticles (20 Tf per particle) to form TfR-targeted Herceptin and TfR-targeted combination CPT/Herceptin nanoparticles, respectively. The solution was again mixed by pipette and allowed to equilibrate for 10 min. Nanoparticle formulations were filtered using a 0.45 μm PTFE membrane Millex syringe filter (Millipore).

Nanoparticle Characterization. Nanoparticles were characterized using a Brookhaven Instruments Corporation (BIC) ZetaPALS. Nanoparticles were diluted in PBS, pH 7.4 and hydrodynamic diameter was measured by dynamic light scattering (DLS) using BIC Particle Sizing Software. Particle formulations were diluted in 10 mM PB, pH 7.4 and zeta potential was measured using BIC PALS Zeta Potential Analyzer software with a target residual of 0.02. Five runs were performed for both the nanoparticle diameter and zeta potential measurements.

Antitumor Efficacy.

Intracardiac (ICD) Brain Metastasis Model. All animals were treated according to the NIH guidelines for animal care and use as approved by the Caltech Institutional Animal Care and Use Committee. BT474-Gluc cells, transduced with an expression cassette encoding Gluc and CFP separated by an internal ribosomal entry site using a lentiviral vector, were obtained from Dr. Jain at Harvard University. BT474-Gluc cells were maintained in RPMI 1640 supplemented with 10% (v/v) FBS in a humidified oven at 37 °C with 5% CO_2. 100,000 BT474-Gluc cells were suspended in 100 μL of RPMI and slowly injected into the left ventricle of female $Rag2^{-/-}$;$Il2rg^{-/-}$ mice. Injections were performed blind, midway between the sternal notch and top of xyphoid process,

and 13% anatomical left of sternum. Successful insertion into the left cardiac ventricle was confirmed by a bright red pulse of blood in the syringe.

Tumor Size Monitoring. Formation of BT474-Gluc brain metastatic tumors was monitored by MRI every third week until macroscopic tumors were visible (~0.2 mm^3 in volume). Tumor growth was then monitored weekly by MRI. Mice were anaesthetized with 1.5–2% (v/v) isoflurane in O_2 at a flow rate of 1–1.5 mL/min. T2-weighted 3D RARE images were acquired to assess the tumor volume. The image acquisition parameters were as follows: echo time: 6.1 ms; repetition time: 250 ms; rapid acquisition relaxation enhanced (RARE) factor: 4; number of averages: 4; field of view: 2.0 cm x 1.2 cm x 0.8 cm; matrix: 200 x 120 x 80 (100 μm isotropic resolution). Tumor volume was determined manually from the T2 hyperintense tumor regions of the brain using Fiji software. Pairwise group comparisons testing for statistically significant differences were performed using the Wilcoxon-Mann-Whitney test in MATLAB.

Treatments. Treatment began when brain metastatic tumors reached ~2 mm^3, as measured by MRI. Mice in each model were randomized into four groups of six mice per group. CPT at a dose of 4 mg/kg (in 20% DMSO, 20% PEG 400, 30% ethanol, and 30% 10 mM pH 3.5 phosphoric acid), Herceptin at 24 mg/kg (in PBS, pH 7.4), TfR-targeted CPT nanoparticles at 4 mg/kg (CPT basis, in PBS, pH 7.4), TfR-targeted Herceptin nanoparticles at 24 mg/kg (Herceptin basis, in PBS, pH 7.4), and TfR-targeted combination CPT/Herceptin nanoparticles at 4 and 24 mg/kg (CPT and Herceptin bases, respectively, in PBS, pH 7.4) were freshly prepared. The different formulations were systemically administered by lateral tail vein injection once per week for 4 weeks. Injections were standardized to 150 μL per 20 g body weight. The control treatment was 0.9% (w/v) saline.

