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pH-Responsive Theranostic Polymer-Caged Nanobins (PCNs): Enhanced Cytotoxicity and T_1 MRI Contrast by Her2-Targeting

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S1. Materials and Instrumentation.

a. Materials. Unless otherwise noted, all reagents and materials were purchased from commercial sources and used as received. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DOPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Doxorubicin (DXR) was purchased from PolyMed Therapeutics, Inc. (Houston, TX). ICP calibration standard solutions of phosphorus (10,085 μg/mL P) and gadolinium (10,012 μg/mL Gd), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDC·MeI), and all other reagents were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). Cholesterol-terminated poly(acrylic acid) (Chol-PAA) was prepared using the previously reported protocol.[S1] Ultrapure deionized water (18.2 MΩ cm resistivity) was obtained from a Millipore system (EMD Millipore, Billerica, MA).

b. Instrumentation. Phosphorus and gadolinium concentrations of the synthesized materials were determined using a Varian Vista MPX (Varian, Inc., Palo Alto, CA) simultaneous inductively coupled plasma optical emission spectrometer (ICP-OES). Intracellular concentrations of gadolinium atoms were measured using a Thermo X-Series II (Thermo Fisher Scientific, Waltham, MA) inductively coupled plasma mass spectrometer (ICP-MS) that is computer-controlled (Plasmalab software) and equipped with an ESI SC-2 autosampler (Thermo Fisher Scientific, Omaha, NE).

Polymer molecular weights were determined relative to polystyrene standards on a Varian PL-GPC 50 Plus (Varian, Inc., Palo Aalto, CA) gel-permeation chromatography (GPC) system equipped with Cirrus software, a PL-AS_RT GPC autosampler, both the RI and UV detectors, Agilent Resipore guard column, and Agilent Mesopore and Resipore columns (both 300 × 7.5 mm in size) in series. HPLC-grade chloroform was used as an eluent at a flow rate of 1.0 mL/min and the instrument was calibrated using polystyrene standards (Aldrich Chemical Co., 6 standards, 2,330-18700 Daltons).

Dynamic light scattering (DLS) and zeta potential measurements were performed on a Zetasizer Nano ZS (Marvern Instruments, Malvern, UK) equipped with a He-Ne laser (633 nm). Non-invasive backscatter method (detection at 173° scattering angle) was used. Correlation data were fitted, using the method of cumulants, to the logarithm of the correlation function, yielding the diffusion coefficient, *D*. The hydrodynamic diameters (D_H) of the bare liposomes (BLs) and PCNs were calculated using *D* and the Stokes-Einstein equation ($D_H = k_BT/3\pi\eta D$, where k_B is the Boltzmann constant, *T* is the absolute temperature, and η is the solvent viscosity (η = 0.8872 cP for water)). The polydispersity index (PDI) of liposomes—represented as $2c/b^2$, where *b* and *c* are first- and second-order coefficients, respectively, in a polynomial of a semi-log correlation function—was calculated by cumulants analysis. Size distribution of vesicles was obtained by non-negative least squares (NNLS) analysis.[S2] Unless noted otherwise, all samples were dispersed in 20 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffered saline (HBS) solution (pH 7.4, 150 mM NaCl) for the measurements. The data reported represent an average of five measurements with ten scans each.

Fourier-transformed nuclear magnetic resonance (NMR) spectroscopy of Chol-PAA was performed on a Varian INOVA-500 MHz spectrometer (Varian, Inc., Palo Alto, CA). Chemical shifts of ^{1}H NMR spectra are reported in ppm against residual solvent resonance as the internal standard ($CHCl_3$ = 7.27 ppm, CHD_2COCD_3 = 2.05 ppm, CHD_2OD = 3.31 ppm, D_2O = 4.8 ppm). Electrospray-ionization mass spectrometric (ESIMS) data were obtained on a Micromass Quattro II triple quadrupole mass spectrometer (Thermo Scientific., West Palm Beach, FL). UV-vis absorption spectra were obtained on either a CARY 300 Bio UV-vis spectrophotometer (Varian, Inc., Palo Alto, CA) or a Lambda 650 UV-vis spectrophotometer (PerkinElmer, Inc., Waltham, MA). Fluorescence emission spectra were obtained on a Jobin Yvon Fluorolog fluorometer (λ_{ex} = 480 nm, slith width = 3 nm for Doxorubicin).

