Long-Circulating Amphiphilic Doxorubicin for Tumor Mitochondria-Specific Targeting

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ABSTRACT: The mitochondria have emerged as a novel target for cancer chemotherapy primarily due to their central roles in energy metabolism and apoptosis regulation. Here we report a new molecular approach to achieve high levels of tumor- and mitochondria-selective delivery of the anticancer drug doxorubicin. This is achieved by molecular engineering which functionalizes doxorubicin with a hydrophobic lipid tail conjugated by a solubility-promoting polyethylene glycol polymer (amphiphilic Doxorubicin or amph-DOX). *In vivo*, the amphiphile conjugated to doxorubicin exhibits a dual function: i) it binds avidly to serum albumin and hijacks albumin's circulating and transporting pathways, resulting in prolonged circulation in blood, increased accumulation in tumor, and reduced exposure to the heart; ii) it also redirects doxorubicin to mitochondria by altering the drug molecule's intracellular sorting and transportation routes. Efficient mitochondrial targeting with amph-DOX causes a significant increase of reactive oxygen species (ROS) levels in tumor cells, resulting in markedly improved antitumor efficacy than the unmodified doxorubicin. Amphiphilic modification provides a simple strategy to simultaneously increase the efficacy and safety of doxorubicin in cancer chemotherapy.

Keywords: Doxorubicin, Mitochondria, Amphiphiles, Chemotherapy, Drug delivery.

■ INTRODUCTION

Anthracyclines, especially doxorubicin (DOX), have broad-spectrum antineoplastic activities and have been extensively used in cancer chemotherapy for more than 40 years.[1-2] However, intrinsic or acquired drug resistance greatly limited the success of DOX in the clinical management of cancers.[2-5] Additionally, like many chemotherapeutic drugs, doxorubicin targets both proliferating cancer and normal cells, such treatment can lead to severe off-target toxicity and side effects,[3-5] especially in patients with advanced disease requiring dose escalation.

An emerging strategy to simultaneously enhance efficacy and reduce toxicity is targeted delivery of doxorubicin to tumor mitochondria,[6-17] the unique cellular organelles that play a central role in the regulation of fundamental tumor cellular functions, including cellular metabolism, adenosine triphosphate (ATP) production, reactive oxygen species (ROS) generation, and apoptosis, among many others.[7-9] Delivery of DOX to mitochondria may bypass the classical resistance pathways, while at the same time improving or maintaining its cytotoxic effects.[9] Mitochondria-targeted anti-cancer therapeutics can eradicate resistant cancer cells through several possible mechanisms.[9, 14-15, 18] For example, mitochondria-specific delivery of doxorubicin or similar anthracyclines has been shown to exert their cytotoxic effects by intercalating mitochondrial DNA[9] or by oxidative damage of DNA, membrane-bound proteins and enzymes,[14-15, 18] resulting in a significantly enhanced cytotoxic effect in cancer cells.

Despite intensive research, to date, no mitochondria-targeting pharmaceutical formulations have been approved clinically. This is in part because *in vivo*, a successful mitochondriotropic delivery requires multi-levels of targeting: it must achieve sufficient circulating time in blood for drug exposure, and must achieve tumor tissue- and tumor cell-specific accumulation followed by mitochondria-specific accumulation.[7-15] Although considerable research attempts have been made to incorporate multiple targeting ligands for mitochondria-targeted delivery,[7-15] many of these strategies fail to overcome the multiple biological barriers *in vivo*. For example, delocalized lipophilic cations (DLCs) are compounds that efficiently accumulate within mitochondria, mainly in response to mitochondria membrane potential.[19-25] However, the intrinsic toxicities associated with DLCs have hampered their clinical development.[26-27] Further, such small molecular compounds fail to achieve the multi-levels of targeting *in vivo*, in some cases, non-specific accumulation in brain, heart, liver, and muscle were observed.[19] Attempting to target mitochondria also includes the use of synthetic peptides and amino-acid-based transporters which either derived from mitochondrial targeting sequence (MTS)[9, 28-30] or comprised of altered lipophilicity and charge that exhibit

strong affinity toward mitochondria.[31-34] The major issues of these peptides are their considerable molecular sizes, poor water solubility, lack of membrane permeability and low serum stability.[8, 23] Another strategy is to make use of emerging biopharmaceutical nanotechnologies, which have demonstrated to offer many advantages compared with traditional small molecular drugs alone. Drug carriers based on nanoparticles are modified with tumor- and/or mitochondria-specific ligands.[13, 35-38] However, multi-level drug targeting nanoparticles require complex designs to increase drug encapsulation efficiency, to evade host immune system, and to release drug upon intracellular exposure.[39]

Here we show a simple amphiphilic modification on doxorubicin (amph-DOX) can overcome multiple biological barriers and selectively target tumor mitochondria *in vivo*. This is achieved by molecular engineering which functionalizes doxorubicin with a lipophilic diacyllipid connected by a polyethylene glycol linker (**Fig. 1A**). This amphiphilic modification fulfills a two-fold purpose: first, amph-DOX reaches and penetrates solid tumor by "hitchhiking" on albumin protein.[40-42] Albumin-binding increases the hydrodynamic size of doxorubicin and prolongs its circulating time in the blood.[41] Albumin-binding also increases DOX's uptake in the tumor by the enhanced permeation and retention (EPR) effect and more importantly, by active metabolic uptake because tumors heavily use albumin as an energy and nutrient source.[40-41] Second, amph-DOX accumulates in mitochondria following tumor cell uptake through a yet unknown mechanism. Compared with free DOX, i.v. injection of amph-DOX heavily accumulates in tumor but not in heart. Efficient mitochondria targeting with amph-DOX causes a significant increase in oxidative stress in tumor mitochondria, resulting in markedly improved antitumor efficacy. Thus, *in vivo*, amphiphilic functionalization improves the doxorubicin molecule's physicochemical properties, which in turn re-defines its bioavailability, organ and subcellular distributions. Amphiphilic modification represents a simple, effective, and nontoxic molecular approach for mitochondria-targeted delivery of doxorubicin *in vivo*.

■ EXPERIMENTAL SECTION

Materials and chemicals. All chemicals were purchased from Sigma-Aldrich and used without further purification unless noted otherwise. Doxorubicin hydrochloride was purchased from LC laboratories, 3-(N-succinimidyloxyglutaryl) aminopropyl, polyethyleneglycolv-carbamyl distearoylphosphatidyl-ethanolamine (DSPE-PEG_{2000}-NHS) was obtained from Biochempeg scientific Inc. Cholesterol polyethylene glycol NHS and DSPE-PEG_{2000}-hydrazide were purchased from Nanocs Inc.

Animals and cells. Animals were housed in the United States Department of Agriculture (USDA)-inspected Wayne State University animal facility under federal, state, local and NIH guidelines for animal care. Female C57BL/6 mice (5-8 weeks) were obtained from the Jackson Laboratory. B16F10, 4T1 cells were purchased from ATCC. The OVCAR-8 human ovarian carcinoma cell line and its doxorubicin resistant derivative NCI/ADR-RES cell line were obtained from NIH. Cells were cultured in complete medium (MEM, 10% fetal bovine serum (Greiner Bio-one), 100 U/mL penicillin G sodium and 100 μg/mL streptomycin (Pen/Strep).

Synthesis and characterization of amph-DOX. 5 mg Doxorubicin hydrochloride (DOX) and 38 mg DSPE-PEG_{2000}-NHS (molar ratio of DSPE-PEG_{2000}-NHS : DOX = 1.5 : 1) were dissolved in 500 μL and 4.5 mL of dimethyl sulfoxide (DMSO), respectively. These two solutions were mixed and activated with 3 μL triethylamine (TEA). After stirred in the dark at 25 ˚C for 24 hours, the solution was dried in a stream of air for 72 h to evaporate DMSO. The remaining reaction residues were dissolved in 5 mL phosphate buffered saline (PBS) buffer (0.1 M, pH = 7.4) with sonication. Amph-DOX was purified by reverse-phase HPLC with a C_4 column (Thermo Scientific, 250 x 4.6 mm, 5 μm). Samples of 100 μL were injected and separations were performed at 25 ˚C using a flow rate of 1.0 mL/min by a liquid chromatography system (Agilent Technologies 1220 Infinity). DSPE-PEG_{2000}-NHS and DOX were detected by measurement of the UV absorbance at 260 nm and 485 nm, respectively. DSPE-PEG_{2000}-DOX was monitored by both wavelengths. A solvent gradient **(Table S2)** with methanol and triethylammonium acetate (TEAA) buffer (0.1 M pH = 7.4) was used for the separation. Amph-DOX was collected (typical retention time: 12 min to 14 min). The solvent was air dried and the final product was dissolved in DMSO and concentration was determined by UV/ VIS spectrophotometry (Thermo Scientific). DSPE-PEG_{2000}-DOX (amph-DOX) was confirmed by ^{1}H-NMR (Varian, 400 MHz) and Mass spectrometry analysis.