Supplemental Figures

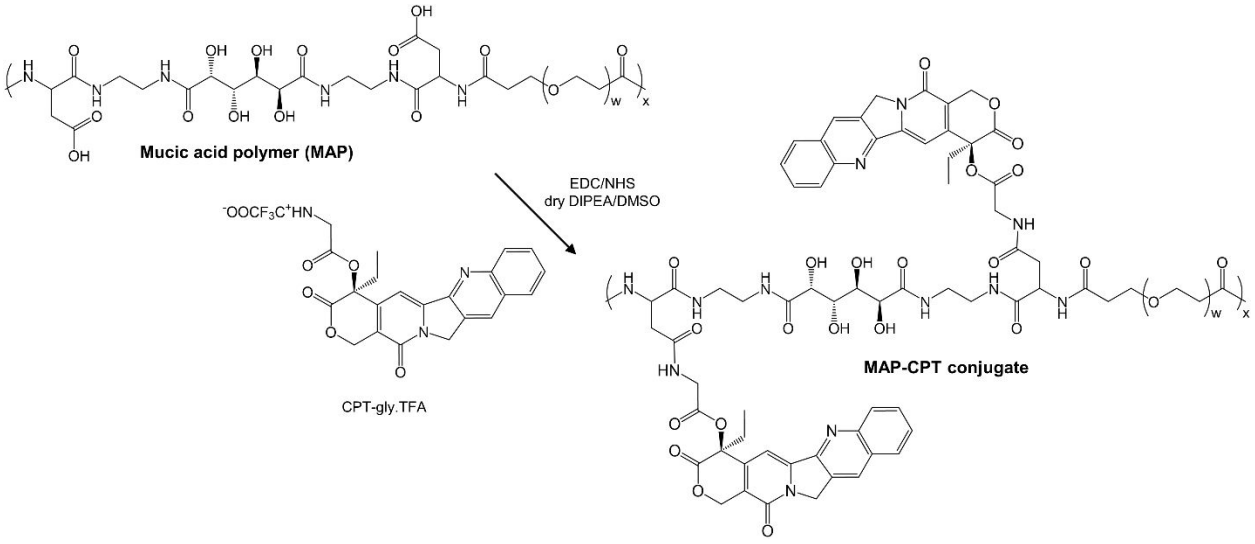

Figure S1. Synthesis of MAP-CPT polymer-drug conjugate. w ~ 82 for 3.4kDa PEG; x ~ 20 for material used in this study.

