

MATERIALS AND METHODS

Materials and Equipment

Phospholipids were purchased from Avanti Polar Lipids, compounds **2** and **5** were supplied by Click Chemistry Tools, and compound **7** was supplied by Macrocylics. The dye DiI12(5)-DS (1,1-diododecyl-3,3,3,3-tetramethyl-indodicarbocyanine-5,5-disulfonic acid) was purchased from AAT Bioquest. All other reagents were purchased from Sigma-Aldrich. All chemicals were used without further purification. Column chromatography was performed on silica gel (SiliCycle Inc., 40–63 μm , 230–400 mesh). ^1H -nuclear MR (NMR) spectra were recorded at room temperature on a Bruker Avance 500 instrument operating at the frequency of 500 MHz. All were internally referenced to the residual solvent peaks, CDCl_3 (7.26 ppm), dimethyl sulfoxide (DMSO)- d_6 (2.49 ppm), or CD_3OD (3.31 ppm). High-resolution mass data were recorded on a Waters LCT Premier XE mass spectrometer.

Radiochemistry

^{89}Zr was produced at Memorial Sloan-Kettering Cancer Center on a TR19/9 variable-beam energy cyclotron (Ebco Industries Inc.) via the $^{89}\text{Y}(p,n)^{89}\text{Zr}$ reaction and purified in accordance with previously reported methods to yield ^{89}Zr with a specific activity of 195–497 MBq/ μg (*1*). Activity was measured using a CRC-15R dose calibrator (Capintec).

High-Performance Liquid Chromatography (HPLC) and Radio-HPLC

HPLC was performed on a Shimadzu HPLC system equipped with 2 LC-10AT pumps and an SPD-M10AVP photodiode array detector. Radio-HPLC was performed using the same Shimadzu system additionally equipped with a Lablogic Scan-RAM Radio-TLC/HPLC detector. Analytic runs were performed on either a C18 Waters Atlantis T3 column (6 \times 250 mm, 5 μm) or a C4 Phenomenex Jupiter column (4.6 \times 250 mm, 5 μm). The solvent systems used were water (0.1% trifluoroacetic acid, solvent A), acetonitrile (0.1% trifluoroacetic acid, solvent B), and methanol/acetonitrile 60:40 (0.1% trifluoroacetic acid, solvent C) with a flow rate of 1 mL/min. Size-exclusion chromatography was performed on a Superdex 10/300 column (GE Healthcare Life Sciences) using phosphate-buffered saline (PBS) as eluent at a flow rate of 1 mL/min.

Cell Culture

The mouse breast cancer cell line 4T1 was obtained from ATCC and grown in Dulbecco modified Eagle medium with a 4.5 g/L concentration of L-glucose, 10% (v/v) heat-inactivated fetal bovine serum, 100 IU of penicillin, and a 100 $\mu\text{g}/\text{mL}$ concentration of streptomycin and purchased from the culture medium preparation facility at Memorial Sloan-Kettering Cancer Center.

Animals

Female homozygous athymic nude NCr mice were obtained from Taconic Laboratories. For xenograft injections, mice were anesthetized with 1%–2% isoflurane gas in 2 L of medical air per minute, before 4T1 cells were injected (1×10^6 cells in 100 μL of Dulbecco modified Eagle medium) subcutaneously and the tumors grown for 10–12 d. For orthotopic injections, the mice were anesthetized with a 150 mg/kg dose of ketamine and a 15 mg/kg xylazine cocktail (10 μL), and an incision was made above the mammary fat pad after sterilization of the region. Then, 4T1 cells (1×10^6 cells in 100 μL of Dulbecco modified Eagle medium) were injected into the mammary fat pad, before the incision was sealed (Vetbond; 3M) and the tumors grown for 8 d. For all intravenous injections,

the mice were gently warmed with a heat lamp and placed on a restrainer, the tail was sterilized with alcohol pads, and the injection was made into the lateral tail vein. All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center and followed National Institutes of Health guidelines for animal welfare.

Serum Stability

A sample of the corresponding radiolabeled liposomal preparation (typically 1.9–2.6 MBq in 40–60 μL of PBS) was added to 400 μL of fetal bovine serum. The mixture was incubated at 37°C for 5 d. Aliquots of 0.3–0.4 MBq were taken at predetermined time points for size-exclusion chromatography analysis by careful integration of the peaks.