Albumin Binding Assay. A gel electrophoresis mobility shift assay was used to detect albumin protein binding with amph-DOX. The solution of free DOX and amph-DOX were incubated with freshly isolated mouse blood for 4 hours at 37 ˚C. The resulting mixtures were separated into two equal volumes. Half of the sample was used for flow cytometry analysis and the other half for fluorescent spectroscopy and gel electrophoresis. Samples were loaded for electrophoresis run under 200 V for 30 min through 0.5% agarose gel. Images were recorded using a digital camera under UV illustration. For the FRET assay, Alexa660 (Thermo Fisher Scientific) labeled bovine serum albumin (BSA-Alexa660) was incubated with 10 μM DOX or amph-DOX in PBS (pH 7.4) for 4 h at 37 ˚C,

after that, samples were analyzed by spectrofluorometer (JASCO FP-6500). DOX or amph-DOX were excited at 470 nm.

***In vitro* cell viability assay.** The antiproliferation activities of the free anticancer drug DOX and the amphiphilic drug amph-DOX against B16F10, 4T1, OVCAR-8 and NCI/ADR-RES cells were evaluated using Alamar Blue assay method. B16F10, 4T1 cells (5×10^4 cells per well) and NCI/ADR-RES cells (1×10^5 cells per well) cultured with 100 μL medium were seeded in 96-well plates, respectively, and incubated overnight to adhere. Cells were incubated with free DOX or amph-DOX at serial doxorubicin concentrations ranging from 0.05 to 10 μM for 24 or 48 h, following by the addition of 10 μL alamarBlue® reagent and incubated for another 1 h. Cells treated with complete medium were used as the controls. Finally, the absorbance was measured at 570 nm with 600 nm as a reference by a microplate reader (Thermo Scientific). The percentage of surviving cells was calculated as the absorbance ratio of treated to untreated cells. The half maximal inhibitory concentration (IC_{50}) was determined from the dose-response curve. All the experiments were carried out in triplicate.

***In vitro* uptake and subcellular distribution.** The cell uptake of free DOX and amph-DOX was examined in B16F10 or NCI/ADR-RES cells by flow cytometry. Cells were seeded to 48-well plate (1×10^6 cells per well) and incubated at 37 ˚C for overnight. The cell medium was removed and replaced with DOX and amph-DOX at a final concentration of 1.0 μM for different time periods. The cells were harvested and washed with 1 × PBS buffer three times and analyzed by flow cytometry using an Attune acoustic focusing cytometer (Applied Biosystems). Each assay was performed in triplicate.

To determine the intracellular distribution of amph-DOX, cells (1×10^4 cells per well) were seeded on a coverslip in 6-well plates and cultured at 37 ˚C for 24h to achieve confluence. For tracking mitochondria by MitoTracker Green FM, cells were treated with free doxorubicin (DOX) or amph-DOX at the concentration of 1 μM (37 ˚C) for 4 h. After treatment, the cells were washed with PBS, fixed with 3% paraformaldehyde (PFA), stained with MitoTracker Green FM (500 nM) (Invitrogen) and DAPI (200 nM) (Invitrogen), and washed with PBS before imaging. For tracking mitochondria by CellLight Mitochondria-RFP BacMam 2.0 (Invitrogen), cells were transfected with 10 μL CellLight reagent for 24 h. After that, the cells were washed with PBS and incubated with free doxorubicin or amph-DOX (1 μM) at 37 ˚C for 4 h. Then the cells were washed with PBS, fixed with 3% paraformaldehyde (PFA), stained with DAPI (200 nM) (Invitrogen) and washed with PBS. Images were captured

by Zeiss confocal microscope (LSM 780) with a 63 × oil-immersion objective. Images were obtained by the following excitation/emission settings: MitoTracker Green (excitation 488 nm, emission 515 nm bandpass filter), doxorubicin (excitation 488 nm, emission 560 nm bandpass filter), CellLight Mitochondria-RFP (excitation 561 nm, emission 585 nm bandpass filter).

Image colocalization analysis. ImageJ (NIH) with Coloc 2 of Fiji's plugin was used for colocalization analysis**.** The colocalization of mitochondria and DOX or amph-DOX was quantified based on the red/green signal intensities and that of nuclei and DOX or amph-DOX was based on red/blue signal intensities. Pearson's (Ps) and Manders' (M1/M2) coefficients were calculated from 1 individual field of view in each of the n = 3 independent experiments (total 12 fields).

Quantification of free DOX and amph-DOX in the intracellular compartments. Cells were plated at a concentration of 1×10^8 with 15 mL of media in 100-mm diameter tissue culture dishes and allowed to grow overnight. DOX and amph-DOX (10 μM) were added and incubated for different time periods. After internalization, the mitochondria and the nuclei were isolated using a mitochondria isolation kit (ThermoFisher Scientific) and a nuclei isolation kit (Sigma), respectively, following manufacturer's instructions.

The amount of DOX or amph-DOX in each fraction was quantified by measuring fluorescence intensity from doxorubicin after solvent extraction. All the experiments were carried out in triplicate.

DOX-induced reactive oxygen species (ROS) measurement. 1×10^6 cells were pre-cultured in 48-well plates for 12 hours, cells were then incubated with DOX or amph-DOX at a final concentration of 1, 5 or 10.0 μM for 4 h. After treatment, cells were washed once with 1 × PBS and incubated 30 min at 37 ˚C in PBS with a final concentration of H2DCFDA at 10 μM. No treatment group was used as a positive control for the quantifications of mitochondrial ROS production. Finally, the cells were washed and analyzed by flow cytometry.

For visualizing intracellular ROS, 1×10^4 cells were plated on coverslip in 6-well plates, and were treated with DOX or amph-DOX (10.0 μM final concentration) for 4 h. After treatment, cells were washed once with 1 × PBS and incubated for 30 min at 37 ˚C in PBS with a final concentration of H2DCFDA at 10 μM. Finally, cells were washed with PBS, stained with DAPI (200 nM) (Invitrogen), MitoTracker Green (500 nM) (Invitrogen) and washed with PBS. Imagines were captured by Zeiss confocal microscope (LSM 780) with a 63 × oil-immersion objective.

***In vivo* pharmacokinetics evaluation.** To measure the pharmacokinetics, 1×10^6 B16F10 melanoma cells suspended in 100 μL of PBS buffer were inoculated s.c. in the flank region of 5-wk-old C57BL/6 mice. When the tumor volume reached ~ 500 mm^3, mice were randomly assigned into three groups (n = 8 mice per group). Free doxorubicin (10 mg/kg) or amph-DOX (10 mg/kg equivalent doxorubicin) were injected into the tumor bearing mice intravenously via the tail vein. Plasma were separated by centrifugation (15,000 × g for 10 min at 4 °C) after blood samples were collected at 30 and 60 min, 2, 4, 6, 12 and 24 h post drug administration (n = 4 at each time point). Sera were diluted three times in PBS and drug concentrations in sera were calculated from standard curve by measuring the fluorescence intensity of DOX in each sample, correcting against sera from blood samples of non-treated animals. The fluorescence intensity was fitted into a calibration curve to determine the DOX concentration. Half-life ($t_{1/2}$) was calculated from DOX concentrations in the area vs. time curve and was fit by one-phase exponential decay (Graphpad prism).

