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Copper−Doxorubicin as a Nanoparticle Cargo Retains Efficacy with Minimal Toxicity

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Abstract: Repeated administration of chemotherapeutics is typically required for the effective treatment of highly aggressive tumors and often results in systemic toxicity. We have created a copper−doxorubicin complex within the core of liposomes and applied the resulting particle in multidose therapy. Copper and doxorubicin concentrations in the blood pool were similar at 24 h (∼40% of the injected dose), indicating stable circulation of the complex. Highly quenched doxorubicin fluorescence remained in the blood pool over tens of hours, with fluorescence increasing only with the combination of liposome disruption and copper trans-chelation. At 48 h after injection, doxorubicin fluorescence within the heart and skin was one-fifth and one-half, respectively, of fluorescence observed with ammonium sulfate-loaded doxorubicin liposomes. After 28 days of twice per week doxorubicin administration of 6 mg/kg, systemic toxicity (cardiac hypertrophy and weight and hair loss) was not detected with the copper−doxorubicin liposomes but was substantial with ammonium sulfate-loaded doxorubicin liposomes. We then incorporated two strategies designed to enhance efficacy, mTOR inhibition (rapamycin) to slow proliferation and therapeutic ultrasound to enhance accumulation and local diffusion. Tumor accumulation was ∼10% ID/g and was enhanced approximately 2-fold with the addition of therapeutic ultrasound. After the 28-day course of therapy, syngeneic tumors regressed to a premalignant phenotype of ∼(1 mm)^3 or could not be detected.

Keywords: Doxorubicin; liposome; toxicity; rapamycin; ultrasound

Introduction

Effective strategies for low-toxicity, multiply administered cancer therapies are uncommonly reported.[1] Encapsulating doxorubicin into liposomes has increased the total tolerated dose,[2−4] while cardiac toxicity, mucositis and palmar−plantar erythrodysesthesia restrict the maximum lifetime dose and limit the clinical dosing schedule to 10−12 mg/kg/week at intervals of two to six weeks.[1,5,6] Unexpected synergies between the cardiotoxicities of anthracyclines and growth

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factors such as anti-ErbB2 antibodies have further increased the need to reduce toxicity.[7] Given the impact of the dose limitations on efficacy, particles with reduced toxicity would facilitate treatment, particularly in recurrence.

To enhance stability of doxorubicin within the particle, we create a complex between the drug and a transition metal, as has previously been reported for doxorubicin with manganese(II) and irinotecan with copper(II).[8−10] Creation of a copper−doxorubicin complex during the loading process is particularly attractive, since the formation of the copper(II)−doxorubicin complex has been associated with oxygen radical-mediated stimulation of DNA strand scission, the stimulation of lipid peroxidation mechanisms and resultant toxicities.[11−13] Formation of a drug−metal complex during loading changes the morphology of the liposomes and subsequently improves circulation lifetime and the accumulation of liposomes in tumors.[14,15] Further, a 1:2 complex of copper and doxorubicin with a stability constant of 10^{16} forms when a neutral pH is created within liposomes.[16,17] Yet, at a low pH, such as the pH encountered within a lysosome or tumor, the stable copper:doxorubicin ratio has been reported to change to 1:1 and the stability of the complex decreases.[17] Here, we track the liposome shell using positron emission tomography (PET) and the drug using multispectral fluorescence in order to assess the pharmacokinetics.

Further, the protective coating of liposomes reduces drug diffusion within the tumor, and the impact of liposomal therapy on clinical efficacy has been modest.[18] We address the dual issues of toxicity and efficacy by applying our stable particle in an aggressive dosing schedule and incorporating two strategies designed to enhance efficacy: mTOR inhibition to slow proliferation[19] and therapeutic ultrasound to enhance accumulation and local diffusion.[20,21] The aggressive syngeneic Met-1 model is known to be sensitive to rapamycin (which is an mTOR inhibitor); however, rapamycin alone is not curative in this model.[22]

Ultrasound, as a source of thermal and mechanical energy, can augment drug delivery by releasing the drug or increasing vascular permeability and thus particle accumulation and diffusion.[20,21] Tumor blood vessels present relatively permeable capillaries that allow macromolecules and small liposomes (100 nm) to leak through open gaps and fenestration due to the enhanced permeability and retention (EPR) effect.[23,24] Heating of the tumor rim, when combined with liposomal drugs, can enhance therapeutic efficacy as was previously demonstrated for radiofrequency (RF) ablation combined with liposomal doxorubicin.[25] Thus, by enhancing the pharmacokinetic profile and the extent of the EPR effect, we demonstrate enhanced efficacy and reduced toxicity in a highly aggressive mouse model of breast cancer.[26,27]

Materials and Methods

A detailed description of the experimental procedures is found in the Supporting Information.

Liposomes and Drug Preparation. Doxil (Ortho Biotech Products, LP Raritan, NJ), a commercial ammonium sulfate-loaded doxorubicin liposome, is used for comparison to experimental preparations. Long-circulating liposomes (LCLs) prepared from HSPC:chol:DSPE-PEG2k (56:39:5), the lipid composition of Doxil, were used in this study.[28]

Copper Liposome Preparation. Liposomes were prepared as described in ref 29. L-α-Phosphatidylcholine, hydrogenated soy (HSPC), 1,2-distearoyl-*sn*-glycero-3-phos-

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phoethanolamine-*N*-methoxy polyethyleneglycol-2000 (DSPE-PEG2k), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The dried lipid was hydrated in 0.3 mL of 100 mM copper(II) gluconate (PURAC, Lincolnshire, IL) including 270 mM triethanolamine (TEA, Sigma, St. Louis, MO), pH 7.4 unless otherwise stated. The multilamellar lipid solution at a final concentration of 50 mg/mL was extruded above the phase transition temperature of the lipid mixture through a polycarbonate membrane with a pore diameter of 100 nm. Copper/TEA-loaded liposomes were then separated from nonencapsulated copper/TEA by passing the extruded liposomal solution through a spin column of Sephadex G-75 (5 × 1 cm, GE Healthcare, Biosciences, Piscataway, NJ) equilibrated with saline (0.9% sodium chloride). The liposomal diameters were ~100 nm (103 nm ±13 nm), as measured using a NICOMP 380 ZLS submicron particle analyzer (Particle Sizing System Inc., Santa Barbara, CA). Lipid concentration was measured using the Phospholipids C assay kit (Wako Chemicals USA, Richmond, VA). Doxorubicin hydrochloride supplied by Sigma (St. Louis, MO) was then loaded, and the resulting liposomes were purified and characterized.