S2. Synthesis

a. Preparation of DXR-loaded bare liposomes (BL_{DXR}). All liposomes were made from a mixture of DPPC/DOPG/cholesterol (56.3/3.6/40.1 mol% ratio). To a 50 mL-round bottom flask was added DPPC (79.5 mg), DOPG (5.5 mg), and cholesterol (29.9 mg), followed by chloroform (1.0 mL) to make a colorless solution. After 30 sec vortexing (Vortex Mixer, American Scientific Products), the solvent was removed by a rotary evaporator (BÜCHI, New Castle, DE). The resulting dry film was further dried under vacuum on a Schlenk line (30 mTorr) overnight. Next, the dry lipid films were hydrated in aqueous ammonium sulfate (6 mL of a 300 mM solution) followed by vigorous vortexing (3 min) and stirring (1 h) to form a dispersion of multilamellar vesicles (MLVs). After being subjected to 10 freeze-thaw cycles, the resulting dispersion of MLVs was extruded ten times through two stacked polycarbonate extrusion membranes (100-nm pore-size) that are maintained at 55 ^{o}C using LIPEX 10ml extruder (Northern Lipids, Burnaby, BC, Canada). The excess ammonium sulfate outside liposome was removed by gel-filtration chromatography with Sephadex G-50 (50 mL) that has been pre-equilibrated with 20 mM HBS solution. To the collected liposome solution (3 mL of a solution with 10.3 mM lipid concentration) was added doxorubicin (DXR, 0.35 equiv of the total lipid content) followed by incubation at 55 °C for 24 h. The excess non-encapsulated DXRs was then removed by gel-filtration chromatography with Sephadex G-50 (50 mL) that has been pre-equilibrated with 20 mM HBS solution.

b. Preparation of alkyne-modified, DXR-loaded polymer-caged nanobins (PCN_{DXR}). The DXR-loaded bare liposome (BL_{DXR}) was subsequently subjected to the PCN fabrication process as reported previously.[S3] An aqueous aliquot of BL_{DXR} (2 mL, 15.22 μmol lipid) was combined with an aqueous solution of Chol-PAA (1.52 μmol; M_n = 10.7 kDa and PDI = 1.12; 10 mol% relative to the lipid concentration) and stirred at room temperature for 24 h. To the resulting solution was added the alkyne-modified diamine cross-linker[S3] (40.32 μmol, 0.25 equiv to the total carboxylic acid groups for a theoretical 50% cross-linking) and EDC·MeI (161.28 μmol, 1.0 equiv to the total carboxylic acid groups) in 20 mM HBS solution (1 mL) followed by 4 h incubation. The PCN_{DXR} was purified from this mixture using a tangential flow filter (500 kDa MWCO, 20 cm^2 surface area, Spectrum Laboratories, Inc., Rancho Dominguez, CA) and 20 mM HBS solution (40 mL). The resulting alkyne-modified PCN_{DXR} can then be used directly in the conjugation with azide-modified Herceptin and gadolinium-tetraazacyclododecanetetra-acetic acid *(vide infra*). The lipid concentration was determined by phosphorous ICP-OES.

c. Preparation of Alexa Fluor 647-labeled, azide-modified Herceptin (azido-Herceptin-AF647). Herceptin (Trastuzumab) was obtained as a generous gift from Dr. Steven T. Rosen and the pharmacy at the Robert H. Lurie Comprehensive Cancer Center of Northwestern University. Herceptin (5-10 mg) in its injection buffer (22 mg mL^{-1} Herceptin, 0.495 mg mL^{-1} L-histidine HCl, 0.32 mg mL^{-1} L-histidine, 20 mg mL^{-1} α,α-trehalose dihydrate, and 0.09 mg mL^{-1} polysorbate 20 with 1.1% benzyl alcohol as a preservative at pH 6) was incubated with 5-fold molar excess of azido-$dPEG_4$-NHS ester crosslinker (Quanta Biodesign, Powell, OH) for 3 h at 37°C. The reaction was then incubated with an Alexa Fluor 647-NHS ester (1.2 molar excess of Herceptin; Invitrogen, Carlsbad, CA) for an additional 3 h at 37°C. The unconjugated Alexa Fluor 647 and azido-$dPEG_4$-NHS crosslinker were then removed by size exclusion chromatography using a Zeba Spin Column (7 kDa MWCO; Thermo Fisher Scientific, Waltham, MA) that has been pre-equilibrated with 20 mM HBS solution. Herceptin and Alexa Fluor 647 concentrations were then measured by UV-vis absorption spectroscopy, at 280 nm (ε = 210,000 M^{-1} cm^{-1}) and 650 nm (ε = 240,000 M^{-1} cm^{-1}), respectively. Herceptin concentration was then confirmed by BCA assay (Thermo Scientific Pierce, Rockford, IL).

d. Preparation of Herceptin- and gadolinium(III)-conjugated, DXR-loaded PCN (Her-Gd^{III}-PCN_{DXR}). To the as-prepared alkyne-modified PCN were conjugated azido-Herceptin-AF647 and 1-(3-azido-2-hydroxypropyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecyl-gadolinium(III) (Gd(III)HPN_3-DO3A)[S4] using copper(I)-catalyzed click ligation as reported previously.[S5] Briefly, azido-Herceptin-AF647 (9.80 μL, 79.38 μg), a mixture of $CuSO_4 \cdot 5H_2O$ (8.71 μL of a 50 mM solution in water), and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA; 5.45 μL of a 100 mM solution in water), an aminoguanidine hydrochloride solution (43.57 μL of a 100 mM solution in 20 mM HBS solution), and a sodium ascorbate solution (21.8 μL of a 100 mM solution in 20 mM HBS solution) were added sequentially to a solution containing the pre-prepared alkyne-modified PCN_{DXR} (1 mL, 7.84 μmol lipid). After 10 min incubation at room temperature, Gd(III)HPN_3-DO3A (3.14 μmol) and a sodium ascorbate solution (21.8 μL of a 100 mM solution in 20 mM HBS solution) were added to the resulting solution and then incubated at room temperature for 2 h. Additional sodium ascorbate (21.8 μL of a 100 mM solution in 20 mM HBS solution) was then added and the reaction was incubated for another 2 h before being purified by gel-filtration chromatography (to remove non-conjugated Herceptin) using a Sepharose CL-4B (15 mL) column that has been pre-equilibrated with 20 mM HBS solution. The collected milky red fraction (1 mL) was then further purified and concentrated using a tangential flow filter (500 kDa MWCO, 20 cm^2 surface area, Spectrum Laboratories, Inc., Rancho Dominguez, CA) and 20 mM HBS solution to remove non-conjugated Gd(III)HPN_3-DO3A. The hydrodynamic diameter of the resulting Her-Gd^{III}-PCN_{DXR} (D_H = 120 ± 20 nm) was measured by DLS. The Gd^{III}-to-lipid ratios were determined by ICP-OES.