***In vivo* biodistribution study.** For *in vivo* biodistribution study, B16F10 tumor (volume ~ 500 mm^3) bearing C57BL/6 mice (n = 8 mice per group) were injected intravenously with either free doxorubicin (10 mg/kg) or amph-DOX (10 mg/kg equivalent doxorubicin). 2 or 24 h after drug administration (n = 4 at each time point), mice were sacrificed and the spleen, heart, brain, lung, kidney, tumor, and liver were collected. Tissue samples were flash frozen and stored at −80 ˚C until extraction. Tissue samples were weighed and homogenized by biomasher II tube (Kimble) and sonicated in 9 parts (v/w) of PBS. In a typical procedure, 200 μL tissue homogenate were extracted with 50 μL 10% Triton X-100 (v/v) and 750 μL 0.75 N HCl in dichloromethane for 12 h at −20 ˚C in the dark. Fluorescence intensity was read and background fluorescence was corrected by subtracting extracts from un-treated animal samples. The concentrations were determined by comparing the fluorescence intensities to a calibration curve established by adding known amounts of doxorubicin to homogenates of un-treated tissue samples.

Confocal microscopy of tumor tissue. Fresh tissue samples were washed with PBS and fixed in Formaldehyde fixation buffer. After 48 h fixation, each tissue was merged in optimal cutting temperature compound, freeze at -80 ˚C in the dark and cut into 10 μm thick tissue sections using a cryostat. The frozen tissue slides were incubated with 100 μL diluted (1 μL MITO-ID® Red in 10 mL 1 × assay buffer, Enzo life sciences) reagent for 30 min and DAPI (200 nM) for additional 15 min. Finally, slides were washed three times by PBS and imaged. Images were captured by Zeiss confocal microscope (LSM 810) with a 63 × oil-immersion objective.

Tumor model. B16F10 (5.0×10^5 cells) were subcutaneously inoculated into the right flank of 5-6-week-old C57BL/6 mice. On day 5 (tumor volume ~ 30 mm^3), mice were i.v. injected with 5 mg/kg doxorubicin hydrochloride or amph-DOX on days 5, 8, and 11. Tumor length and width were measured with digital calipers, and the tumor volume was calculated using the following equation: Tumor volume (V) = length × $width^2/2$.

Statistical analysis. Comparisons of mean values of two groups were performed using unpaired Student's t tests. To analyze the statistical difference between groups, a one-way analysis of variance (ANOVA) with Bonferroni post-test was used. All the values were expressed as means ± standard error of mean. GraphPad Prism software was used for all the statistical analyses. ***P<0.001, **P<0.01, *P<0.05. NS, not significant.

■ RESULTS AND DISCUSSION

Amph-DOX binds to serum albumin in blood. Anticancer drug delivery based on endogenous serum proteins is an attractive 'self-delivering' approach in targeting cancer cells *in vivo*.[40-41] We recently developed an 'albumin-hitchhiking' molecular approach which uniquely delivers subunit vaccines to lymph nodes after subcutaneous injection.[42] In this approach, subunit vaccines are conjugated to diacyllipid-polyethylene glycol, a structurally optimized albumin-binding domain and following subcutaneous injection, accumulate in the draining lymph nodes by binding to and transporting with endogenous albumin.[42] Diacyllipid-polymer self-assembles into micelles in aqueous buffer.[43] However these micelles are kinetically unstable, especially in the presence of lipid-binding albumin.[42-44] In addition to albumin binding, these amphiphilic molecules also exhibit intrinsic affinity toward plasma membrane, as demonstrated by the rapid uptake and confined intracellular membrane-domain-selective accumulation.[42, 45-46] Thus, in the presence cells and serum, there exists a complicated three-way equilibrium: amph-DOX forms micelles, but amph-DOX can also insert its diacyl tails into cell membranes or bind to albumin protein. This three-way equilibrium is delicately controlled by 1), the concentrations of albumin; 2), the molecular weight (or length) of both lipid tails and PEG.[42-43, 47] We showed that the equilibrium shifts toward albumin binding when a long diacyl lipid (≥ 16 carbons) and a long polyethylene glycol (≥ 36 ethylene glycol units) is used.[42] In order to translate this 'albumin-hitchhiking' vaccine approach to deliver anti-cancer drugs, we modified doxorubicin with a structure optimized amphiphilic albumin-binding diacyl lipid linked by a polyethylene glycol linker (**Fig. 1A and Fig. S1**). We hypothesize that the amphiphilic functionalization alters doxorubicin's physicochemical properties,

which in turn re-defines its bioavailability, organ and subcellular distributions, improves its therapeutic efficacy and reduces DOX-associated toxicity.

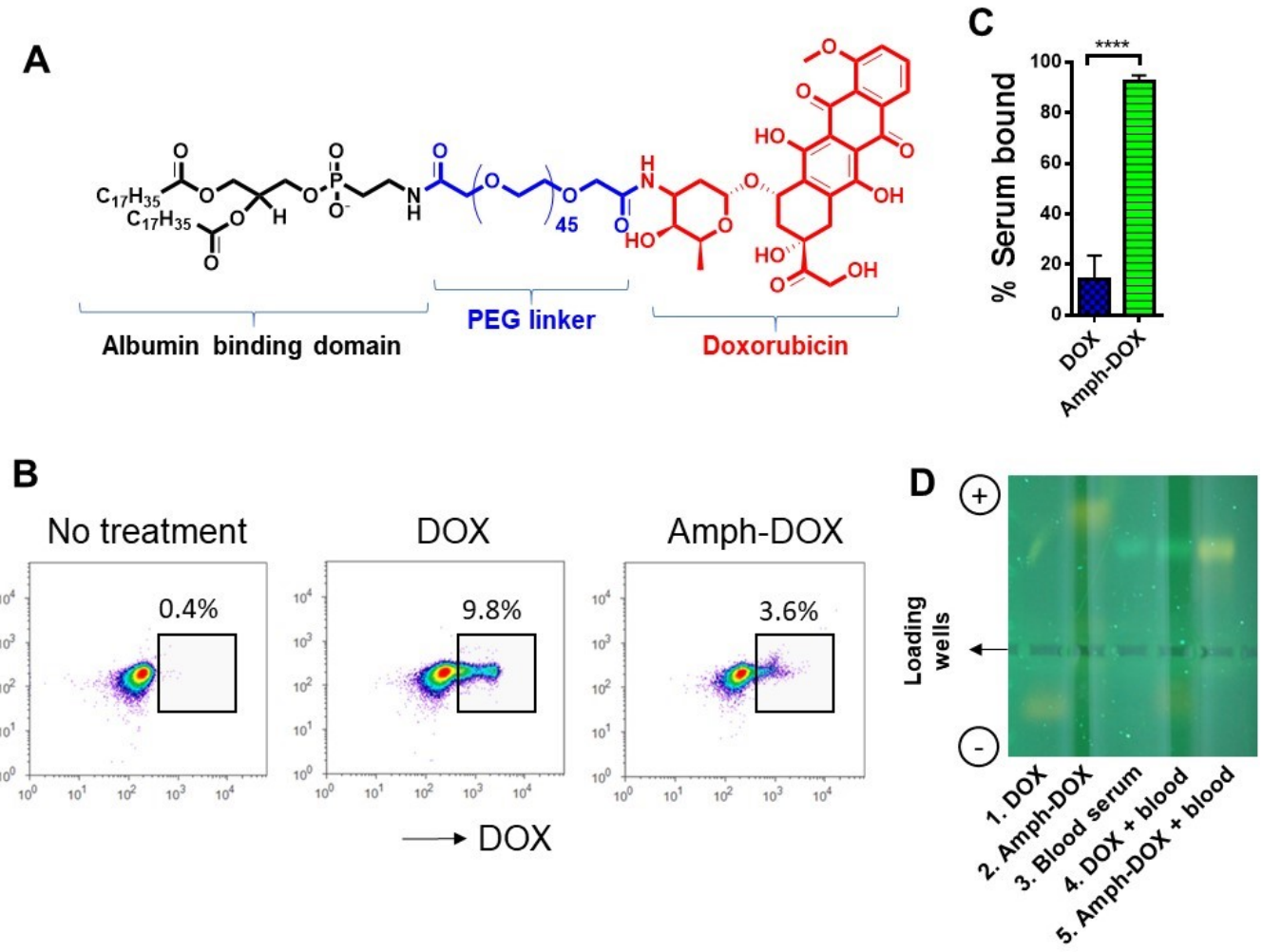

Figure 1. Amph-DOX binds to albumin in blood. (*A*) Molecular structure of amphiphilic doxorubicin (amph-DOX). (*B-D*) amph-DOX, but not free DOX binds to serum albumin in blood. Mouse blood samples were incubated with 0.5 μM DOX or amph-DOX for 4 h, after centrifuge, cells were analyzed by flow cytometry (*B*) and sera were analyzed by gel electrophoresis (*D*). DOX concentrations in serum were quantified by fluorescence spectroscopy (*C*).