Protocol. All animal handling was performed in accordance with University of California, Davis (UCD), Animal Use and Care Committee guidelines. Efficacy studies involved 80 animals, randomized between 10 groups (ultrasound only, copper-doxorubicin (CuDox) liposomes, rapamycin, Doxil, CuDox liposomes + ultrasound, CuDox liposomes + rapamycin, CuDox liposomes + rapamycin + ultrasound, Doxil + rapamycin, doxorubicin, diluent only). Toxicity studies involved 32 animals randomized between 4 groups (doxorubicin, CuDox liposomes, Doxil, and control). Mice bearing bilateral Met-1 tumors of 4−6 mm in longitudinal diameter (≥100 mm^3) were injected intravenously with either free or liposomal doxorubicin (~6 mg doxorubicin/kg body weight and ~32 mg lipid/kg body weight) twice a week with a total doxorubicin injected dose of 267 mg/m^2 over 4 weeks and compared to control animals which received saline. For rapamycin, animals were treated by intraperitoneal (ip) injection of (~0.9 mg rapamycin/kg body weight) three times per week over the entire period of treatment. For combined treatments with ultrasound, one tumor per animal was insonified for 2 min at 42 °C postinjection. The ultrasound pulses consisted of 100-cycle bursts at 1.5 MHz center frequency and 1.2 MPa peak negative pressure, with variable pulse-repetition frequency (PRF) ranging from 100 Hz up to 5 kHz.

***In Vivo* Imaging.** Images of circulating doxorubicin were acquired using the Maestro hyperspectral imaging system (Cambridge Research & Instrumentation, Inc., Woburn, MA). Animals were then euthanized and perfused with saline at 24 or 48 h post systemic administration of the drug, and the accumulation of doxorubicin or copper in tissues and organs was imaged and quantified *ex vivo*.

To study the systemic circulation and tumor accumulation of liposomal vehicles, mice were systemically injected with liposomes labeled with ^{64}Cu-BAT lipid to track the liposomal lipid shell and imaged using PET.[30] A near-infrared fluorophore in the drug core validated accumulation of intact particles.

Results

We report for the first time that the copper−doxorubicin complex, when loaded within a liposome, has substantial advantages in reducing systemic toxicity. Further, we exploit this reduced toxicity to create an efficacious therapy through repeated administration over a multiweek regimen and augment the therapy with rapamycin and/or ultrasound.

Copper−Doxorubicin Complex Quenches Fluorescence and Demonstrates Enhanced Stability at Neutral pH. Doxorubicin loading increased with the ratio of copper to doxorubicin, reaching a maximum at a 1:2 molar ratio with 100% loading efficiency (Supplementary Methods and Supplementary Figure S1). Loading also increased linearly with the intraliposomal copper concentration, up to 0.6 mg doxorubicin per mg lipid (Supplementary Figure S1c). In all following studies, particles were loaded using 100 mM copper-gluconate and 270 mM TEA, achieving a final ratio of 0.2 mg of doxorubicin per mg of lipid in order to facilitate a comparison with Doxil.

Although doxorubicin loading increased in proportion to the TEA gradient, intraliposomal doxorubicin fluorescence was quenched (Figure 1a). For copper−doxorubicin liposomes, full restoration of doxorubicin fluorescence was observed only with the combination of Triton X-100, transchelation with EDTA and incubation at 55 °C for 1 h, indicating that doxorubicin was associated with copper (Figure 1a,b). Even in the presence of serum albumin, EDTA and elevated temperature were required to achieve transchelation and restore fluorescence (Figure 1b). Alternatively, for liposomes that did not contain copper, the fluorescence intensity of released doxorubicin was not affected by the addition of EDTA (Figure 1a,b). Long-term *in vitro* stability of doxorubicin encapsulation was also assessed; free doxorubicin was not detected during a 30-day *in vitro* incubation of either copper−doxorubicin liposomes or Doxil at 37 °C.

Fluorescence of both Doxil and copper−doxorubicin liposomes in plasma remained quenched after 24 hours of circulation (Figure 1c). Upon addition of Triton X-100, fluorescence of doxorubicin was fully restored for Doxil, whereas, a combination of Triton X-100 and EDTA at elevated temperatures was required to restore the fluorescence of doxorubicin for copper−doxorubicin liposomes as shown previously in the *in vitro* stability assay (Figure 1b). Thus, the copper−doxorubicin complex circulates stably within liposomes and remains associated in plasma after release from liposomes. Trans-chelation kinetics of copper from copper−doxorubicin complex in the presence of albumin, one of the major trans-chelating components of blood, was strongly

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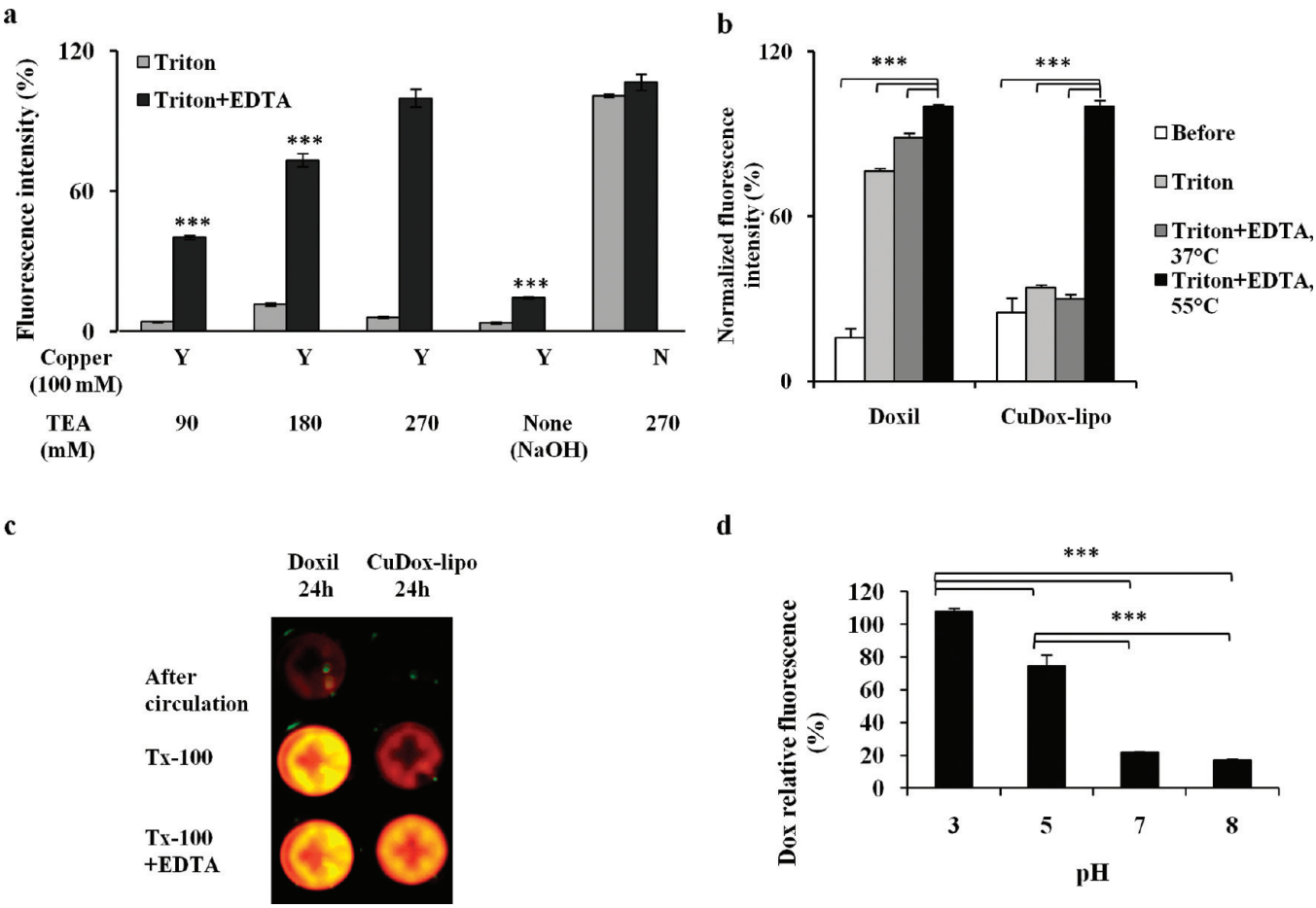