e. Quantification of Herceptin conjugated to a PCN. The number of Herceptin conjugated per PCN was determined by UV-vis spectroscopy after proteolysis. Herceptin-conjugated PCN solution (80 μL) was incubated with Pronase solution

(Sigma-Aldrich Chemical Co., Milwaukee, WI; 40 μL, 10 mg mL^{-1}) at 42 °C for 4 h. Digested Herceptin was then separated from intact PCNs using an Amicon Ultra centrifugal filter (50 kDa MWCO; EMD Millipore, Billerica, MA). The concentration of Alexa Fluor647 was determined by measuring the 650 nm absorbance of the filtrate and was used as a proxy for the calculation of Herceptin concentration. The number of Herceptin antibodies conjugated to a PCN was calculated on the basis of the approximate number (80,000) of phospholipid molecules/liposome (100 nm in diameter).[S6]

S3. DXR-Release Assay from Her-Gd^{III}-PCN_{DXR}.

The PCN (Her-Gd^{III}-PCN_{DXR}; 1 mM lipid) was incubated in a 2 mL quartz SUPRASIL fluorescence cell (Hellma Cells Inc., Plainview, NY) containing either 20 mM acetate buffer (pH 5.0, 150 mM NaCl) or 20 mM HEPES buffer (pH 7.4, 150 mM NaCl) at 37 °C with magnetic stirring. While the fluorescence from the encapsulated DXR in the PCN was largely self-quenched due to its high concentration, the DXR released out of the PCN showed strong restored fluorescence which was measured as a function of incubation time.[S3] The DXR fluorescence (F_f) for the 100% DXR release value was measured after adding aqueous Triton X-100 (5 vol%, 10 μL) to totally break up the PCN. The drug release percentage (%) was calculated as a ratio of F_t/F_f where F_t is the DXR fluorescence at a specific time point.

S4. General Cell Culture Conditions

a. Media and Cell Culture Reagents. All BD-brand cell culture consumables (flasks, plates, and serological pipettes) were purchased from VWR Scientific (Radnor, PA). All media, buffers, serum, and dissociation reagents were purchased from Invitrogen (Carlsbad, CA).

b. Cell Lines and Conditions. SK-BR-3 and MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231-Her2 cells which stably express Her2 receptors, as well as transfection vector control cells (MDA-MB-231-Vector), were obtained as a generous gift from Dr. Vincent Cryns at the University of Wisconsin School of Medicine and Public Health.[S7] SK-BR-3 cells were cultured using McCoy's 5A medium supplemented with 10% FBS, 1.5 mM L-glutamine, penicillin (100 unit mL^{-1}), and streptomycin (0.1 mg mL^{-1}). MDA-MB-231, MDA-MB-231-Her2, and MDA-MB-231-Vector cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 vol% FBS, 4 mM L-glutamine, penicillin (100 unit mL^{-1}), and streptomycin (0.1 mg mL^{-1}). All cell culture experiments were carried out using the aforementioned cell-specific media in an incubator operating at 37 °C and in a humidified atmosphere containing 5.0 vol% CO_2. All cells were harvested using a TrypLE Express (1×) solution. Unless otherwise specified, all compounds/PCN *in vitro* incubations were carried out at 37 °C in an incubator with a humidified atmosphere containing 5.0 vol% CO_2.

S5. *In vitro* Cytotoxicity Assays

a. Dose-responsive cell viability. SK-BR-3, MDA-MB-231, MDA-MB-231-Her2, and MDA-MB-231-Vector cells (4×10^4 cells/well for SKBRs and 2×10^4 cells/well for the others) were plated in 48 well plates and incubated for 24 h. Media in the wells were then replaced with pre-prepared media containing the appropriate drug formulation (0.15 mL of solution at the appropriate DXR concentrations). After 24 h incubation at 37 °C, the cells were treated with a TrypLE Express (1×) solution (0.1 mL/well), harvested, transferred to 1.5 mL microcentrifuge tubes, and centrifuged at 1000 g for 5 minutes. After removing the supernatant via aspiration, the resulting cell pellets were washed in DPBS (3 × 0.1 mL/tube) and resuspended in media (0.1 mL/tube).