The amph-DOX was synthesized and purified as previously reported (**Fig**. **S1**).[48] Due to the molecular similarities between amph-DOX and DSPE-PEG_{2000}-NHS, the complete separation of amph-DOX after reaction by a preparative HPLC was not practical (**Fig. S1**). However, we found that DSPE-PEG_{2000}-NHS or its hydrolyzed product did not affect the subsequent experiments. The self-assemble and albumin-binding properties were

demonstrated by dynamic size scattering (DLS), transmission electron microscopy (TEM) and Förster resonance energy transfer (FRET) (**Fig. S2**). To test whether amph-DOX can bind to albumin in blood, free DOX or amph-DOX were incubated with freshly isolated mouse blood for 4 hours at 37°C. The partition of DOX between serum albumin and blood cells was subsequently analyzed and quantified by fluorescence spectroscopy, gel electrophoresis, and flow cytometry. Upon incubation with freshly isolated blood, free DOX was detected in 9.8% of the blood cells, which was almost 3 times more than that of amph-DOX (3.6%) (**Fig. 1B**). This observation suggests free DOX interacts with erythrocytes, consistent with previous publications.[49-52] In contrast, despite being in the possession of lipophilic diacyl lipid tail, amph-DOX had less association with the cells in the blood. Fluorescence measurements by spectroscopy indicated that around 92% of amph-DOX and 18% of free DOX remained in the blood serum (**Fig. 1C**). Further, gel electrophoresis analysis **(Fig. 1D and Fig. S3)** indicated that the vast majority of the amph-DOX in serum bound to serum albumin, showing a light-yellow fluorescent band co-migrated with albumin (**Fig. 1D,** lane 5). This band was distinct from albumin as pure serum showed a major albumin band with green autofluorescence under ultraviolet light (254 nm) (**Fig. 1D,** lane 3). In contrast, free DOX incubated with blood migrated as a single band toward the negative electrode (**Fig. 1D,** lane 4), indicating a lack of interaction with albumin. These data strongly suggest that, unlike unmodified DOX, which extensively interacts with erythrocytes,[49-52] amph-DOX binds to albumin protein in whole blood and warrants further investigation of using this 'albumin-hitchhiking' platform for targeted drug delivery.

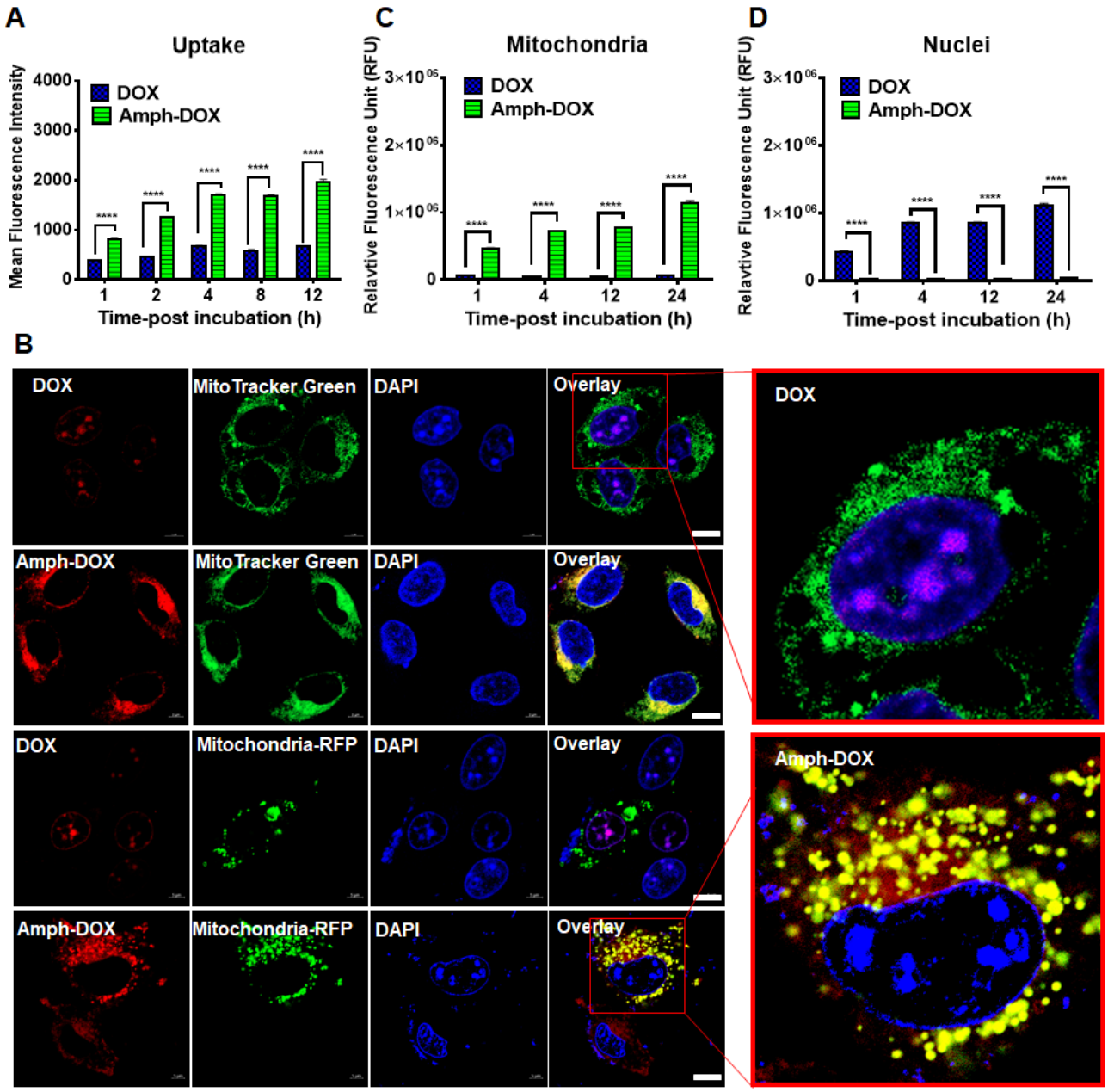

Figure 2. Amph-DOX selectively accumulates in mitochondria *in vitro*. (*A*) Kinetics of amph-DOX or DOX internalization showing amph-DOX is quickly internalized by B16F10 cells. The uptake is analyzed by measuring the mean fluorescence intensity by flow cytometry. (*B*) Confocal microscope characterization of B16F10 cells showing the cellular uptake and intracellular distribution of free doxorubicin or amph-DOX (concentration of 1 μM) at 4 h. B16F10 cells were treated with free DOX, and amph-DOX (red) and stained for mitochondria (green) by Mito-Tracker Green (upper two panels) or Mitochondria-RFP (lower two panels). Cell nuclei were stained with DAPI (blue). Note that some cells were not transfected in the Mitochondria-RFP treated group. Scale bar = 10 μm. (*C and D*) Quantification of DOX or amph-DOX in (*C*) mitochondria and (*D*) nuclei of B16F10 cells. 1 x 10^8 cells

were incubated with 10 μM DOX or amph-DOX for 1, 4, 12, 24 h. Mitochondria and nuclei were isolated by isolating kits and DOX fluorescence were quantified by fluorescence spectroscopy after solvent extraction.