Figure 1. Loading optimization and *in vitro* characterization of copper−doxorubicin in long-circulating liposomes (LCLs). (a) Fluorescence intensity of doxorubicin encapsulated in LCLs with an increasing TEA gradient, released by Triton X-100 in the presence or absence of 10 mM EDTA. (b) Effect of EDTA and heat in trans-chelation of doxorubicin from copper, as assessed by fluorescence. Copper−doxorubicin (CuDox) liposomes and Doxil were incubated in complement-preserved human serum in the presence or absence of 0.25% Triton X-100 for 1 h. (c) Fluorescence images of plasma isolated from mice 24 h post injection of either Doxil or CuDox liposomes, before and after the addition of TritonX-100 at 37 °C or TritonX-100 with 10 mM EDTA at 55 °C for 1 h. (d) Dissociation of copper from CuDox complex in 0.5 mM BSA solutions as a function of pH at 37 °C. Statistical analyses were performed using one-way ANOVA followed by a Tukey post hoc test. ***, $p < 0.001$.

dependent on pH and exhibited a significantly lower dissociation rate (≤20%) at pH values of 7 and higher over a period of 48 h, $p < 0.001$ (Figure 1d). In contrast, copper trans-chelation increased as the pH decreased below 7 with a rapid dissociation of copper (≥75%) observed at pH ≤ 5 (Figure 1d).

Cryo-electron microscopy verified the presence of precipitation as a dotted and diffuse structure of the copper−doxorubicin complex uniformly distributed inside the liposomes (Figure 2a.i left), which was substantially different from the needle-like precipitate formed by ammonium sulfate loading of doxorubicin[31,32] or the subtle precipitate of copper alone (Figure 2a.i right). Given the molar ratio of 1:2, the hypothesized structure for the liposomal copper:doxorubicin complex is schematically depicted (Figure 2a.ii).

***In Vitro* Cytotoxicity of Copper−Doxorubicin Liposomes Is Enhanced.** When evaluated with the Met-1 cell line, the cytotoxicity of the copper−doxorubicin liposomes (IC50 of 0.33 ± 0.16 μM, $n = 12$) was greater than that of Doxil (IC50 of 1.72 ± 0.85 μM, $n = 6$), $p < 0.001$, whereas free doxorubicin exhibited the lowest IC50 value (0.02 ± 0.01 μM, $n = 15$) (Figure 2b, Supplementary Figure S1d). Empty liposomes and copper liposomes (each tested with an equal lipid concentration) had no effect on cell viability. Delivery of copper and doxorubicin in two separate liposomal formulations (copper liposomes and Doxil) did not change the IC50 value of Doxil.

Copper−Doxorubicin Liposome Stability Is Associated with Reduced Systemic Toxicity. *In vivo* stability was assessed by serial imaging of fluorescent doxorubicin,

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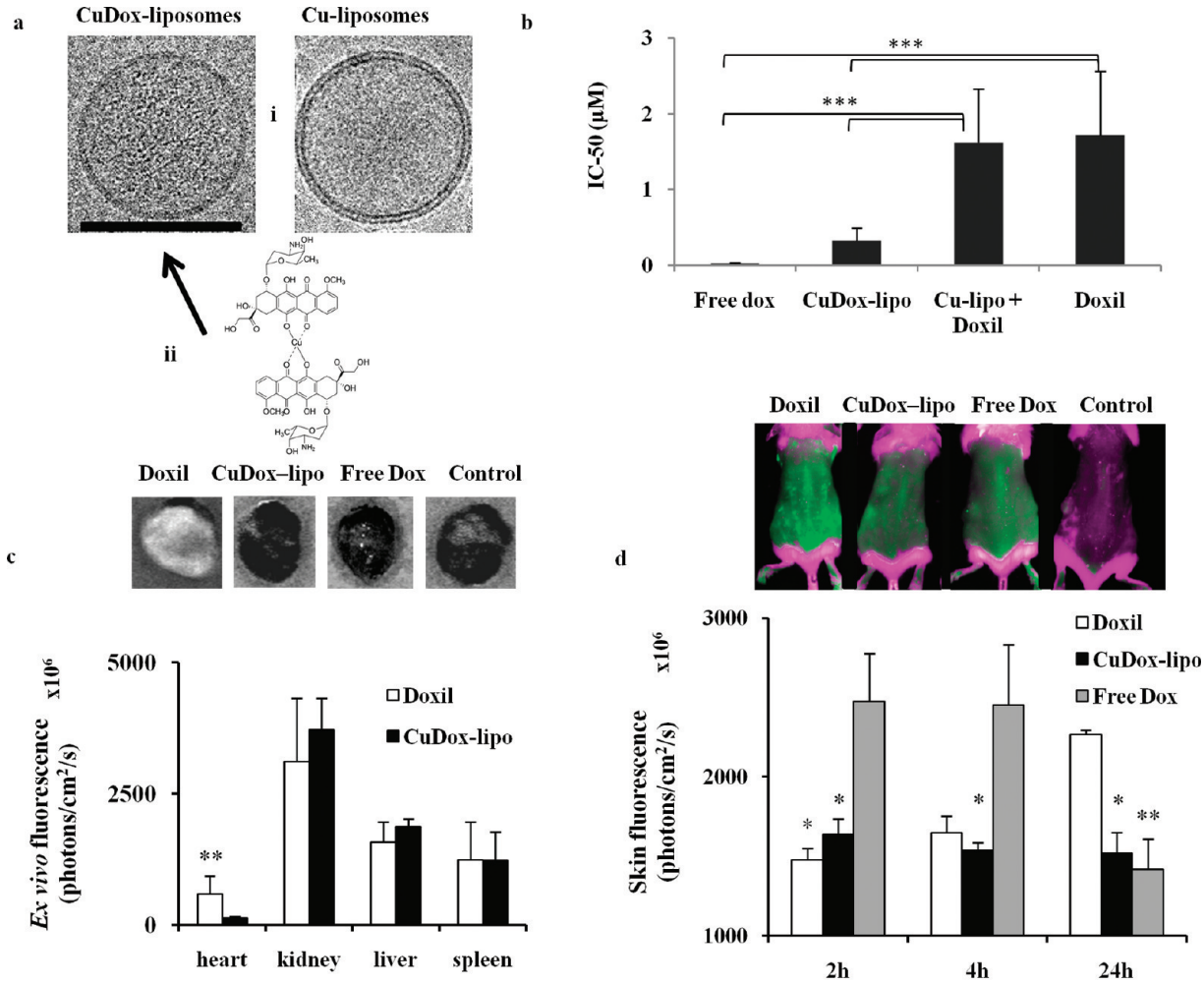