Cell viability was determined utilizing a Guava EasyCyte Mini flow cytometer (EMD Millipore, Billerica, MA). Guava assay distinguishes between viable and non-viable cells based on the differential cell membrane permeability of two DNA-binding dyes in the Guava ViaCount Reagent (EMD Millipore, Billerica, MA). In brief, the resulting resuspended cell solutions (0.1 mL) were mixed with Guava ViaCount reagent (0.3 mL) and allowed to stain for 5-20 min followed by acquisition of the data using the Guava EasyCyte Mini flow cytometer. The relative cell survival percentages compared to the DXR-free control were plotted against the drug concentration in logarithmic scale. The reported data represent an average of three measurements from different batches. The dose-effect profiles were obtained by Sigmoidal DoseResp fitting using OriginPro 8 (OriginLab, Northampton, MA) and the half-maximal inhibitory concentration (IC_{50}) values were determined on the basis of the fitted data.

Table S1. Half-maximum inhibitory concentration (IC_{50}) values of DXRs in different drug formulations against SK-BR-3, MDA-MB-231-Her2, MDA-MB231, and MDA-MB-231-Vector cells.

	IC_{50} [DXR] (μM)		
Cell lines	Free DXR	Gd^{III}-PCN_{DXR}	Her-Gd^{III}-PCN_{DXR}
SK-BR-3	2.57 ± 0.81	65.2 ± 1.05	4.47 ± 1.02
MDA-MB-231-Her2	2.32 ± 1.08	7.54 ± 1.12	3.36 ± 1.10
MDA-MB-231	2.08 ± 0.92	7.94 ± 1.02	8.32 ± 1.19
MDA-MB-231-Vector	2.28 ± 1.01	7.70 ± 0.89	7.76 ± 0.94

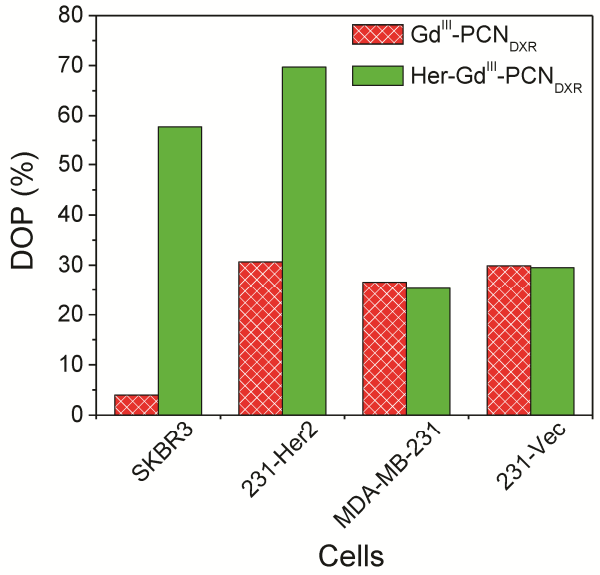

Figure S1. Relative degree of potentiation (DOP) of the encapsulated DXRs in the targeted (Her-Gd^{III}-PCN_{DXR}; red left bars) and the non-targeted PCNs (Gd^{III}-PCN_{DXR}; green right bars) compared to the free drug (calculated as [IC_{50} (free drug)/IC_{50} (drug in delivery vehicle)] × 100) for each cell line.

b. Cellular DXR-uptake measurement. SK-BR-3 cells (1×10^5 cells/well) were plated in 24 well plates and incubated for 24 h. Media in the wells were then replaced with pre-prepared media containing the appropriate drug formulation (0.25 mL of solution at the appropriate DXR concentrations). After 24 h incubation at 37 °C, the cells were treated with a TrypLE Express (1×) solution (0.1 mL/well), harvested, transferred to 1.5 mL microcentrifuge tubes, and centrifuged at 1000 g for 5 minutes. After removing the supernatant via aspiration, the resulting cell pellets were washed in DPBS (3 × 0.1 mL/tube) and resuspended in media (0.15 mL/tube). The cell suspension was mixed with aqueous Triton X-100 (10 vol%, 0.15 mL) and then shaken at 500 rpm and 37 °C for 23 h to completely decompose the cells and the internalized PCNs, followed by fluorescence measurement (excitation wavelength = 480 nm; emission wavelength = 588 nm; slit width = 10 nm). The total number of the collected cells was obtained with Guava ViaCount Assay.

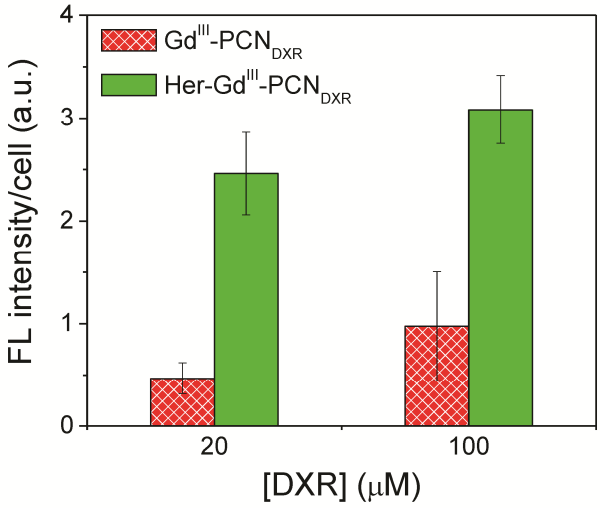

Figure S2. Relative cellular DXR-uptake profile of Gd^{III}-PCN_{DXR} (red left bars) and Her-Gd^{III}-PCN_{DXR} (green right bars) by SK-BR-3. Cells were incubated in media containing Gd^{III}-PCN_{DXR} and Her-Gd^{III}-PCN_{DXR} at given DXR concentration for 24 h at 37 °C.