Blood Half-Life

Healthy female NCr mice (8–10 wk old and 15–20 g in weight, $n = 6$) were injected with 0.19 ± 0.02 MBq (range, 0.16–0.21 MBq; 3–4 μmol of lipid) of liposome preparation in 200 μL of PBS solution. Blood was sampled from the saphenous vein at predetermined time points (5 min, 30 min, 2 h, 6 h, 20 h, and 26 h), and radioactivity was measured on a Wizard 2470 Automatic Gamma Counter (Perkin Elmer). Measurements were performed in triplicate, and the radioactivity content was calculated as the mean %ID/g \pm SD.

Biodistribution Studies

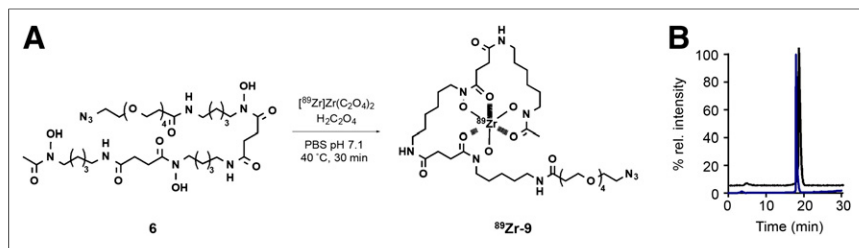
Biodistribution experiments were conducted on female NCr nude mice (6–10 wk old and 15–20 g in weight, $n = 21$) bearing 4T1 breast xenografts. The radiolabeled liposome preparation (0.65 ± 0.03 MBq; range, 0.60–0.70 MBq; 0.4–0.6 μmol of lipid, in 200 μL of PBS solution) was administered via the lateral tail vein and allowed to circulate for various times (2, 24, and 48 h), after which the mice were sacrificed and the organs perfused. The radioactive content in tissues of interest, (blood, tumor, large and small intestines, stomach, kidneys, brain, bone, liver, lungs, heart, skin, spleen, bladder, and tail) was measured using a Wizard 2470 Automatic Gamma Counter, and the tissue-associated activity was calculated as the mean %ID/g \pm SD.

Autoradiography

After sacrifice, liver, spleen, tumor, and muscle tissues were excised and embedded in optimal-cutting-temperature mounting medium (Sakura Finetek) and frozen on dry ice, and a series of 10- μm frozen sections was cut. To determine radiotracer distribution, digital autoradiography was performed by placing tissue sections in a film cassette against a phosphor imaging plate (BASMS-2325; Fujifilm) for 48 h at -20°C . Phosphor imaging plates were read at a pixel resolution of 25 μm with a Typhoon 7000IP plate reader (GE Healthcare). After autoradiographic exposure, the same frozen sections were then used for immunohistochemical staining and imaging.

Staining and Microscopy

Tissue sections (10 μm , frozen) were stained for Iba1 with anti-Iba1 rabbit polyclonal antibody (Wako) followed by a biotinylated goat antirabbit secondary antibody (VECTASTAIN ABC kit; Vector Labs), followed by Alexafluor 568-tyramide for fluorescent signal (VECTASTAIN ABC kit). Additional 4,6-diamino-2-phenylindole (DAPI) staining was performed using 4',6-diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich). All sections were counterstained with hematoxylin and eosin solution. All images were obtained using an EVOS FL Auto digital inverted fluorescence microscope (Life Technologies). Fluorescent images were obtained at the $\times 4$ objective, whereas bright-field images were obtained at both the $\times 4$ and the $\times 20$ objectives. On stained sections, Iba1 fluorescence was observed using a Texas Red light cube (excitation [Ex], 585/29; emission



SUPPLEMENTAL FIGURE 3. (A) Radiosynthesis of ^{89}Zr -9. (B) HPLC chromatogram showing ultraviolet (absorption at 220 nm, front) and radioactive (back) traces of mixture of ^{89}Zr -9 and reference compound 9, demonstrating coelution.

[Em], 624/40; EVOS LED light cube; Life Technologies), and DAPI fluorescence was observed using a DAPI light cube (Ex, 357/44; Em, 447/60; EVOS LED light cube). On sections containing DiIC, fluorescence was observed using a Cy5 light cube (Ex, 628/40; Em, 692/40; EVOS LED light cube).