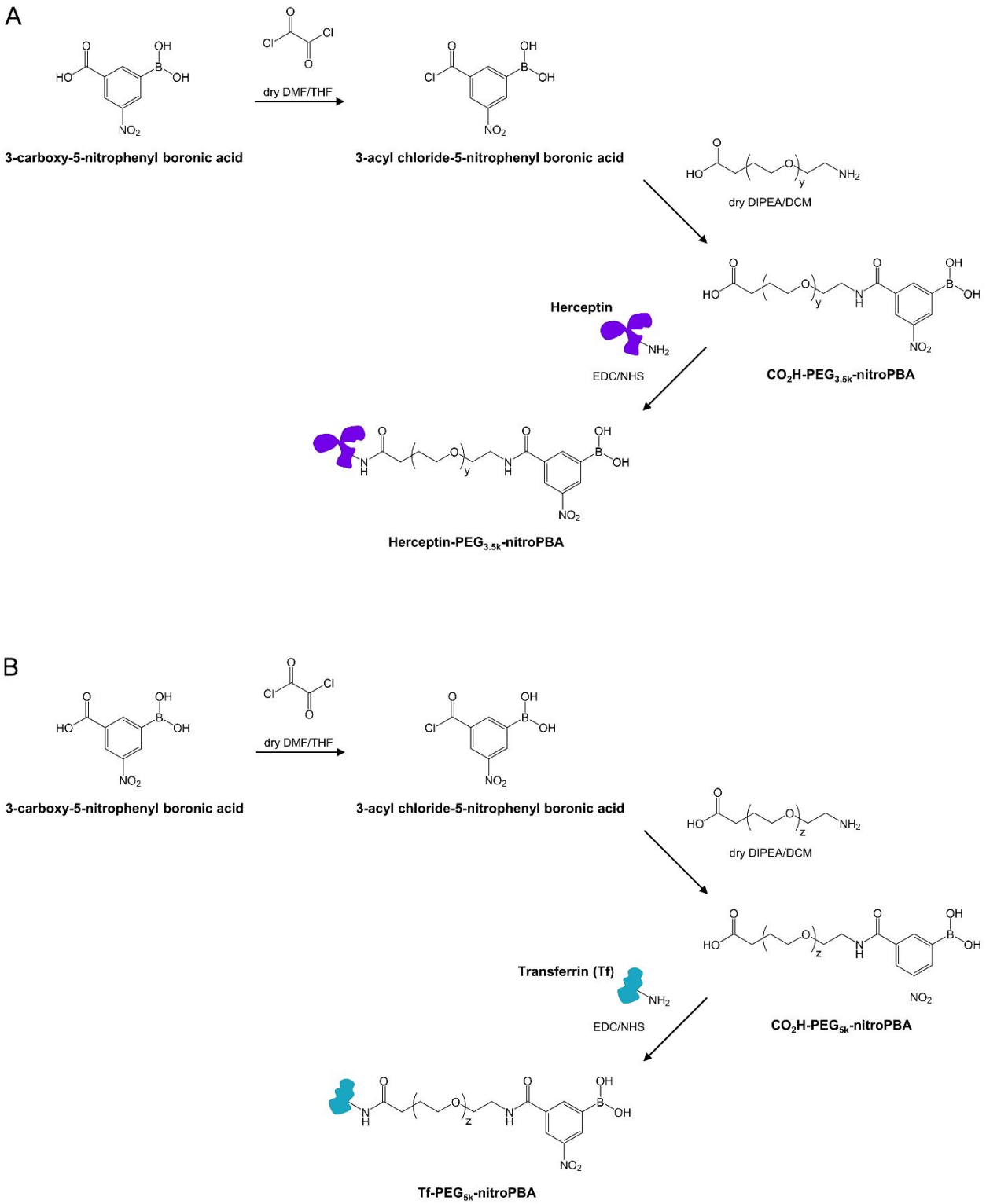

Figure S2. Synthesis of nitroPBA conjugates. (**A**) Herceptin-$PEG_{3.5k}$-nitroPBA. y ~ 84 for 3.5kDa PEG. (**B**) Tf-PEG_{5k}-nitroPBA. z ~ 120 for 5kDa PEG.

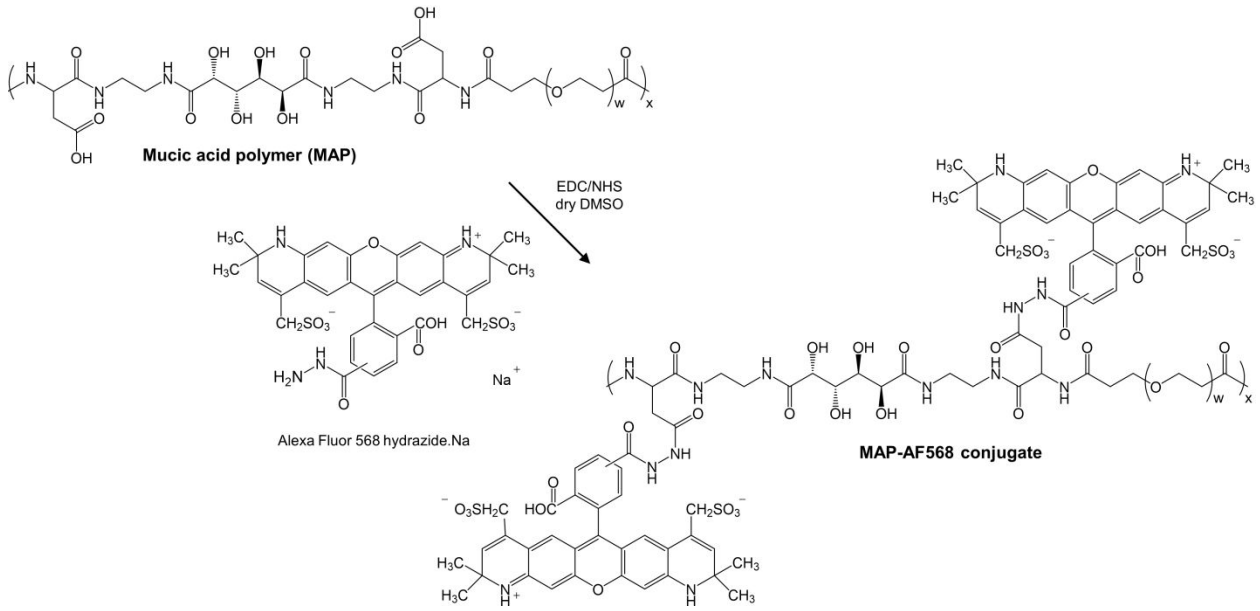

Figure S3. Synthesis of MAP-AF568 polymer conjugate. w ~ 82 for 3.4kDa PEG; x ~ 20 for material used in this study.