S6. *In vitro* Celluar Gd^{III}-uptake Measurements

a. Gd^{III}-treated cells and their collection. Cellular Gd^{III}-uptake was quantified using an updated literature procedure.[S8] Specifically, SK-BR-3, MDA-MB-231, MDA-MB-231-Her2, and MDA-MB-231-Vector cells (1×10^5 cells/well for SK-BR-3 and 5×10^4 cells/well for the others) were plated in 24 well plates and incubated overnight. Cells were then incubated in the appropriate media with Her-Gd^{III}-PCN, Gd^{III}-PCN, or Gd^{III}-DOTA at 10 and 50 μM of Gd^{III} for 4 h and 24 h. Following incubation, cells were washed with DPBS (4 × 0.5 mL/well) and treated with a TrypLE Express (1×) solution (0.2 mL/well). The harvested cells were added to 1.5 mL microcentrifuge tubes and centrifuged at 1000 g for 5 min followed by removing the supernatant via aspiration. The resulting cell pellets were washed with DPBS (0.5 mL/tube) and then resuspended in media (0.2 mL). The total numbers of the collected cells were analyzed with a Countess Automated Cell Counter (Invitrogen, Carlsbad, CA) after staining with Trypan Blue (10 μL, 0.4%; Invitrogen, Carlsbad, CA).

b. Cellular Gd^{III}-uptake measurement by ICP-MS. Gd^{III} quantification was accomplished by carrying out ICP-MS analyses of acid digested samples. Specifically, ACS trace-metal-grade nitric acid (0.1 mL, ~70%; Sigma-Aldrich Chemical Co., Milwaukee, WI) was added to cell suspensions (0.15 mL) in 15 mL centrifugal tubes, vortexed for 10 sec, and placed at 70 °C for 4 h to allow for complete sample digestion. To the resulting digested solutions were added a multielement internal standard (15 μL, 1.0 ppm of In, Bi, Ho, ^{6}Li, Tb, Y, and Sc) and 18.2 MΩ cm deionized H_2O (2.735 mL) followed by 10 sec vortexing. Individual Gd^{III} elemental standards were prepared at 0.10, 0.50, 1.0, 5.0, 10, and 20 ppb concentrations with nitric acid (2.3 vol%) and internal standard (5.0 ppb). ICP-MS analyses were performed on a computer-controlled (Plasmalab software) Thermo X-Series II ICP-MS instrument (Thermo Fisher Scientific, Waltham, MA) equipped with an ESI SC-2 autosampler (Thermo Fisher Scientific, Omaha, NE). Each sample was acquired using 1 survey run (10 sweeps) and 3 main (peak jumping) runs (100 sweeps). The isotopes selected were ^{156,157}Gd, ^{115}In, and ^{165}Ho where indium and holmium were chosen as internal standards for data interpolation. The amount of cellular Gd^{III}-uptake was calculated by dividing the measured Gd^{III} amounts by the previously quantified number of cells.