PET/CT Imaging

Female nude NCr mice (8–10 wk old, $n = 8$) bearing 4T1 breast tumors were injected with 9.3 ± 1.5 MBq (range, 7.8–11.5 MBq) of ^{89}Zr -liposomes (3–4 μmol of lipid) in 200–250 μL of PBS solution via the lateral tail vein. At predetermined time points (2, 24, 48, and 120 h), the animals were anesthetized with a mixture of isoflurane (Baxter Healthcare) and oxygen gas (2% for induction and 1% for maintenance), and scans were then obtained using an Inveon PET/CT scanner (Siemens Healthcare Global). Whole-body PET static scans recording a minimum of 50 million coincident events were performed, with a duration of 10–20 min. The energy and coincidence timing windows were 350–700 keV and 6 ns, respectively. The image data were normalized to correct for nonuniformity of response of the PET, dead-time count losses, positron branching ratio, and physical decay to the time of injection, but no attenuation, scatter, or partial-volume averaging correction was applied. The counting rates in the reconstructed images were converted to activity concentrations (%ID/g of tissue) by use of a system calibration factor derived from the imaging of a mouse-sized water-equivalent phantom containing ^{89}Zr . Images were analyzed using ASI-Pro VMTM software (Concorde Microsystems). Activity concentration was quantified by averaging the maximum values in at least 5 regions of interest drawn on adjacent slices of the tissue of interest (2). Whole-body standard low-magnification CT scans were obtained with the x-ray tube setup at a voltage of 80 kV and current of 500 μA . The CT scan was acquired using 120 rotational steps for a total of 220°, yielding an estimated scan time of 120 s with an exposure of 145 ms per frame.

Near-Infrared Imaging

Fluorescence imaging was performed on an IVIS Spectrum (Caliper) system (Perkin Elmer). Fluorescence images were acquired with excitation and emission wavelengths of 650 and 670 nm, respectively, and using auto acquisition times. Data were quantified as radiant efficiency.

Preparation of Liposomes

All liposome preparations used in the present work were obtained by the sonication method. Briefly, a lipid film was prepared by evaporating a chloroform solution containing the corresponding lipids in the desired proportion. The resulting film was hydrated with PBS (typically 10 mL) and sonicated for 25 min using a Biologics, Inc., 150 V/T Ultrasonic Homogenizer working at 30% power output. After quick centrifugation, size and Z-potential measurements were performed on a Malvern NanoSeries Z-Sizer and a Zeta PALS analyzer (Brookhaven Instruments Corp.), respectively. Liposomes containing the synthesized lipids were concentrated using a Millipore 100-kDa VivaSpin tube and washed twice with PBS.

Ready-to-label fluorescent liposome, DiIC@DFO-L, was prepared by addition of 1% of the fluorophore 1,1-diododecyl-3,3,3-tetramethylindodicarbocyanine-5,5-disulfonic acid (DiIC12 (5)-DS; excitation, 650 nm; emission, 670 nm) and 0.3% DSPE-DFO to the initial lipid mix at the expense of DPPC. After the sonication step, the liposome was concentrated by spin filtration (Vivaspin 100-kDa molecular-weight-cut-off tube) and washed with PBS. No free dye was found in the filtrate, suggesting a complete incorporation to the liposome. The binding stability of the dye to the liposome in PBS was assessed by

size-exclusion chromatography and was found to be excellent (>99%) over a period of 5 mo.

Synthesis of 3-(((23-(11,12-dehydrodibenzo[b,f]azocin-5(6H)-yl)-4,20,23-trioxo-7,10,13,16-tetraoxa-3,19-diazatricosyl)oxy)(hydroxyphosphoryl)oxy)propane-1,2-diyl distearate (3)