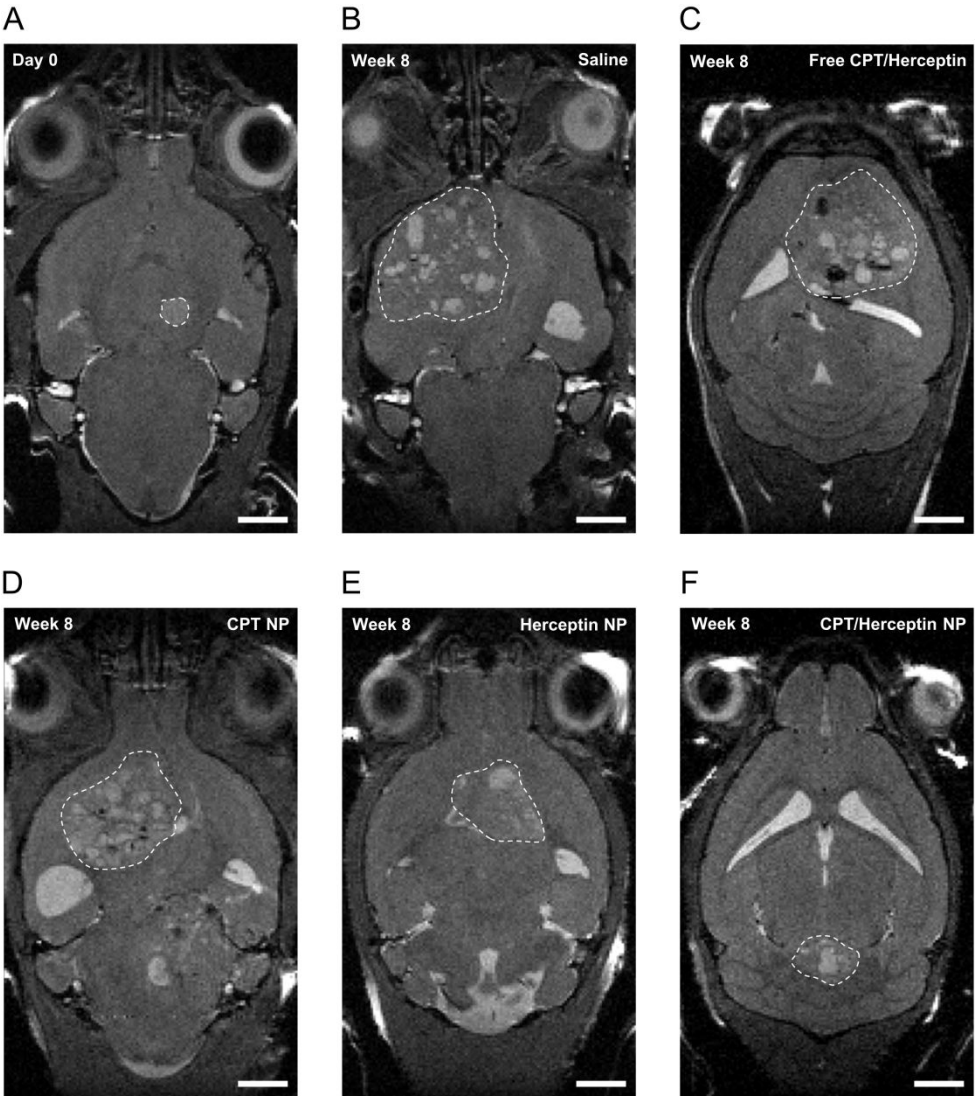

Figure S4. Representative MRI images of metastatic brain tumors at the start of treatment (**A**), and 8 weeks after the start of treatment with saline (**B**), free CPT and Herceptin (**C**), TfR-targeted CPT nanoparticles (**D**), TfR-targeted Herceptin nanoparticles (**E**) and TfR-targeted combination CPT/Herceptin nanoparticles (**F**). Dotted lines denote tumor margins. Formulations were systemically administered weekly for 4 weeks at a dose of 4 and/or 24 mg/kg (CPT and/or Herceptin bases, respectively). Scale bar, 2 mm; NP, nanoparticle.