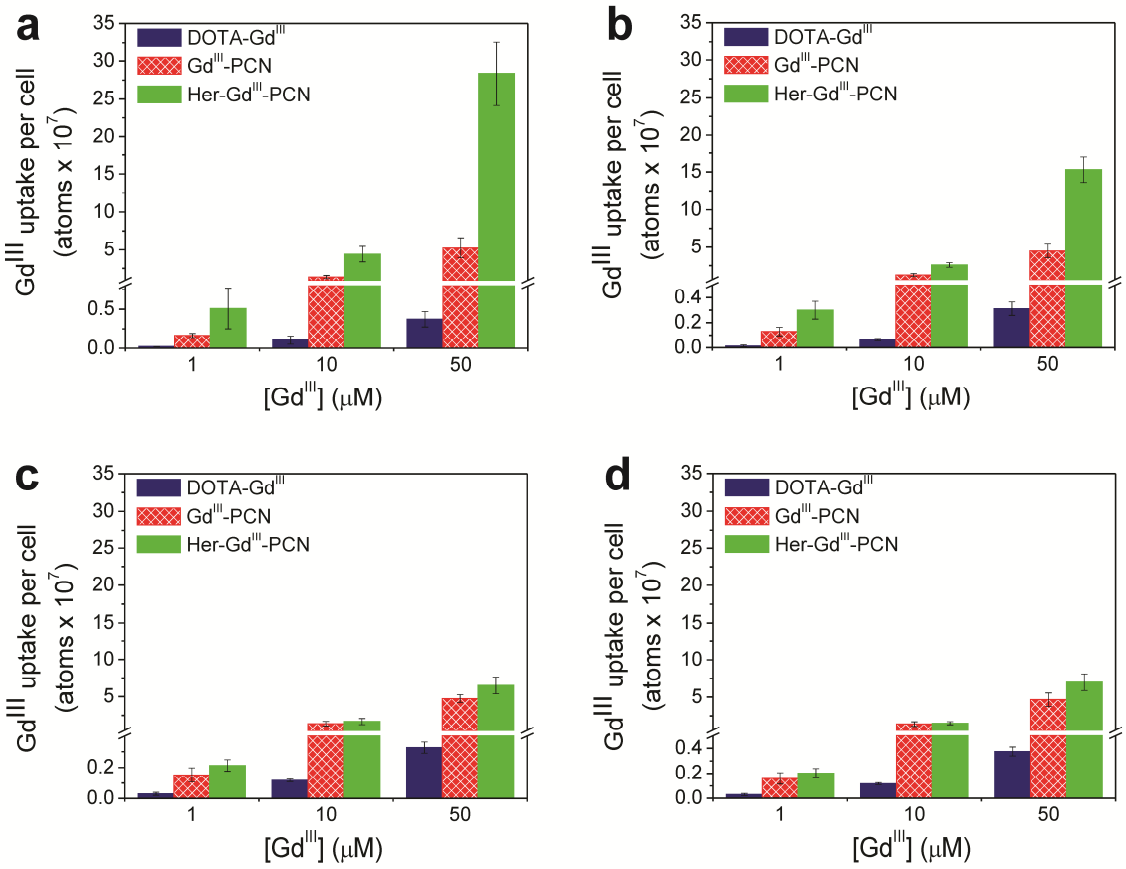

Figure S3. Cellular Gd^{III}-uptake profile of free DOTA-Gd^{III} (blue left bars), Gd^{III}-PCN (red middle bars), and Her-Gd^{III}-PCN (green right bars) by (a) SK-BR-3, (b) MDA-MB-231-Her2, (C) MDA-MB-231, and (d) MDA-MB-231-Vector cells. Cells were incubated in media containing free DOTA-Gd^{III}, Gd^{III}-PCN, or Her-Gd^{III}-PCN at given Gd^{III} concentration for 4 h at 37 °C.

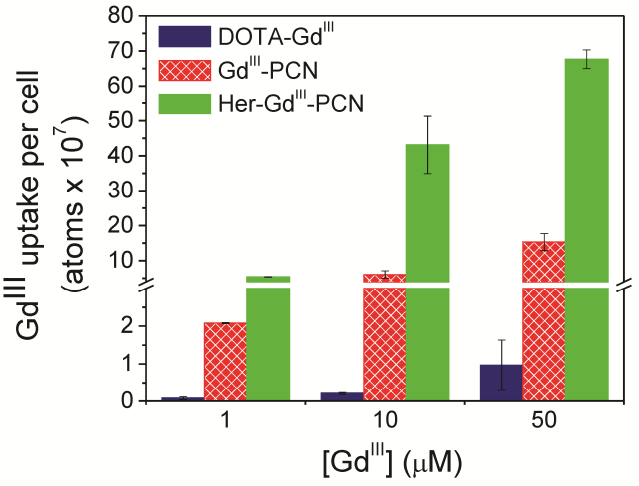

Figure S4. Cellular Gd^{III}-uptake profile of free DOTA-Gd^{III} (left blue bars), Gd^{III}-PCN_{DXR} (middle red bars), and Her-Gd^{III}-PCN_{DXR} (right green bars) by SK-BR-3 cells. Cells were incubated in media containing free DOTA-Gd^{III}, Gd^{III}-PCN_{DXR}, or Her-Gd^{III}-PCN_{DXR} at given Gd^{III} concentration for 24 h at 37 °C. We note that more than half of the cells were already decomposed by the time we analyzed the culture.

S7. MR Imaging and T_1 Analysis

For cell pellets MR imaging, SK-BR-3, MDA-MB-231-Her2, and MDA-MB-231-Vector cells (1×10^6 cells/well for all) were plated in 60 cm^2 cell culture dish and incubated overnight. Cells were then incubated in the appropriate media with Her-Gd^{III}-PCN, Gd^{III}-PCN, or Gd^{III}-DOTA at 50 μM of Gd^{III} for 24 h. Following incubation, cells were washed with DPBS (4 × 1 mL/dish) and treated with a TrypLE Express (1×) solution (1 mL/dish). The harvested cells were added to 15 mL centrifugal tubes and centrifuged at 1000 g for 5 min followed by removing the supernatant via aspiration. The resulting cell pellets were washed with DPBS (0.5 mL/tube) and then resuspended in media (1 mL). The total number of the collected cells was analyzed with a Countess Automated Cell Counter after treatment with Trypan Blue Stain (10 μL, 0.4%, Invitrogen, Carlsbad, CA). The resulting cell suspensions were added to 5¾″ flame-sealed Pasteur pipets and centrifuged at 100 g and 4.0 °C for 5 min. The bottoms of the flame-sealed pipets were cut away with a glass scribe to form small capillaries that were then imaged in a 23 mm mouse-head coil.