Amph-DOX selectively accumulates in mitochondria *in vitro*. To investigate the uptake and intracellular distribution of amph-DOX related to DOX parent compound in cancer, murine melanoma B16F10 cells were incubated with amph-DOX or DOX in the presence of bovine serum and analyzed by flow cytometry and confocal laser scanning microscopy (CLSM). The melanoma model was selected due to its intrinsic resistance to DOX.[53] *In vitro*, amph-DOX showed a rapid and enhanced uptake, reaching high levels of DOX concentration 1 hour after incubation in B16F10 cells (**Fig. 2A**). In cells treated with free DOX, the drug concentration slowly increased over 12 hours, reaching 30% of the level of that treated with amph-DOX (**Fig. 2A**) when assayed by flow cytometry. The subcellular locations of amph-DOX in B16F10 cells were subsequently determined by confocal microscopy. As expected, free DOX exhibited strong nuclear accumulation following drug exposure, determined by using the intrinsic DOX fluorescence (**Fig. 2B**). In contrast, amph-DOX fluorescence was mainly confined in the mitochondria (**Fig. 2B**), demonstrated by analyzing the fluorescence colocalization with MitoTracker Green FM (Invitrogen), a mitochondria-specific dye (**Fig. 2B,** upper two panels). The mitochondria-selective accumulation of amph-DOX was unexpected, as our previous amphiphilic oligonucleotides were mainly confined within the endolysosomal compartment.[42, 54] Analysis of LysoTracker Green (a lysosome-specific dye) colocalization by confocal laser scanning microscopy showed little overlap with amph-DOX (**Fig. S4**), suggesting amph-DOX does not accumulate within lysosomes. To verify the mitochondria accumulation, we used CellLight Mitochondria-RFP BacMam 2.0 (Invitrogen) to stain the mitochondrial matrix (**Fig. 2B** lower two panels). CellLight Mitochondria-RFP is a highly selective transfection-based approach which targets the red fluorescent protein to the mitochondria in live cells. Quantitative analysis using the ImageJ "Coloc 2" plug-in revealed significant spatial overlap between amph-DOX and both mitochondria dyes in B16F10 cells (Pearson coefficient, 0.57; Manders coefficient, 0.874/0.992; **Table S1**). For unmodified DOX, weak correlation of the red signals and the mitochondrial staining was demonstrated by low coefficient values (Pearson coefficient, 0.26; Manders coefficient, 0.196/0.039; **Table S1**).

Because DOX fluorescence is dramatically quenched upon DNA intercalation, the uptake quantification measured by flow cytometry (Fig. 2A) might not reflect the DOX concentrations after being delivered to different

subcellular locations. To verify the enhanced uptake and distribution results, we isolated the mitochondria and the nuclei from B16F10 cells, and the DOX concentrations were quantified by fluorescent spectroscopy after solvent extraction. Free DOX reached between 30%-70% of the uptake from amph-DOX at different time points (**Fig. S5A**). The uptake differences between flow cytometry and fluorescence spectroscopy are most likely due to the fluorescence quenching of DOX by different levels of DNA intercalation. Consistent with our confocal results, unmodified DOX accumulated primarily in the nuclei, accounting for 72% of the fluorescence within the cells in 24 h (**Fig. 2C, D** and **Fig. S5**). In contrast, in cells treated with amph-DOX, approximately 40% of the intracellular DOX fluorescence was in isolated mitochondria (**Fig. 2C, D and Fig. S5**). Though a fraction of the DOX might be lost during organelle isolation, these data clearly demonstrated the selective mitochondria accumulation in tumor cells after treatment with amph-DOX. Enhanced uptake and selective mitochondria accumulation of amph-DOX were not restricted to B16F10 cells, as similar intracellular distribution was observed in mouse breast tumor 4T1 cells (**Fig. S6**).

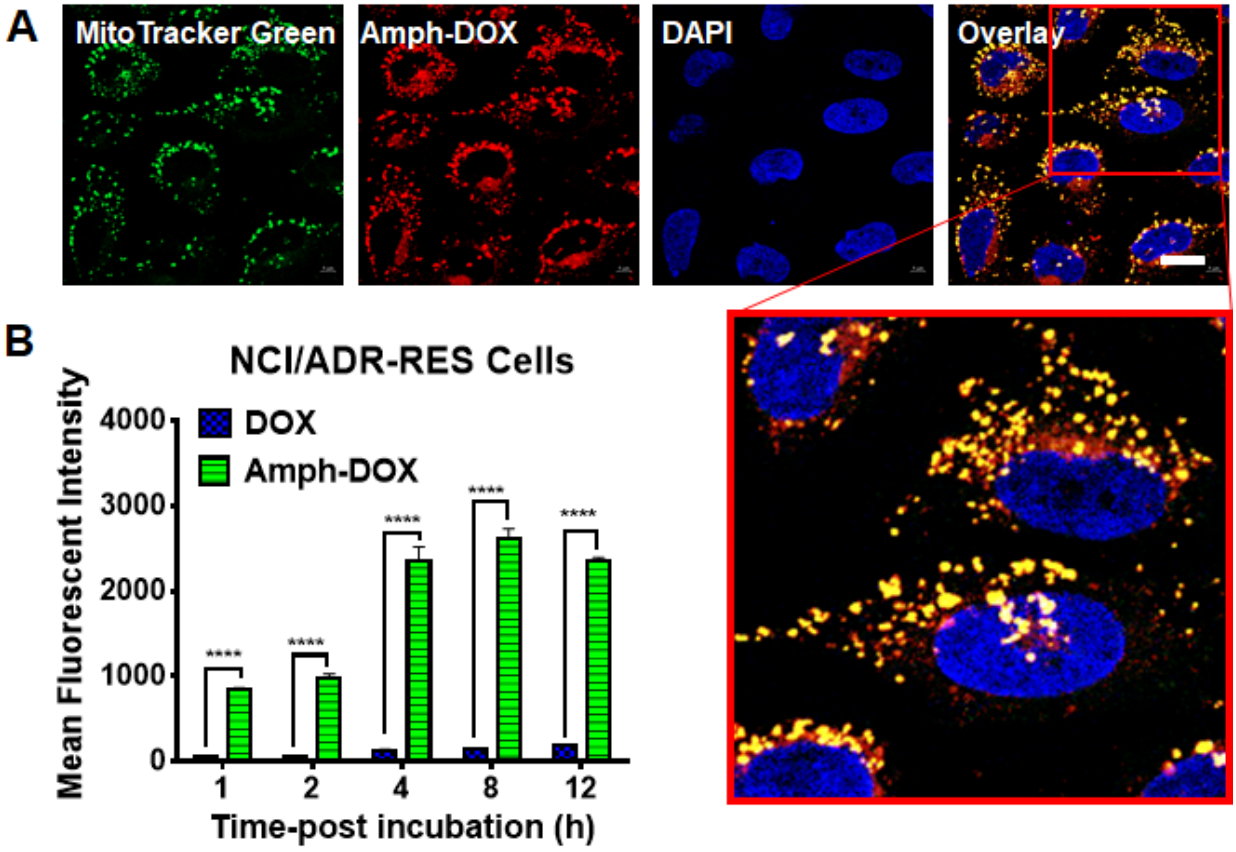

Figure 3. Amph-DOX enhances DOX uptake and accumulates in mitochondria in drug-resistant NCI/ADR-RES cells *in vitro*. *(A)* Confocal microscope characterization of NCI/ADR-RES cells showing the intracellular distribution of amph-DOX at 24 h. (scale bar: 10 μm) (*B*) Kinetics of amph-DOX or DOX uptake in NCI/ADR-RES cells.

Drug resistant cancer cells are known to overexpress P-glycoprotein (P-gp), which acts as an efflux pump and reduces Doxorubicin's uptake and retention.[3, 55] To test whether amph-DOX can increase intracellular net drug uptake in DOX resistant cells, human ovarian adenocarcinoma (NCI/ADR-RES) cells[56] were used to incubate with amph-DOX, or soluble DOX, and the DOX uptake, intracellular distribution were analyzed as before. These cells were established to resist doxorubicin treatment.[56] Similar to previous observation, DOX uptake in NCI/ADR-RES cells remained low throughout the incubation, presumably due to the P-gp mediated DOX efflux.[57] In contrast, rapid DOX uptake and prolonged retention were observed in cells treated with amph-DOX (**Fig. 3A, B**). Confocal microscopy analysis confirmed that in these DOX-resistant cells, amph-DOX selectively accumulated in mitochondria (**Fig. 3A**). These results demonstrate that efficient uptake and mitochondria accumulation can be achieved in DOX resistant cells, suggesting a plausible mechanism to overcome the drug-induced resistance.