Figure 2. Pharmacokinetics of copper−doxorubicin liposomes and comparison with control vehicles. (a) (i) Cryo-EM images of LCLs encapsulating copper−doxorubicin (left panel) and copper only (right panel) under 100 mM copper/270 mM TEA intraliposomal condition. Scale bar represents 100 nm. (ii) Schematic presentation of the hypothesized molecular interaction between copper and doxorubicin upon loading into liposomes. (b) IC50 values of free and liposomal doxorubicin were calculated using GraphPad. (c) *Ex vivo* hyperspectral fluorescence intensity of the organs of mice 24 h after injection of copper−doxorubicin (CuDox) liposomes or Doxil. Mice were perfused with saline immediately prior to organ harvesting and imaging. Inset view of heart fluorescence at 48 h after injection (white indicates higher fluorescent intensity). (d) *In vivo* hyperspectral fluorescence intensity of the skin of mice after injection of CuDox liposomes or Doxil. Statistical significance is compared to the highest value in each time point. Inset images acquired at 24 h after injection, green indicates doxorubicin, and pink indicates tissue background. Statistical analyses were performed using one-way ANOVA followed by a Tukey post hoc test (b, d) and Student's *t* test (c). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

PET labeling of the liposomal shell, and ICP-MS measurements of copper accumulation. At 24 h after the injection of copper−doxorubicin liposomes, the concentration of doxorubicin and copper in plasma were 43.3 ± 3.8% ID/cc ($n = 6$) and 39.1 ± 6% ID/cc ($n = 6$) of the initial dose, indicating a stable association of doxorubicin with copper in circulation. The concentration of doxorubicin in Doxil in the blood pool was higher 24 h after injection with ~50% of the initial dose continuing to circulate ($p < 0.01$).

Following organ perfusion with saline and excision at 24 h after injection, fluorescence was similar for Doxil and copper−doxorubicin liposomes in organs associated with drug clearance (spleen, liver, kidney). However, in the heart, fluorescence resulting from copper−doxorubicin liposomes was one-fifth that resulting from Doxil administration, $p < 0.01$ (Figure 2c). Skin fluorescence increased to a greater extent following Doxil administration than with Cu−doxorubicin liposomes or free doxorubicin, increasing with time for Doxil and decreasing with time for free drug and Cu−doxorubicin liposomes, $p < 0.05$ (Figure 2d). A unique spectrum, associated with intact Doxil liposomes, was detected within the skin by multispectral optical imaging (Supplementary Figure S2a,b), indicating that the increased fluorescence resulted at least partially from intact liposomes.

Doxorubicin-associated toxicity was then assessed with an aggressive four-week dose schedule of 6 mg/kg of

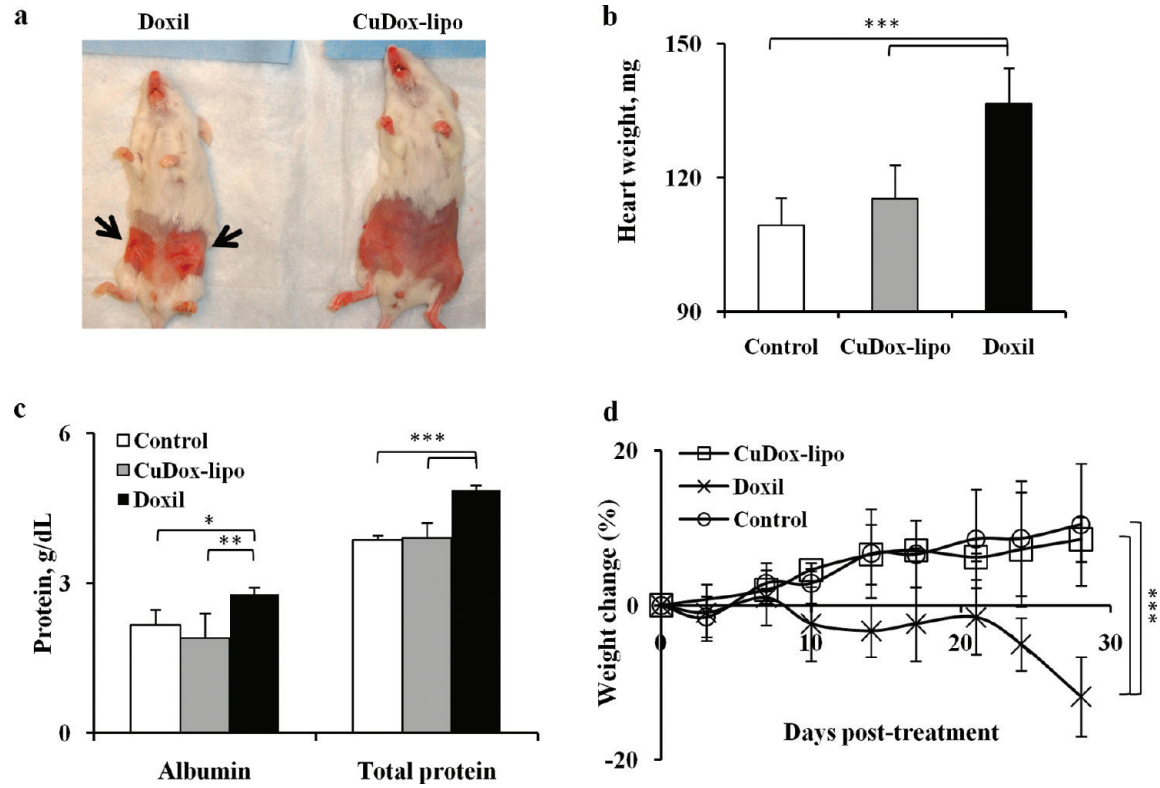

Figure 3. Toxicity of copper−doxorubicin (CuDox) liposomes and Doxil assessed over 28 day administration of 6 mg/kg (33.4 mg/m^2) twice per week (total of 266.7 mg/m^2). (a) Images of Doxil-treated and CuDox liposome-treated mice. Arrows show redness over the tumor region of Doxil-treated animal. Heart weight (b) and protein (albumin and total protein) measurement (c) for mice injected with either CuDox liposomes or Doxil. (d) Weight change of mice treated with either CuDox liposomes, Doxil or saline control, over 28 days of treatment. Statistical analyses were performed using one-way ANOVA at the end of the treatment period followed by a Tukey post hoc test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