T_1- and T_2-weighted MR images as well as corresponding T_1 and T_2 measurements of SK-BR-3 cell pellets were acquired on a wide-bore BioSpec 9.40 T MR imager fitted with shielded gradient coils (Bruker BioSpin, Billerica, MA) using a RF RES 400 1H 075/040 quadrature transmit-receive volume coil (Bruker BioSpin, Billerica, MA) at 25 °C. Spin-lattice relaxation times (T_1) were measured using a rapid-acquisition rapid-echo (RARE) T_1-map pulse sequence, with static TE (10 ms) and variable TR (150, 250, 500, 750, 1000, 1500, 2500, 4000, 6000, 8000, and 10000 ms) values. Imaging parameters were as follows: field of view (FOV) = 25 × 25 mm^2, matrix size (MTX) = 256 × 256, number of axial slices = 4, slice thickness (SI) = 1.0 mm, averages (NEX) = 3, acquisition time = 2 h 46 min, and RARE Factor = 2. T_1 analysis was carried out using the image-sequence analysis tool in Paravision 5.0 pl3 software (Bruker BioSpin, Billerica, MA) with mono-exponential curve-fitting of image intensities of selected regions of interest (ROIs) for each axial slice. Spin-spin relaxation times (T_2) were measured using a multi-slice multi-echo (MSME) T_2-map pulse sequence, with static TR (5000 ms) and 64 fitted echoes in 10 ms intervals (10, 20,..., 640 ms). Imaging parameters were as follows: field of view (FOV) = 25 × 25 mm^2, matrix size (MTX) = 256 × 256, number of axial slices = 4, slice thickness (SI) = 1.0 mm, averages (NEX) = 3, and acquisition time = 48 min. T_2 analysis was carried out using the image-sequence analysis tool in Paravision 5.0 pl3 software (Bruker BioSpin, Billerica, MA) with mono-exponential curve-fitting of image intensities of selected regions of interest (ROIs) for each axial slice.

T_1-weighted MR images of MDA-MB-231-Her2 and MDA-MB-231-Vector cell pellets were acquired on an 89 mm bore size PharmaScan 7.05 T MR imager fitted with shielded gradient coils (Bruker BioSpin, Billerica, MA) using a RF RES 300 1H 089/038 quadrature transmit-receive volume coil (Bruker BioSpin, Billerica, MA) at 25 °C. Images were acquired using a rapid-acquisition rapid-echo (RARE) pulse sequence. Imaging parameters were as follows: TE = 8.075 ms, TR = 250, field of view (FOV) = 25 × 25 mm^2, matrix size (MTX) = 128 × 128, number of axial slices = 3, slice thickness (SI) = 1.0 mm, and averages (NEX) = 1.

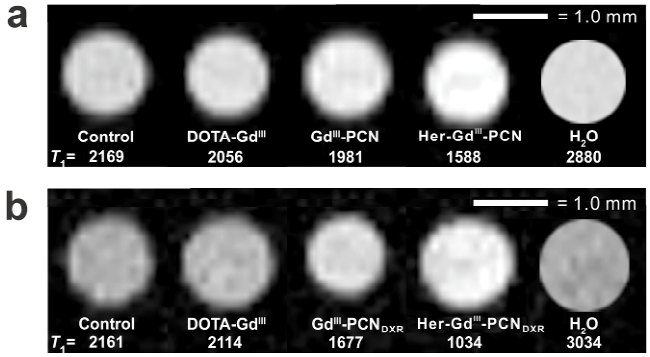

Figure S5. T_1-weighted MR images (at 9.4 T (400 MHz) and 25 °C) of SK-BR-3 cells incubated for 24 h with media alone (control), or with Gd-containing ([Gd^{III}] = 50 μM) formulations (free DOTA-Gd^{III}, Gd^{III}-PCN, or Her-Gd^{III}-PCN) , with (a) or without (b) DXR. This image is the gray-scale version of the color-mapped Figure 3 in the main text.

Table S2. Cellular Gd^{III} content and T_1 values (as well as SNR and CNR values) of SK-BR-3 cell pellets from the MR images shown in Figure S5a. Error in table represents ± one standard deviation of the mean of 4 MR slices (thickness = 1.0 mm).

	Cellular Gd^{III}-uptake (atoms × 10^7 per cell)	T_1 (ms)	SNR[1]	CNR[2] (versus DOTA-Gd^{III}-treated cells)
Untreated Cells	–	2428 ± 199	61.88 ± 4.93	-4.31 ± 0.33
DOTA-Gd^{III}	2.8	2181 ± 123	66.02 ± 4.67	–
Gd^{III}-PCN	11.8	2166 ± 186	69.21 ± 6.33	3.20 ± 0.29
Gd^{III}-Her2-PCN	51.1	1671 ± 79	76.36 ± 5.05	10.35 ± 0.68
H_2O	–	2972 ± 68	64.79 ± 4.89	-1.22 ± 0.092

[1] Signal-to-noise (SNR) ratios calculated from image acquired in Figure S5a (TR = 500 ms and TE = 10 ms) and is an average of 4 slices.
[2] Contrast-to-noise (CNR) ratios were calculated versus cells incubated with DOTA-Gd^{III} using the image acquired in Figure S5a (TR = 500 ms and TE = 10 ms) and is an average of 4 slices.