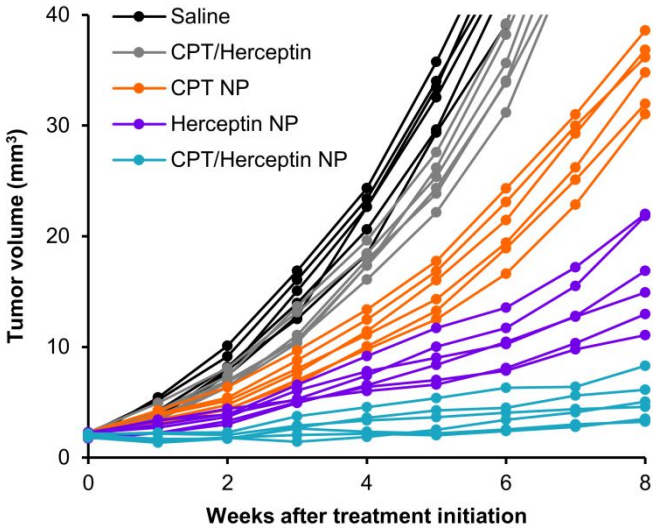

Figure S5. Individual tumor growth curves of BT474-Gluc metastatic brain tumors treated with CPT and Herceptin (gray, 4 and 24 mg/kg, respectively), TfR-targeted CPT nanoparticles (orange, 4 mg CPT/kg), TfR-targeted Herceptin nanoparticles (purple, 24 mg Herceptin/kg) and TfR-targeted combination CPT/Herceptin nanoparticles (blue, 4 mg CPT/kg and 24 mg Herceptin/kg) compared to saline (black). NP, nanoparticle. Animals dosed on weeks 0,1,2 and 3.

Supplemental Tables

Table S1. Properties of MAP polymer, MAP-CPT polymer-drug conjugate, and MAP-CPT nanoparticle.

Material	Property	
MAP polymer	d*n*/dc (mL/g)	0.14
	MW[a] (kDa)	68
	Polydispersity[b]	1.26
MAP-CPT conjugate	Wt % CPT	12.1
MAP-CPT nanoparticle	# conjugates/nanoparticle	2-3[2]

[a]MW, molecular weight determined as $(M_w + M_n)/2$; M_w, weight average molecular weight; M_n, number average molecular weight.
[b]Polydispersity determined as M_w/M_n.

Table S2. Nanoparticle formulations and characteristics.

Formulation	Nanoparticle diameter, pH 7.4, nm	Zeta potential, pH 7.4, mV
TfR-targeted CPT nanoparticle	31.6 ± 1.3	-1.07 ± 0.48
TfR-targeted Herceptin nanoparticle	34.1 ± 1.9	-0.38 ± 0.75
TfR-targeted combination CPT/Herceptin nanoparticle	29.8 ± 1.6	-1.22 ± 0.64

Data shown for hydrodynamic diameter and zeta potential are the average of 5 measurements ±1 SD.

Table S3. Antitumor efficacy in $Rag2^{-/-};Il2rg^{-/-}$ mice bearing human BT474-Gluc breast cancer metastatic brain tumors.

	Mean tumor volume (mm^3)	Median tumor volume (mm^3)	*P* vs. saline
Saline	89	89	-
CPT and Herceptin (4 and 24 mg/kg, respectively)	72	74	0.0087
TfR-targeted CPT nanoparticle (4 mg CPT/kg)	35	35	0.0022
TfR-targeted Herceptin nanoparticle (24 mg Herceptin/kg)	17	16	0.0022
TfR-targeted combination CPT/Herceptin nanoparticle (4 mg CPT/kg and 24 mg Herceptin/kg)	5	5	0.0022

Data provided are mean and median tumor volumes at the end of the study. *P* values were calculated using the Wilcoxon-Mann-Whitney test.

References

1. Wyatt, E.A., and Davis, M.E. (2019) Method of establishing breast cancer brain metastases affects brain uptake and efficacy of targeted, therapeutic nanoparticles. *Bioeng. Tranls. Med. 4*, 30-37.
2. Han, H., and Davis, M.E. (2013) Targeted nanoparticles assembled via complexation of boronic-acid-containing targeting moieties to diol-containing polymers. *Bioconjug. Chem. 24*, 669–677.