doxorubicin liposomes twice per week, which is equivalent to 66.7 mg/m^2 per week. Animals receiving Doxil at this relatively high dose demonstrated fur loss and a skin rash as early as 7−10 days post-treatment; such effects were not observed with the equivalent dose copper−doxorubicin liposomal therapy throughout a 28-day course of treatment (Figure 3a). Doxil-treated animals showed a significant increase in heart weight ($p < 0.001$), circulating albumin ($p < 0.05$) and total protein ($p < 0.001$) and a significant weight loss ($p < 0.001$), as compared to control mice and mice treated with copper−doxorubicin liposomes (Figures 3b, 3c, 3d). Leucopenia was observed with each liposomal doxorubicin group; however, the effect was greater with Doxil. Red and white blood cell counts following the administration of a control diluent, copper−doxorubicin liposomes or Doxil were 7.6 ± 0.7 ($n = 5$), 4.5 ± 0.3 ($n = 7$), 3.1 ± 0.1 M/μL ($n = 5$) for red blood cells and 5.7 ± 2.1, 3.6 ± 1.1, 1.9 ± 0.6 K/μL for white blood cells, respectively.

Efficacy of Copper−Doxorubicin Liposomes Demonstrated in Multicomponent Regime. In initial studies with copper doxorubicin liposomes or Doxil with 3 mg/kg (~17 mg/m^2) biweekly dosing, growth of the Met-1 tumor continued with only a small extension of survival (data not shown). Thus, the dose was increased to 6 mg/kg (~33 mg/m^2) and the treatments were incorporated into a multitreatment regime with rapamycin, which is also known to be efficacious in the Met-1 line. Also, we recognize that the penetration of liposomal particles within solid tumors is problematic, and thus, we added ultrasound to improve the accumulation and diffusion of the particles and drug within the tumor. In our study, ultrasound was applied immediately after injection, with a goal of increasing tumor accumulation (Figure 4a). With the mechanical index (MI) of 0.9 applied here, changes in vascular permeability are not produced when short (1−2 cycle) imaging pulses are applied; however, in this study, long pulses were employed and controlled such that an increase in tumor temperature to 42 °C was achieved and maintained for two minutes. Immediately after insonation, the tumor blood vessel diameter increased to 25.5 ± 25.3 μm ($n = 4$, Figure 4b, upper left panel) compared to 11.6 ± 6.9 μm for untreated tumors ($n = 4$, Figure 4b, upper right panel), $p < 0.001$. In the absence of drug, insonifying one of two bilateral tumors did not change the tumor growth rate (Figure 4b, lower panels).

As assessed with PET and optical imaging, the concentration of liposomes within the tumors peaks between 18 and 20 h after injection (Figure 4c, Supplementary Figure S2c). Copper (assessed by ICP-MS) accumulated in tumors

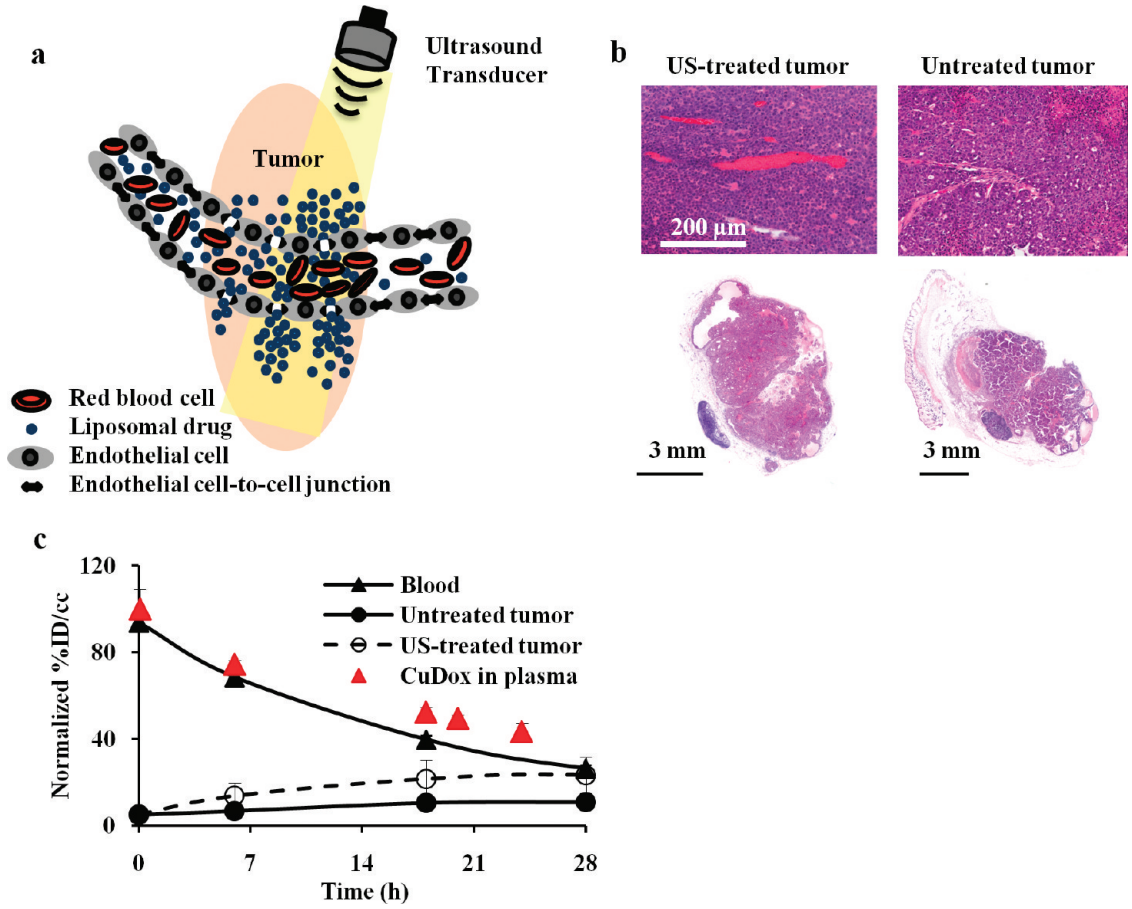

Figure 4. Enhanced accumulation of copper−doxorubicin liposomes using therapeutic ultrasound (US). (a) Concept image of locally enhanced extravasation and accumulation of liposomal chemotherapeutic in solid tumors by ultrasound. (b) H&E histological images of insonified tumor (upper left panel) compared to untreated tumor (upper right panel) demonstrating vascular dilation and engorgement produced by ultrasound, tumor insonified for 2 min at 42 °C over a 10 day period of treatment with insonation repeated at day 1, 4, and 8 (lower left panel) compared to untreated tumor with the same period of treatment (lower right panel). (c) Blood stability and tumor accumulation of the lipid shell of LCLs quantified by positron emission tomography (PET) and compared with stability of copper−doxorubicin in blood as quantified by copper (ICP-MS) and indicated by % injected dose.

