**Materials and Methods**

***Reagents***

Cyclophosphamide monohydrate, doxorubicin hydrochloryde, Cremophor ® EL and human insulin were from SIGMA-ALDRICH, Inc. (St. Louis, MO). Paclitaxel and ifosfamide were from Selleck Chemicals (Houston, Texas). Eribulin was from Eisai Inc. (Woodcliff Lake, NJ). FITC-conjugated lectin was from Vector Laboratories, Inc. (Burlingame, CA). Acetic acid, methanol, and acetonitrile were from EMD Chemicals Inc. (Gibbstown, NJ). HPLC-grade water and trifluoroacetic Acid (TFA) were from J. T. Baker (Phillsburg, NJ). Cholesterol and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (PEG-DSPE) were from Avanti Polar Lipids Inc. (Alabaster, Alabama). Hydrogenated soy phosphatidylcholine (HSPC) was from Lipoid (Newark, NJ). Fetal Bovine Serum (FBS) was from Tissue Culture Biologicals (Long Beach, CA). RPMI, Leibovitz’s L-15, DMEM, F12 media, Trypsin-EDTA (0.25%), Geneticin and penicillin G/streptomycin sulphate were from Gibco (Invitrogen, Grand Island, NY). 4-hydroxy-cyclophosphamide preparation kit was from Toronto Research Chemicals (Toronto, Canada). Estrogen pellets (0.74 mg; 60-day release) were from Innovative Research of America (Sarasota, FL). OCT (Optimal Cutting Temperature) compound was from Tissuetek Sakura Finetek USA Inc. (Torrance, CA). Hoechst 33342 trihydrochloride trihydrate, ProLong Gold, DiIC18(5)-DS (DiI5), and PrestoBlue® Cell Viability Reagent were from Invitrogen (Carlsbad, CA). Goat anti-mouse Alexa Fluor® 555, goat anti-rabbit Alexa Fluor® 555 were from Molecular Probes (Eugene, OR). Goat anti-mouse Alexa Fluor® 488, goat anti-rabbit Alexa Fluor® 647, rabbit anti-human cleaved caspase 3, rabbit anti-human cleaved PARP, and SignalStain® Antibody diluent were from Cell Signaling Technology (Danvers, MA). Goat anti-hamster Alexa Fluor® 647 was from Jackson ImmunoResearch (West Grove, PA). Mouse anti-human phospho-histone H2AX and armenian hamster anti-mouse CD31 were from Millipore (Billerica, MA). Mouse anti-human cytokeratin and EnVision+ System-HRP labeled polymer anti-rabbit were from DAKO (Carpinteria, CA). Rabbit anti-human HER2 (clone SP3) was from Fisher Scientific (Pittsburg, PA). Rabbit anti-collagen type I was from AbCam (Cambridge, MA). Cyanine 5 Tyramide was from Perkin Elmer (Boston, MA). 64CuCl2 was obtained from the cyclotron facility at the Mallinckrodt Institute of Radiology (Washington University School of Medicine; St. Louis, MO). The chelator, 4-DEAP-ATSC, was synthesized by Albany Molecular Research, Inc. (AMRI; Albany, NY). Synthesis and characterization of 4-DEAP-ATSC was reported elsewhere (1)**.**

***Evaluation of cell viability***

Cells (BT474-M3, NCI-N87, SUM190 and MDA-MB-361) were plated in a 96-well plate at 5000 cells/well. 4-hydroxy-cyclophosphamide was prepared from its precursor, 4-hydroperoxy-cyclophosphamide, according to the manufacturer instructions. Cells were incubated with 4-hydroxy-cyclophosphamide (~ 1 uM) for 72 hrs and viability was evaluated by PrestoBlue Assay.

***Preparation of liposomes***

The lipid components were HSPC, Cholesterol, and PEG-DSPE (3:2:0.3, mol:mol:mol). Liposomes were prepared as previously described (2–6) with the exception that buffer exchange was performed by tangential flow filtration to remove unencapsulated doxorubicin, using a KrosFlo Research II Pump and MidiKros Filter Modules (Spectrum Labs, Rancho Dominguez, CA). Liposome size was measured by dynamic light scattering using a ZetaSizer Nano-ZS (Malvern, Worcestershire, UK) and was determined to be 75-115nm. Each experimental group was dosed with the exact same formulation of HER2-tPLD.

***Image Analysis***

The rule sets are available upon request. γ-H2AX and cleaved caspase 3-stained images were analyzed by identifying the nuclei in the Hoechst layer, and by subsequently subdividing them into γ-H2AX-positive, cleaved caspase 3-positive, γ-H2AX and cleaved caspase 3-double positive, or γ-H2AX and cleaved caspase 3-double negative based on the intensities of the γ-H2AX and cleaved caspase 3 staining. To quantify the extent of cleaved PARP positive tumor cells, nuclei were segmented in the Hoechst layer, and tumor cells (cytokeratin positive) were distinguished from non-tumor cells/stroma (cytokeratin negative). The tumor cells were then classified into PARP-positive or-negative based on the intensity of the cleaved PARP staining. To quantify vascular changes, blood vessels were segmented in the CD31 layer. MVD was determined by dividing the number of blood vessels by the tumor area (mm2) or by dividing the total vessel area (mm2) by the total tumor area (mm2). Blood vessels were classified into perfused (FITC-lectin + CD31 +) or non-perfused (FITC-lectin - CD31 +). The % of perfused blood vessels was determined by dividing the perfused blood vessels by the total vessels (CD31 +). Blood vessels were subdivided into small (66-110 um2), medium (110-438 um2), and large (>438 um2) based on their area, and they were then classified into perfused or non-perfused as described above. Collagen type I deposition was quantified by segmentation of collagen objects based on the collagen signal. Collagen deposition was expressed as % of collagen area over total tumor area. HER2 expression was quantified as described in (7). The interstitial space area, expressed as a percent of total tumor area, was quantified by dividing the area not occupied by any cellular component by the total tumor area. The % of doxorubicin-positive nuclei was determined on frozen tumor sections by segmenting the nuclei based on the Hoechst signal and subsequently classifying them into doxorubicin-positive or -negative classes based on the intensity of the doxorubicin signal within the nucleus. The distribution of liposomes relative to the vasculature was quantified by identifying perfused blood vessel based on the FITC-lectin signal. Perfused blood vessels were further classified into “High Delivering” or “Low Delivering”, based on a threshold of 1100 mean fluorescence intensity (MFI) in the liposome channel (DiI5), corresponding to a 5-fold DiI5 MFI value above background MFI (~220). Subsequently, new objects were generated, concentric to the blood vessels (each 10-um wide), and finally the liposome DiI5 MFI was calculated within each vessel-concentric object.

**References**

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