Table S3. Cellular Gd^{III} content and T_1 values (as well as SNR and CNR values) of SK-BR-3 cell pellets from the MR images shown in Figure S5b. Error in table represents ± one standard deviation of the mean of 4 MR slices (thickness = 1.0 mm).

	Cellular Gd^{III}-uptake (atoms × 10^7 per cell)	T_1 (ms)	SNR[1]	CNR[2] (versus DOTA-Gd^{III}-treated cells)
Untreated Cells	–	2121 ± 114	27.78 ± 6.98	1.01 ± 0.25
DOTA-Gd^{III}	2.3	2043 ± 107	26.77 ± 2.86	–
Gd^{III}-PCN	17.5	1633 ± 103	26.27 ± 2.39	3.18 ± 0.25
Gd^{III}-Her2-PCN	73.2	1056 ± 189	30.04 ± 8.77	5.11 ± 0.93
H_2O	–	2992 ± 81.1	22.48 ± 0.91	-2.45 ± 0.099

[1] Signal-to-noise (SNR) ratios calculated from image acquired in Figure S5b (TR = 500 ms and TE = 10 ms) and is an average of 3 slices.
[2] Contrast-to-noise (CNR) ratios were calculated versus cells incubated with DOTA-Gd^{III} using the image acquired in Figure S5b (TR = 500 ms and TE = 10 ms) and is an average of 3 slices.

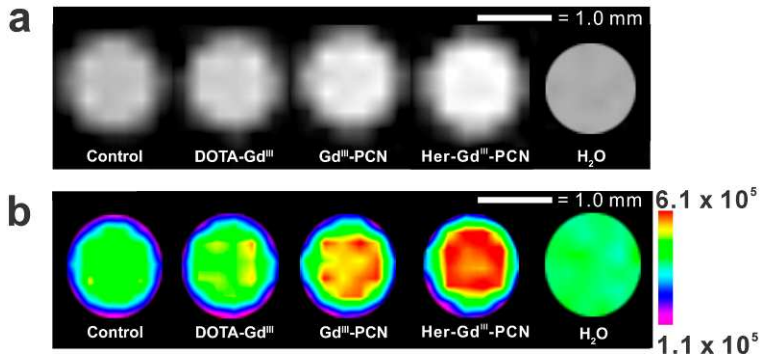

Figure S6. (a) T_1-weighted MR images (at 7 T and 25 °C) of MDA-MB-231-Her2 cells incubated for 24 h with media alone (control) or with Gd-containing ([Gd^{III}] = 50 μM) formulations (free DOTA-Gd^{III}, Gd^{III}-PCN, or Her-Gd^{III}-PCN). (b) The corresponding image-intensity color map for panel (a) where the maximum and minimum signal intensities are shown in the color-mapped calibration bar on the right side.

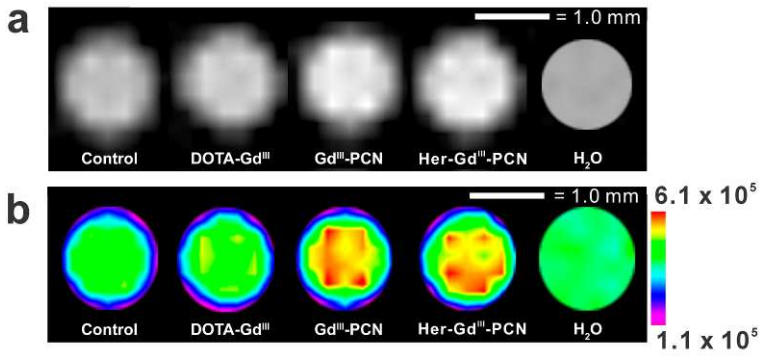

Figure S7. (a) T_1-weighted MR images (at 7 T and 25 °C) of MDA-MB-231 cells incubated for 24 h with media alone (control) or with Gd-containing ([Gd^{III}] = 50 μM) formulations (free DOTA-Gd^{III}, Gd^{III}-PCN, or Her-Gd^{III}-PCN). (b) The corresponding image-intensity color map for panel (a) where the maximum and minimum signal intensities are shown in the color-mapped calibration bar on the right side.

Author Contributions

B.J.H., R.W.A., and S.T.N. conceived the experiments presented herein. A.J.C. synthesized and characterized Chol-PAA polymers. P.L.H synthesized and characterized azido-Herceptin-AF647. D.J.M synthesized and characterized Gd(III)HPN_3-DO3A. B.J.H. synthesized and characterized Her2-targeted and non-targeted PCNs. B.J.H and E.P.S. did cellular cytotoxicity and Gd^{III}-uptake studies. B.J.H and K.W.M. did in vitro MR imaging studies. T.V.O. supervised E.P.S. and P.L.H. and T.J.M supervised K.W.M. S.T.N. supervised the project. B.J.H. wrote the initial draft of the paper with inputs from E.P.S. and K.W.M. B.J.H. and S.T.N. finalized the manuscript. All authors have given approval to the final version of the manuscript.

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