similarly over time, reaching a maximum of 10% ID/g at 18−20 h postinjection. The concentration of liposomes within the insonified tumor increased by approximately 2-fold compared with the contralateral tumor, as quantified using PET (Figure 4c) and optical imaging (Supplementary Figure S2c). Further, the concentration of copper also increased by approximately 2-fold in the insonified tumor, reaching a maximum of 15% ID/g which translates to 20 μg of doxorubicin/g of tumor, $p < 0.05$. Doxorubicin fluorescence was also evident within tumors, increasing as a function of time following the injection of both Doxil and copper−doxorubicin formulations.

A ten day course of therapy was next applied to compare the efficacy of free doxorubicin, copper−doxorubicin liposomes (with and without ultrasound), ultrasound only, and systemic injection of a saline control therapy (Figure 5a). The treatment with free doxorubicin showed a therapeutic effect when compared with the saline control ($p < 0.001$). Tumor growth suppression was greater for copper−doxorubicin liposomes than free doxorubicin ($p < 0.001$).

The therapeutic effect of copper−doxorubicin liposomes was then tested in an aggressive multidose 28-day treatment, in combination with rapamycin and ultrasound and appropriate single therapy control groups. Tumor longitudinal diameter ranged from 4 to 6 mm prior to treatment and was similar in all groups. Animals treated with diluent (control group) or rapamycin survived only 18 and 24 days post-treatment, respectively, whereas all animals receiving sole or combination therapy with liposomal doxorubicin or Doxil survived the entire 28-day course of treatment (Figure 5b). All therapies suppressed the tumor growth as compared to control after 18 days of treatment ($p < 0.001$). The *in vivo* efficacy of Doxil was similar to the efficacy of copper−doxorubicin liposomes in the two subgroups that were evaluated, which were liposomal doxorubicin alone (not shown) and combined therapy with liposomal doxorubicin and rapamycin (Figure 5b). Suppression of tumor growth was observed in all copper−doxorubicin liposome-treated animals (Figure 5b, $p < 0.001$); however, average tumor growth was ~700% with copper−doxorubicin liposomes and was reduced to <200%,

