SUPPLEMENTAL MATERIALS AND METHODS

Dorsal Skin Fold Window Chamber

A circular incision in the dorsal skin fold was excised, along with corresponding fascia, and a window chamber was surgically implanted. For FaDu tumor injection, 15-25µL of single-cell suspension tumor cells at concentration of 8x10^6 cells/mL were injected into the opposing skin fold. A circular glass coverslip was placed over the incision for later imaging. Imaging studies were performed 7-14 days following surgery, when the tumors were approximately 2mm in diameter and perfused tumor vessels were apparent.

Liposome Preparation

This study utilized two different liposome formulations, the primary TSL formulation, used for evaluating drug kinetics and penetration, and a second formulation prepared in Rotterdam, Netherlands to corroborate intravascular release of drug in eNOS-GFP mice. The first liposome preparation consisted of 99.9mol% of DPPC, MSPC, and DSPE-PEG2000 with corresponding mole percentages of 85:9.8:5.2, along with 0.5mol% fluorescein DHPE. TSLs were similarly prepared at the Erasmus Medical Center, Netherlands, but with the mole percentages 90:10:4 (DPPC:MSPC:DSPE-PEG2000). These phospholipids were purchased from Avanti Polar Lipids Inc (Alabaster, AL), with corresponding chemical nomenclature 1,2-dipalmitoyl-sn-glycerol-3-phosphocholine (DPPC), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (MSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000). The fluorescein DHPE was purchased from Invitrogen, Inc as N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt. The phospholipids were massed and dissolved
together in chloroform and methanol (4:1 volume ratio) in a small round-bottom flask, and the solvent was removed using a roto-evaporator (Buchi Rotovapor: R-124 roto evaporator, Flawil, Switzerland) under a vacuum for one hour. The lipid film was then dried overnight under a vacuum. The dried lipids were hydrated at 55°C for 30min with citrate buffer (300mM, pH 4) containing 1µM of dissolved MSPC to a lipid concentration of 100mg/mL. The hydrated lipid vesicles were extruded 2 times through a polycarbonate membrane of 200nm pore size and then 8 times through a polycarbonate membrane of 100nm pore size at 55°C and 400psi nitrogen pressure (approximately 25atm) by using a water-jacketed extruder (Northern Lipids Inc, Vancouver, Canada). This procedure resulted in the formation of unilamellar liposomes averaging about 116nm +/- 3nm in diameter. Following extrusion, the liposomes were immediately placed into 4°C until doxorubicin loading.

Doxorubicin loading was achieved by the remote pH gradient method (33). The extruded liposome formulation was pH adjusted to 7.4 through the addition of Sodium Carbonate buffer (0.5M, pH 11). Doxorubicin (5mg/mL) was combined in a ratio of 10mg lipid: 0.5mg doxorubicin and further incubated for 30min, with gentle agitation every 5min. Following loading, the liposomes were immediately transferred to a pre-cooled glass tube on ice, left to rest for 10min on ice followed by 5min at room temperature. The remaining unencapsulated doxorubicin was removed from the liposome samples by gel filtration using Sephadex G-50. Liposomes were added to Sephadex G-50 columns (prepared by centrifuging the solution of Sephadex G-50 in HEPES buffer (pH 7.4) at 1100g for 12min at room temperature) and centrifuged for 10min at 780g (Legend Sorval X1R, Thermo Scientific, Germany). Sephadex column separation was repeated twice. Final concentration of the liposome-encapsulated doxorubicin was estimated using the spectrofluorophotometer (RF-1501, Shimadzu, Japan) with
5μL of Triton X-100 (10% aqueous solution) in 1mL HEPES buffer. The fluorescence was compared with a doxorubicin standard concentration curve with excitation and emission wavelengths of 480 and 550nm, respectively. The size of the doxorubicin-loaded liposomes was measured using a particle size analyzer (Brookhaven Instruments Corporation, US).

Confocal Image Acquisition

Doxorubicin was imaged using 458 laser excitation and LP 560 emission filter, and fluorescein was imaged using 488 laser excitation and BP 505-530 emission filter. Images were acquired using an in-plane resolution of 512 by 512 pixels with a field of view of 460 by 460μm (20X objective) and an optical slice thickness of 10.1μm.

Images were acquired using a LSM 510 with 488 nm laser excitation for GFP fluorescence and 543nm laser excitation for doxorubicin fluorescence (BF 505-550, LP 585, 10X and 20X objective, pinhole 284μm). Zeiss LSM image browser was used for image post-processing.

Confocal Image Analysis

Vascular masks were created from a custom-designed mask algorithm using MATLAB software (MathWorks, Natick, MA) on 8-bit images in a TIFF format. Briefly, the computer algorithm first median filtered the images and then defined multiple thresholds and evaluated the connectivity of pixels to be added with each increasing threshold step, in order to reduce noise. Images were then morphologically closed, holes were filled, and small volumes scrapped to define a continuous tumor vasculature from the initial rhodamine-dextran images. Vascular and extravascular fluorescence intensities were determined from the average intensity in pixels located within or outside of the vascular mask, respectively, with background subtraction. Doxorubicin data was adjusted for fluorescein bleed through and normalized to the maximum
vascular intensity (expressed as \( \%I_{\text{vasc,max}} \)). Normalization was used to permit averaging of the data across animals and remove tissue absorption effects due to differences in tissue depth.

**Histology Image Analysis**

Mice were placed into custom hyperthermia holders, with the tumor-bearing leg wrapped in a plastic bag to prevent direct water contact with the leg. Mice were placed into a water bath set at 43.5°C (resulting in average flank tumor temperature above 41.4°C), with the tumor-bearing leg inserted just far enough into the water to fully cover the tumor volume. A fan was placed angled towards the mice during hyperthermia to prevent body temperature elevation.

Tumors were sectioned at a thickness of 10µm. Doxorubicin was imaged using a TRITC fluorescence cube set and 500ms exposure time. Immediately after imaging, the slides were placed in 20°C methanol and fixed for 30min. They were then placed in PBS, blocked with 5% Donkey Serum for 1hr, washed and incubated with CD31 primary antibody at 1:100 overnight at 4°C. The slides were washed 5 times with PBS and then the CD31 Alexa 488 secondary antibody was applied at a concentration of 1:1000 for 1hr. Slides were then washed 3 times with PBS and imaged for CD31 using a FITC filter (485/20 BF Ex, 525/25 BF, Em) with an exposure time of 300ms.