Structural requirements of amphiphiles in mitochondrial trafficking. Targeting subcellular organelles via lipid modification on drugs have been extensively studied in the past.[58] It is generally believed that the lipid structure governs the intracellular sorting mechanisms and thus determines where the lipid-modified molecules localize within the cell.[58] However, no lipid has been shown to selectively accumulate in mitochondria. As amph-DOX exhibits an overall negative charge (**Fig. 1D**, lane 2), it is unlikely amph-DOX is concentrated in mitochondria in response to negative transmembrane potentials. To investigate the possible mechanisms for mitochondrial accumulation, we first set out to determine the uptake mechanisms of amph-DOX and compared that with DOX encapsulated DSPE-PEG_{2000} micelles (micelle-DOX).[59] Although micelle-DOX enhanced the levels of DOX uptake in B16F10 cells, it was primarily accumulated in the nuclei (**Fig. S8**). In addition, amph-DOX employs multiple uptake mechanisms in typical cell culture conditions (**Fig. S9**). To determine the role of albumin in the uptake and intracellular distribution, cell culture experiments were repeated in the absence or presence of FBS. *In vitro*, uptake of amph-DOX was inversely proportional to FBS content at first two hours, reflecting the shift of equilibrium toward cellular membrane insertion at low albumin concentrations (**Fig. S10, A, B**). However, after longer time incubation, similar levels of uptake were observed for amph-DOX in the presence or absence of FBS in B16F10

cells (**Fig. S10**, **B**). At low albumin concentrations, amph-DOX equilibrate between albumin-binding state and membrane insertion state (**Fig. S10, C, D**), both of which showed significantly better cellular uptake than free DOX. It is worth to point out that these *in vitro* uptake assays may not accurately reflect the *in vivo* process as the blood albumin concentration is ~10 times higher than that in cell culture medium. Nevertheless, amph-DOX accumulated in the mitochondria in the absence of FBS (**Fig. S10, E**), suggesting albumin is not involved in the intracellular sorting and trafficking of amph-DOX, and that the intracellular release of amph-DOX from albumin/amph-DOX complex is highly possible (**Fig. S11**). Finally, it appeared that intact amph-DOX conjugate traffics to mitochondria as similar amphiphilic-DOX linked via an acid labile hydrazone bond showed both mitochondrial and nuclear accumulation (**Fig. S12**).

To investigate whether amphiphilic modification via diacyl lipid PEG can be a generalizable approach for mitochondria-specific targeting, we modified fluorescein with the same amphiphilic PEG and tested its intracellular uptake in B16F10 cells. Interestingly, no mitochondria accumulation of amph-Fluorescein was observed (**Fig. S13**). To determine whether DSPE lipid is required in the mitochondria targeting, we conjugated DOX to cholesterol-PEG_{2000}. Unlike DSPE lipid which is negative charged, cholesterol is neutral and is less hydrophobic. Similar to amph-DOX, cholesterol-PEG_{2000}-DOX selectively accumulates in mitochondria (**Fig. S14**). These data suggest that amphiphilic modification on DOX can alter its intracellular distribution, and that the mitochondria accumulation can tolerant the amphiphilic structure to a certain degree. This observation rules out the possibility that amph-DOX is sorted and transported by lipid-specific proteins, instead, it favors the notion that the unique chemical and biophysical properties of amphiphilic DOX conjugates have key roles in their intracellular trafficking and distribution. Although the detail structure-function relationship remains unclear (e.g., whether PEG plays a role), it appeared the amphiphiles and DOX contributed jointly to the overall physicochemical characteristics which govern the mitochondria targeting. Perhaps amphiphilic modification alters the overall hydrophilic/hydrophobic balance of DOX and subsequently affect its permeability, diffusion, and membrane partition. Together, these results clearly demonstrated that *in vitro*, amphiphilic modification on DOX enhanced the cellular uptake and selectively targeted DOX to mitochondria.

Amph-DOX enhances antiproliferation efficacy by increasing reactive oxygen species levels in cancer cells. Targeting doxorubicin to mitochondria has recently been shown to enhance the cytotoxicity toward a number

of tumor cells.[9-14] To examine the impact of amphiphilic DOX modification on the antiproliferation efficacy, the viabilities of several cancer cells, including drug-resistant NCI/ADR-RES cells were evaluated. Exposure of cells to amph-DOX caused a concentration-dependent toxicity, with an IC_{50} value of 0.2 μM in B16F10 cells, as compared with 2.0 μM in cells treated with free DOX (**Fig. 4A**). Similarly, treatment with amph-DOX reduced the IC_{50} values in both OVCAR-8 cells (DOX sensitive, 0.1 μM as compared to 1.0 μM with free DOX) and the DOX resistant NCI/ADR-RES cells (0.5 μM as compared to 1.8 μM with free DOX) (**Fig. 4B**). It is worth to point out that DSPE-PEG_{2000}-NHS or its hydrolyzed derivative exhibits negligible toxicity (**Fig. S15**), suggesting amph-DOX exerts its cytotoxic effects via DOX instead of amphiphilic polymer. These results clearly demonstrated that amph-DOX was considerably more effective than free DOX in both drug sensitive and drug-resistant cell lines.

The cytotoxic effect of doxorubicin is thought to be mediated primarily by nuclear DNA intercalation to disrupt topoisomerase-II-mediated DNA repair.[1] However, oxidative damage of mitochondria functions has been observed *in vitro* following delivery of DOX to mitochondria.[10] To gain insight into the source and potential mechanism of amph-DOX inducing ROS generation in cancer cells, we analyzed the production and spatial distribution of the intracellular ROS. B16F10 cells were continuously exposed to amph-DOX at different concentrations and intracellular levels of ROS were measured after different times of drug exposure. By using the 2',7'-dichlorodihydrofluorescein diacetate probe (H2DCFDA) that detects multiple ROS species within the cells, we observed significant increases in intracellular ROS levels in cells treated with amph-DOX compared with free DOX (**Fig. 4D, E**). ROS production was dominantly amph-DOX in origin as demonstrated by colocalization of amph-DOX and dichlorofluorescein staining using confocal microscopy (**Fig. 4E**). As amph-DOX accumulates in mitochondria (**Fig. 2B, Fig. 3A**), our results suggest that mitochondria are the locations of amph-DOX induced ROS response in cancer cells. Together, these data qualify amph-DOX as a promising drug for cancer chemotherapy, which significantly increases anti-cancer potency, through effective uptake of DOX to tumor cells and more importantly, through mitochondria-selective accumulation and ROS production.