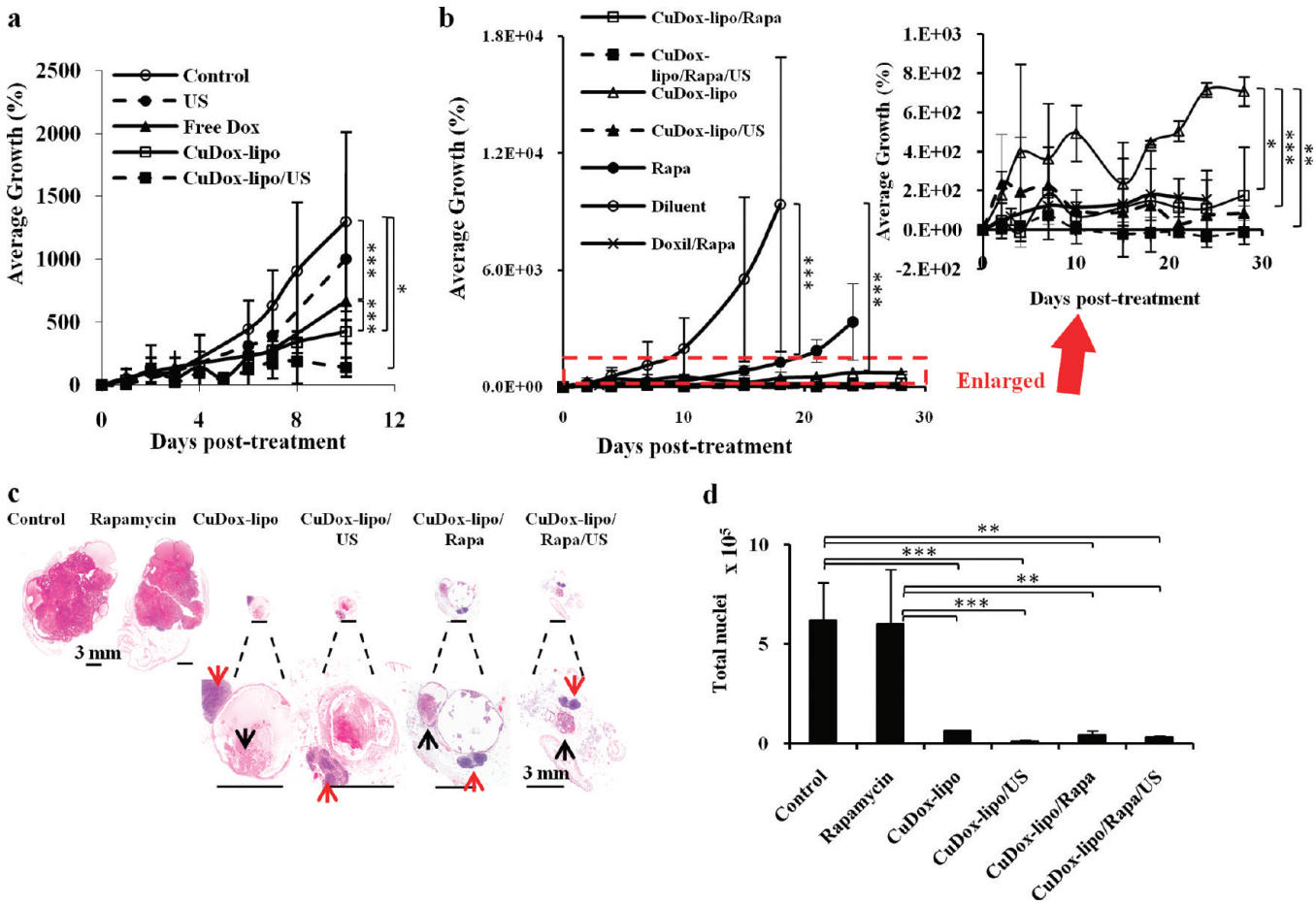

Figure 5. *In vivo* treatment efficiency including ultrasound (US), free doxorubicin (free Dox), rapamycin and copper-doxorubicin liposomes (CuDox-lipo) in Met-1 tumor mice. (a, b) Percent tumor growth as a function of days post-treatment, over 11-day treatment cycle (a) and over 28-day treatment cycle (b). Initial tumor diameter was 4−6 mm. Each mouse was injected intravenously with either free or liposomal doxorubicin (~6 mg of doxorubicin/kg body weight equivalent to ~33 mg/m^2) and compared to control animals that received either iv injection of saline (a) or intraperitoneal (ip) injection of diluent (b). A subset of animals were treated by ip injection of ~0.9 mg rapamycin/kg body weight three times per week over the entire period of treatment. For treatment with therapeutic ultrasound, one tumor per animal was insonified for 2 min at 42 °C postinjection. Statistical analyses were performed using mixed models as described in the Statistical Analysis section of the Supporting Information. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ are the significance between growth curves. H&E (c) and immunohistochemical quantification of total nuclei (d) of tumors treated with therapies post 28 days of treatment compared to control at 18 days post ip injection of diluent. Statistical analyses were performed using one-way ANOVA followed by a Tukey post hoc test (d). **$p < 0.01$, ***$p < 0.001$. Red arrows indicate lymph nodes within fat pad; black arrows indicate remaining tumor.

85% or −11% with the addition of rapamycin ($p < 0.05$), ultrasound ($p < 0.001$) or rapamycin plus ultrasound ($p < 0.01$), respectively (Figure 5b,c).

Histological Measurements Confirm Efficacy with Liposomal Doxorubicin and Enhancement with Rapamycin and Ultrasound. Histological sections obtained from tumors confirmed the efficacy of therapy; however viable tumor assessed by histology was smaller than the diameter measured by ultrasound at the end of the treatment due to the presence of cysts (Figure 5c). As a result of copper−doxorubicin liposomes and rapamycin or ultrasound therapy, a cystic, epithelial phenotype with reduced proliferation (as compared with control tumors) was observed (Figures 5c and 6). Mammary lymph nodes within the sections provided a control for proliferation. With the combination of copper−doxorubicin liposomes and therapeutic ultrasound, viable tumor was not detected in a subset of tumors (Figure 5c). Total tumor nuclei were reduced in all treatments with copper−doxorubicin liposomes as compared to diluent injection, $p < 0.01$ (Figure 5d). Compared to control tumors, CD31 and Ki67 were reduced and apoptosis increased with copper−doxorubicin liposomes alone or in combination with rapamycin and therapeutic ultrasound, $p < 0.05$ (Figure 6a−c). Contrast ultrasound imaging confirmed the reduced vascularity observed in treated compared with control tumors (Figure 6c.i,