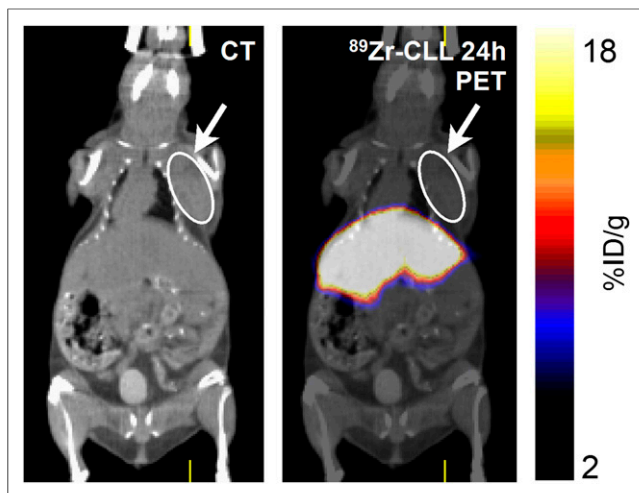
A solution of DBCO *N*-hydroxysuccinimide ester (2, 12.8 mg, 19.7 μmol), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE, 1, 16.2 mg, 21.7 μmol), and diisopropylethyl amine (5.4 μL) in dry dichloromethane (2 mL) was prepared in a round-bottom flask equipped with a condenser. The system was purged with nitrogen, and the mixture was stirred at 40°C for 15 h. The resulting solution was chromatographed on silica gel, using gradient elution from neat dichloromethane to dichloromethane/methanol 5:1 to obtain the desired product as a pale yellow solid (14.4 mg, 57% yield). $^1\text{H-NMR}$ (CDCl_3): 0.90 (t, 6H), 1.26 (bs, 56H), 1.60 (bs, 4H), 2.31 (t, 4H), 2.54 (m, 4H), 3.32 (m, 2H), 3.47 (m, 4H), 3.58 (m, 12H), 3.57 (d, 1H), 3.71 (t, 2H), 3.99 (bs, 4H), 4.10 (m, 1H), 4.36 (m, 1H), 5.07 (d, 1H), 5.15 (m, 1H), 7.23 (m, 2H), 7.29 (m, 2H), 7.36 (m, 3H), 7.58 (m, 1H), 7.64 (m, 1H), 11.3 (bs, 1H). High-resolution mass spectrometry time-of-flight electrospray ionization (HRMS TOF ES) [$\text{M}]^-$: m/z 1280.8057 (calculated for $\text{C}_{71}\text{H}_{115}\text{N}_3\text{O}_{15}\text{P}$ 1280.8066).

Synthesis of *N*¹-(1-azido-15-oxo-3,6,9,12-tetraoxa-16-azahenicosan-21-yl)-*N*¹-hydroxy-*N*⁴-(5-(*N*-hydroxy-4-((5-(*N*-hydroxyacetamido)pentyl)amino)-4-oxobutan-amido)pentyl)-succinamide (6)

A suspension of DFO mesylate (5, 33.8 mg, 51 μmol), PEG₄-azide *N*-hydroxysuccinimide ester (4, 20 mg, 51 μmol), and diisopropylethyl amine (15.0 μL) in dry dimethylformamide (0.7 mL) was stirred at 40°C–45°C for 7 h under nitrogen. After cooling to room temperature, diethyl ether (1 mL) was added and the mixture was kept at 4°C for another hour. The solid was then filtered and washed thoroughly with methanol to furnish the pure product as a white solid (6.5 mg, 15% yield). $^1\text{H-NMR}$ (CD_3OD): 1.31 (m, 6H), 1.48 (m, 6H), 1.59 (m, 6H), 2.05 (s, 3H), 2.40 (m, 6H), 2.72 (m, 4H), 3.12 (m, 6H), 3.39 (t, 2H), 3.56 (m, 6H), 3.61 (m, 12H), 3.63 (m, 2H), 3.68 (t, 2H). HRMS TOF ES [$\text{M}+\text{Na}]^+$: m/z 856.4724 (calculated for $\text{C}_{36}\text{H}_{67}\text{N}_9\text{O}_{13}\text{Na}$ 856.4756).

Synthesis of 3-((hydroxy(2-(3-(4-(3-(3,14,25-trihydroxy-2,10,13,21,24-pentaaxo-3,9,14,20,25-pentaazatri-ocantan-30-yl)thioureido)phenyl)thiourea)ethoxy)phosphoryl)oxy)propane-1,2-diyl distearate (8)

To a solution of DFO-NCS (7, 6.0 mg, 8.0 μmol) in dimethyl sulfide (0.5 mL) was added 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE, 1, 9.9 mg, 13.2 μmol), chloroform (0.5 mL), and diisopropylethyl amine (20.0 μL). The mixture was stirred at 50°C under nitrogen for 3 d, after which chloroform was evaporated and 1 M Tris was added (0.5 mL). Half an hour later, the suspension was filtered and the resulting solid washed with 1 M Tris (2 \times 1 mL), water (3 \times 1 mL), and dichloromethane (3 \times 1 mL). The white solid was dried to afford the title product (11.1 mg, 85%). $^1\text{H-NMR}$ (Tris salt, $\text{CDCl}_3/\text{DMSO}-d_6$): 0.91



SUPPLEMENTAL FIGURE 4. PET/CT imaging of ^{89}Zr -CLL showing CT only (left) and PET/CT fusion (right) at 24 h after injection. Arrow indicates location of tumor.