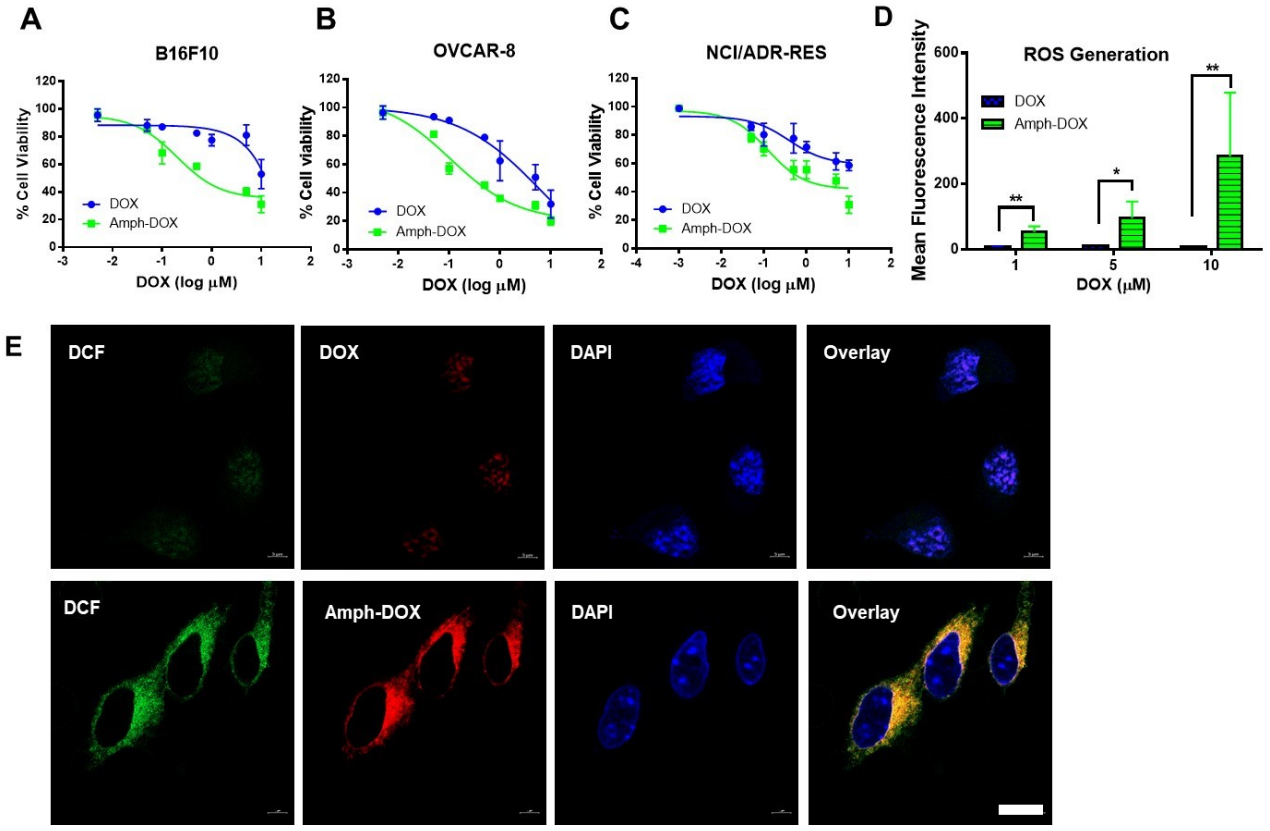

Figure 4. Amph-DOX induces strong cytotoxicity *in vitro* by stimulating massive production of ROS in mitochondria. (*A and B*) *in vitro* cytotoxicity of free DOX and amph-DOX against B16F10 (*A*), OVCAR-8 (*B*), or NCI/ADR-RES (*C*) cells 24 h after exposure. 1 x 10^6 cells were incubated with amph-DOX or free DOX with varying concentrations for 24. Cell viability was determined by alamarBlue viability assay. (*D and E*) Intracellular levels of ROS induced by DOX and amph-DOX. (*D*) Flow cytometer analyses of ROS production in 1 x 10^6 B16F10 cells treated with 1, 5 and 10 μM DOX or amph-DOX, and (*E*) Confocal microscopy images of B16F10 cells incubated with 10 μM DOX and amph-DOX (red) for 4h, after which H2DCFDA (DCF, green) was added at a final concentration of 20 μM for 30 min. scale bar = 10 μm.

Amphiphilic conjugation markedly prolongs the circulation time, enhances tumor accumulation and improves the therapeutic anti-tumor efficacy of Doxorubicin. Drugs associated with albumin are known to have long blood residence time.[36,37,39] To test whether the albumin-binding amph-DOX has prolonged serum half-life, mice were injected i.v. with amph-DOX, or free DOX. At various time points following injection, blood samples were collected from the tail for DOX measurements. *In vivo*, free DOX was rapidly cleared from the plasma and its concentration was dropped below detectable level after 60 min (**Fig. 5A**). In contrast, amph-DOX exhibited

much higher serum concentrations after injection and had superior blood retention, with a half-life in blood increased to 3.0 h (**Fig. 5A**). The area under the concentration-time curve (AUC) of amph-DOX was increased approximately 60-fold compared with that of free doxorubicin (**Fig. 5A**).

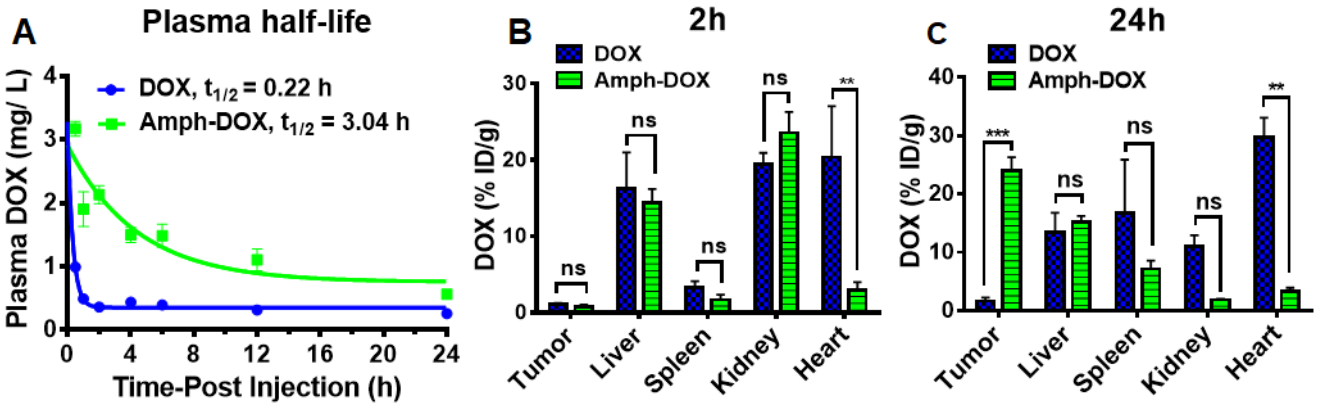

Figure 5. *In vivo* plasma pharmacokinetic analysis and biodistribution of amph-DOX in C57BL/6 mice bearing B16F10 tumor. (*A*) Plasma pharmacokinetic curves of amph-DOX and DOX. Doxorubicin concentrations in plasma as a function of time following a single dose of free doxorubicin (10 mg/kg) or amph-DOX (10 mg/kg equivalent doxorubicin). The values are the mean ± SEM (n = 4). (*B and C*) Tissue (tumor, liver, spleen, kidney, and heart) accumulation of doxorubicin at 2 h (*B*) and 24 h (*C*) following a single dose of free doxorubicin (10 mg/kg) or amph-DOX (10 mg/kg equivalent doxorubicin) (n = 4).

Albumin-bound DOX is also expected to accumulate in tumor via multiple mechanisms: i) due to EPR effect, DOX-albumin complex accumulates in tumor instead of normal tissues; ii) It is well known that tumor tissues utilize albumin as a source of amino acid and energy to fuel their growth.[40-41] In contrast, the uptake of drug bound to albumin in normal tissues is expected to be low due to the FcRn mediated albumin recycling pathway.[60] iii) albumin has an extraordinarily broad tissue penetration capability (by receptor-mediated transcytosis) in both normal and disease conditions.[40-41] Compared with free DOX, i.v. injection of amph-DOX led to 14-fold increases in s.c. B16F10 tumor (mouse melanoma) 24 h post injection (**Fig. 5C**). Importantly, amph-DOX resulted in a significantly lower tissue accumulation of DOX compared to free DOX treatment in the heart (**Fig. 5B, C**), where DOX can cause cardiotoxicity, suggesting amph-DOX might lead to a reduction of the potential short-term and long-term side effects of the drug.