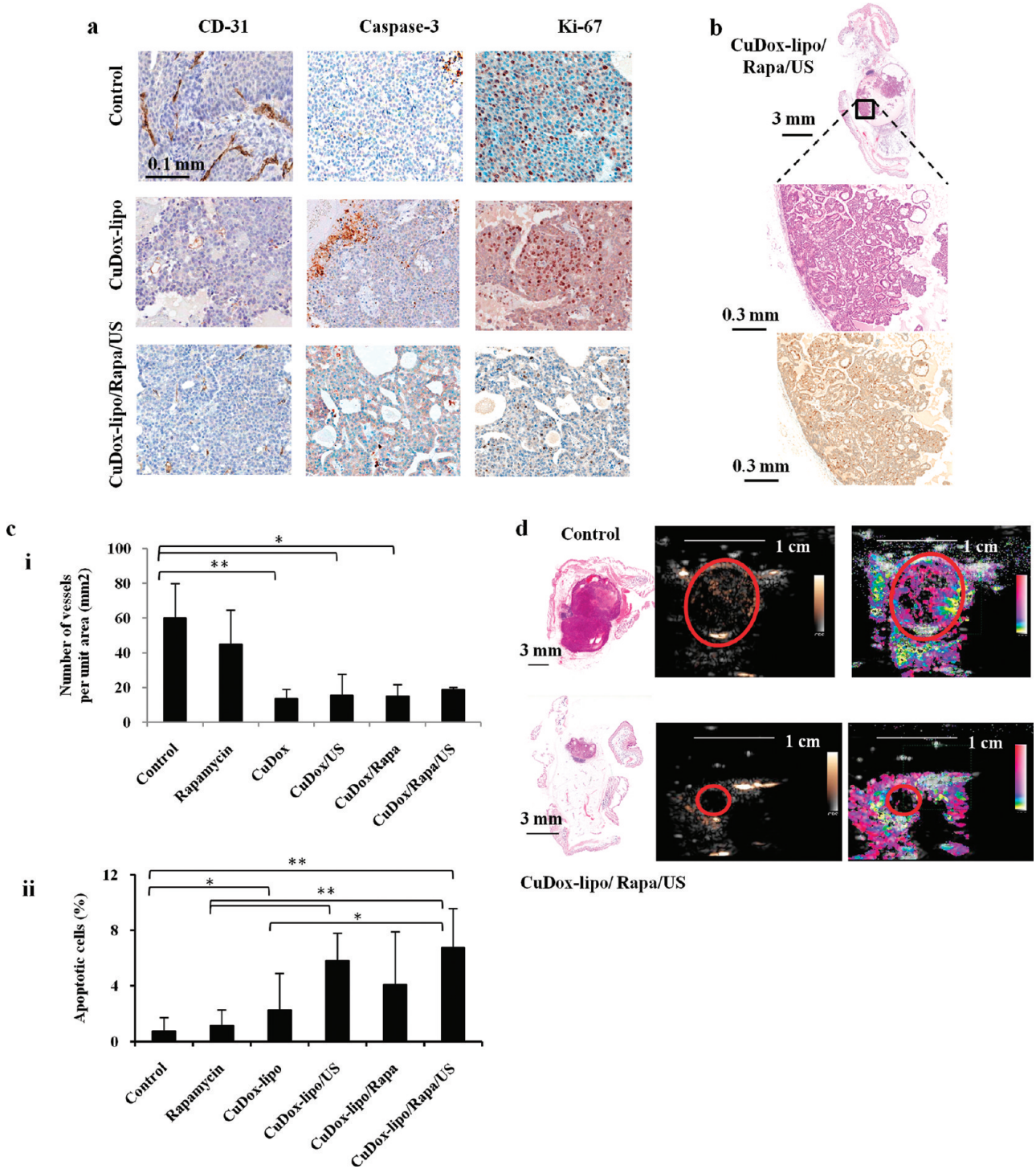

Figure 6. Histology and immunohistochemistry of tumors treated with sole or combined therapies using copper−doxorubicin liposomes. (a) Immunohistochemistry of selected tumors treated with copper−doxorubicin liposomes (CuDox-lipo) or combined therapy with rapapmycin and ultrasound (each at 28 days post onset of treatment) compared to control tumor at 18 days after ip injection of diluent. (b) Histological images of a tumor treated with copper−doxorubicin liposomes combined with rapamycin and ultrasound (CuDox/Rapa/US) H&E (upper), magnified H&E of tumor indicating areas with change in tumor phenotype (middle), anti Ki-67 image (lower). (c) Immunohistochemical quantification of number of vessels per unit area (i) and percent apoptotic cells (ii) across different therapies post 28 days of treatment compared to control at 18 days post ip injection of diluent. (d) H&E (left), corresponding ultrasound (US) contrast agent images (middle) and parametric US images (right) from a diluent-treated control (upper panels) and CuDox/Rapa/US-treated tumor at 28 days (lower panels) with tumor circled in red. In US contrast agent images, density of contrast agent (yellow) is proportional to vascular density. Parametric US images indicate fast flowing arteries in yellow, slow flow in capillaries in pink. Statistical analyses were performed using one-way ANOVA followed by a Tukey post hoc test. *$p < 0.05$, **$p < 0.01$.

Figure 6d), where functional vasculature was not detected in the copper−doxorubicin liposomes plus rapamycin plus therapeutic ultrasound-treated tumors. Gold indicates the presence of contrast agent within the control tumor in the middle panels, and yellow indicates fast flow within the control tumors in the right upper panel (Figure 6d).

Discussion

Long-circulating copper-doxorubicin particles were repeatedly injected at intervals of 3−4 days without evident toxicity to skin or cardiac tissue. To simulate clinical scenarios, long-term studies of anthracycline cardiotoxicity in animals are required; we use such a study to confirm the combined efficacy and safety of our particle.[7] The treatment of highly aggressive tumors with initial volumes above 100 mm^3 and doubling times of a few days is challenging. Doxorubicin must be efficiently and uniformly delivered, and sufficient time must be available for doxorubicin-initiated inhibition of DNA biosynthesis. Here, rapamycin reduced tumor proliferation and therapeutic ultrasound enhanced delivery; each significantly improved the efficacy of liposomal doxorubicin.

We loaded a stable drug−metal complex, rather than the free drug, and demonstrated an improved therapeutic profile. The presence of copper−doxorubicin precipitates inside the liposomes was confirmed by cryo-electron microscopy, and was distinct from the fine precipitate in copper liposomes[9] and needle-like doxorubicin crystals within Doxil.[31,32] Doxorubicin fluorescence was quenched by the interaction of doxorubicin with copper and was restored only by the combination of liposome disruption and trans-chelation, thus confirming both the complex formation and its extended stability in circulation. In the presence of albumin, copper trans-chelation from the copper−doxorubicin complex was pH dependent; dissociation was low at physiological pH but rapid in an acidic environment. Thus, stability was optimized for minimal toxicity in circulation and maximal efficacy in tumors. The stability of the copper−drug complex was further indicated by the concentration of copper assessed by ICP-MS, which corresponded well with the doxorubicin fluorescence and liposomal shell concentration, as quantified by PET.

Toxicities associated with frequent, multiple injections of Doxil have been mitigated clinically by changes in the schedule of administration.[1] With copper−doxorubicin liposomes, the toxicity to skin, heart and other organs from multiple injections was lower than with Doxil. As in ref 33, no evidence of copper toxicity was detected. We hypothesize several potential mechanisms for the reduced toxicity. First, spectroscopic studies have shown that copper forms two different complexes with doxorubicin, Cu:Dox (1:2) at pH 6−8.5 as demonstrated in our study and Cu:Dox (1:1) below pH 6.[17] The lipid membrane permeability of the intact copper−doxorubicin complex is expected to be reduced as compared with doxorubicin. Second, anthracyclines generate reactive oxygen species during complexation with copper or iron and the resulting radicals have been assumed to be the primary mechanism for the cardiac toxicity of doxorubicin.[7] The creation of the copper−doxorubicin complex before injection ultimately reduces reactive oxygen species that would otherwise be produced by formation of a metal−doxorubicin complex *in vivo*. Third, the accumulation of extraliposomal copper−doxorubicin within the heart is expected to be small, since a primary determinant of cardiac accumulation is lipophilicity. Finally, the creation of a low pH environment within the liposomal interior is a component of many active loading methods;[34−36] however, during extended circulation in the higher pH of the blood pool, this pH gradient can reverse and transport of the drug through the bilayer may be enhanced.[34,37] In the strategy reported here, such a gradient is not required to achieve drug loading and thus extended stability is expected. Still, the plasma concentration was ∼10% lower than that of Doxil at 24 h and many factors could influence the relative circulation stability. Thus, reduced blood circulation may play a role in the reduced toxicity.

We demonstrated that direct optical imaging and spectroscopy of doxorubicin is a viable tool, as also discussed in ref 38. At 24−48 h following the administration of Doxil, doxorubicin fluorescence in the skin was several times higher than with copper−doxorubicin or free doxorubicin and trends of accumulation or clearance were assessed. With the administration of Doxil, the spectrum indicated encapsulated drug within the skin and enhanced accumulation in the heart.

Insonation of one tumor with a 2 min increase in temperature to 42 °C resulted in engorgement of blood vessels with red blood cells and enhanced the accumulation of liposomes and of copper within the insonified tumor. The resulting accumulation of ∼20 μg of doxorubicin/g of tumor exceeds the reported therapeutic concentration of doxorubicin.[39,40] While ultrasound alone did not reduce tumor growth or enhance survival, the combination of copper−doxorubicin liposomes with ultrasound was efficacious ($p < 0.001$), greatly reducing or eliminating viable tumor cells after 28 days of treatment.

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The Met-1 syngeneic model is a well-perfused epithelial cancer. The vascular response to ultrasound is expected to decrease in poorly perfused tumors.

The opportunity to deliver relatively large quantities of doxorubicin with reduced toxicity was exploited here to achieve a regression of our highly aggressive tumor model. The antitumor activity of copper−doxorubicin liposomes alone was similar to that of the ammonium sulfate-loaded doxorubicin liposomes, although with reduced toxicity. With this single therapy, tumor growth was decreased as compared with the saline control ($p < 0.001$). Efficacy was further enhanced by the combination of copper−doxorubicin liposomes with rapamycin ($p < 0.05$) or ultrasound ($p < 0.001$). Altogether the results suggest that the copper−doxorubicin complex preserved the anticancer activity of doxorubicin, reduced toxicity and facilitated a multidose strategy producing regression or tumor elimination.

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Supporting Information Available: Additional experimental details and figures as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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