(t, 6H), 1.25 (bs, 62H), 1.37 (m, 4H), 1.52 (m, 12H), 1.99 (s, 3H), 2.22 (m, 4H), 2.35 (m, 4H), 2.64 (m, 4H), 3.04 (m, 4H), 3.47 (bs, 8H), 3.51 (s, 6H), 3.68 (m, 2H), 3.82 (bs, 2H), 3.88 (bs, 2H), 4.05 (m, 1H), 4.28 (m, 1H), 5.07 (m, 1H), 5.10 (bs, 3H), 7.22 (d, 2H), 7.34 (bs, 3H), 7.59 (s, 1H), 7.66 (bs, 4H), 7.72 (bs, 1H), 9.23 (s, 1H), 9.50 (bs, 1H), 9.69 (s, 1H), 9.76 (s, 1H), 9.82 (bs, 1H). HRMS fast atom bombardment $[\text{M-H}]^-$: m/z 1498.8977 (calculated for $\text{C}_{74}\text{H}_{133}\text{N}_9\text{O}_{16}\text{PS}_2$ 1498.9049).

Synthesis of ^{89}Zr -9

Precursor **6** (5–10 μg in DMSO) was dissolved in PBS (200 μL). Activity (^{89}Zr -oxalate in 1 M oxalic acid) was added, followed by addition of an equal volume of 1 M sodium carbonate to adjust pH to 7.1–7.4. The solution was vortex-mixed and reacted at 40°C for 30 min. The product was purified by HPLC using a C4 Phenomenex Jupiter column, using solvents A and C and gradient elution from 5% to 100% C over 27 min. The retention time of ^{89}Zr -9 was 16.1 min, and its identity was established by coelution with the reference cold

compound (Supplemental Figure 3B). The radiochemical yield was $44\% \pm 15\%$ ($n = 7$), and the radiochemical purity was more than 98%. The collected fraction containing ^{89}Zr -9 was concentrated to dryness in vacuo and used in the following step.

Synthesis of ^{89}Zr -CLL

Over the isolated ^{89}Zr -9 (18–37 MBq), a solution of DBCO-L (typically 0.5–1.0 mL, 5–10 μmol of lipid) in PBS was added. A 1 M sodium carbonate solution was used to adjust pH to 6.8–7.4. The mixture was sonicated and shaken at 400 rpm at 30°C in a ThermoMixer (Eppendorf) for 16 h and finally loaded onto a 100-kDa nominal molecular weight limit Amicon centrifugal filter (Millipore). The solution was concentrated by centrifugation at 4,000g and washed at least twice with 0.5 mL of PBS, until the activity in the filtrate was constant. The resulting concentrate was diluted with PBS to the desired volume. The radiochemical yield was $14\% \pm 4\%$ ($n = 3$), and the radiochemical purity was more than 95%, as established by size-exclusion chromatography. Dynamic light scattering was also measured.

Synthesis of ^{89}Zr -SCL

To a solution of DFO-L (typically 0.5–1.0 mL, 5–10 μmol of lipid) in PBS was added ^{89}Zr -oxalate in 1 M oxalic acid (18–37 MBq), followed by addition of an equal volume of 1 M sodium carbonate to adjust pH to 7.1–7.4. The mixture was shaken at 400 rpm at 30°C in a ThermoMixer for 4 h and finally loaded onto a 100-kDa NMWL Amicon centrifugal filter (Millipore). The solution was concentrated by centrifugation at 4,000g and washed at least twice with 0.5 mL of PBS, until the activity in the filtrate was constant. The resulting concentrate was diluted with PBS to the desired volume. The radiochemical yield was $80\% \pm 10\%$ ($n = 7$), and the radiochemical purity was more than 99%, as established by size-exclusion chromatography. Dynamic light scattering was also measured.

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