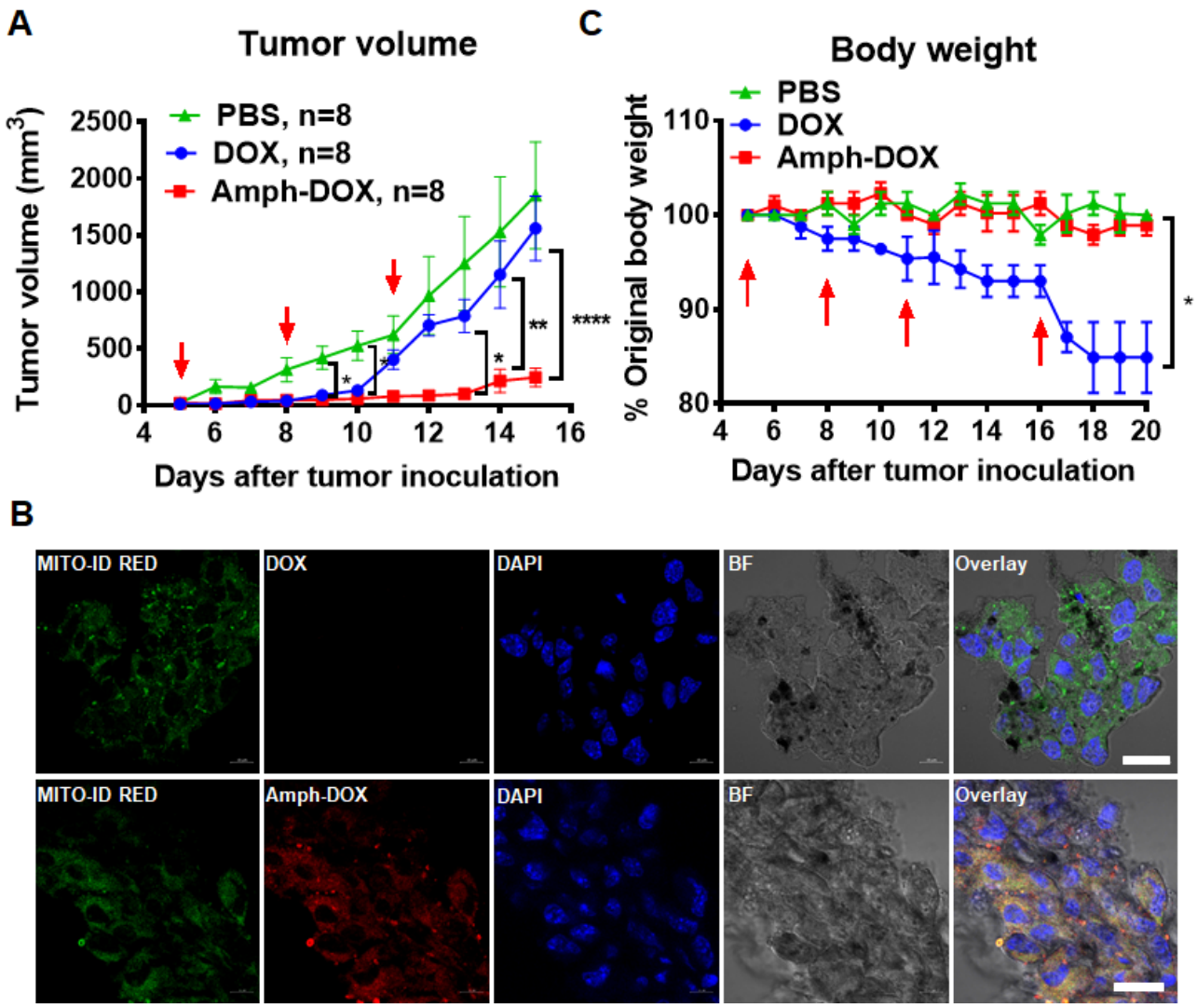

Figure 6. ***In vivo*** **anti-tumor activity of amph-DOX in B16F10 tumor.** (*A*) C57BL/6 mice (n = 8 per group) were injected with doxorubicin hydrochloride, or amph-DOX (3 × 5 mg/kg doxorubicin), or saline on days 5, 8 and 11 after tumor innoculation. Tumor volumes were measured on a daily basis during the experimental period. (*B*) Confocal laser scanning microscopy (CLSM) images of frozen sections of B16F10 tumor tissues. Tumor tissues were isolated at the end of tumor therapeutic period (day 16). Tumor sections were labeled and imaged. Images show mitochondria (green, stained with MITO-ID RED), nuclei (blue, stained by DAPI) and overlay (scale bar = 10 μm). (*C*) Tumor free C57BL/6 mice were treated with DOX or amph-DOX (10 mg/kg equivalent doxorubicin) at days 5, 8, and 11, and a final dose of 20 mg/kg on day 16. Body weight of mice were monitored (n = 8).

Next, the antitumor activities of amph-DOX was evaluated by therapeutical treatment of C57BL/6 mice bearing melanoma tumor. A total of 5×10^5 B16F10 cells were subcutaneously implanted into mice. Mice received three injections of 5 mg/kg of free DOX, or equivalent amph-DOX, or saline on days 5, 8, and 11. As shown in **Fig. 6A**,

administration of free DOX only caused a transient regression of B16F10 tumor at the early stage of the treatment, and tumor quickly resumed growth. However, mice treated with the same doses of amph-DOX markedly delayed the growth of s.c. implanted B16F10 tumor (**Fig. 6A, Fig. S16**). To examine whether amph-DOX accumulates in tumor mitochondria *in vivo*, tumors were isolated 24 and 48 h after injection, sectioned, and stained with MITO-ID, a mitochondria-selective dye suitable for fixed cells. Accumulation of amph-DOX was observed in tumor mitochondria 24 h and 48 h post injection (**Fig. 6B, Fig. S17**), suggesting an improved EPR effect. In contrast, under the same conditions, soluble DOX fluorescence in the tumor section was undetectable. Treatment with amph-DOX also diminished doxorubicin-related losses in total body weight in tumor-free mice (**Fig. 6C**). Histopathological analysis of heart section (on day 15) of mice after three injections (on days 5, 8 and 11) of amph-DOX showed no sign of heart muscle damage and no acute cardiotoxicity, similar to those with no treatment control (**Fig. S17**). However, DOX treated animals showed noticeable, albeit mild damage to cardiac tissue, characterized by increased cytoplasmic vacuolization and distorted myocardial cell arrangement (**Fig. S18**). Taken together, these data strongly suggest amph-DOX is able to bind albumin protein in blood, prolong circulating time, accumulate in tumor mitochondria, and inhibit tumor growth. Though the long-term cardiotoxicity cannot be determined by our model, the reduced mouse cardiac tissue accumulation and no cardiomyocyte pathology also suggests a favorable cardiosafety profile in the preclinical model.

■ CONCLUSION

The physicochemical properties appear to have important consequences for the behavior of anthracyclines in biological systems. In this work, we described a simple molecular approach to deliver doxorubicin to tumor mitochondria *in vivo*. We showed that in mice, diacyl lipid conjugation on doxorubicin linked with a PEG linker uniquely achieves tissue-, cellular-, and mitochondria-selective accumulation of doxorubicin, and significantly enhances the antitumor efficacy of the drug. This new type of molecular anticancer drug conjugate features several favorable advantages as therapeutic options in cancer therapy: i) This approach uses a simple molecular conjugate to achieve multiple levels of targeting *in vivo*. First, the amphiphilic DOX can reach and penetrate solid tumor by 'hitchhiking' on albumin protein.[40-41] Compared with soluble DOX, albumin-drug complex exhibits increased hydrodynamic size, prolongs DOX's circulating half-life, and retargets the drug to the tumor by both passive and active targeting mechanisms.[36-39] Second, amphiphilic DOX accumulates in mitochondria following tumor cell uptake through a yet unknown mechanism. Several long circulating doxorubicin formulations exist in clinical or

preclinical studies (e.g., liposomal DOX: Doxil; DOX-albumin covalent conjugate: Aldoxorubicin).[61-62] However, none of these formulations is able to selectively target mitochondria. Unlike many of the previous mitochondriotropic ligands, which are concentrated in mitochondria in response to negative transmembrane potentials,[7-15, 19-21] our amphiphilic drug conjugate has a completely different structure. Our approach thus challenges current paradigms in mitochondria targeting, providing a new mechanism to potentiate the efficacy and safety for future mitochondria drug design. ii) Our molecular approach is carrier-free. Amphiphilic DOX relies on endogenous albumin protein for tumor targeting and intracellular sorting mechanisms for mitochondria targeting. Anti-cancer drug delivered via endogenous protein particles has the potential to hold the key advantages while completely avoid the side effects (e.g., immunogenicity) associated with exogenous carriers. iii) Targeting doxorubicin to mitochondria enables a mechanism to overcome the drug efflux mediated resistance by delivering doxorubicin to intracellular organelle where the drug efflux protein cannot access.[9] iv) Compared with proteins or nanoparticles, the molecular conjugate is fully synthetic, which is favored in production, cost, stability, safety and in principle, could be readily translated to clinical cancer chemotherapy. Altogether, the results presented here demonstrate amphiphilic modification on doxorubicin which targets doxorubicin to mitochondria is an effective approach to simultaneously enhance the drug's potency and safety. This approach might be applicable to many other anthracyclines in cancer chemotherapy.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Synthesis and characterization of amph-DOX, characterization self-assemble and albumin-binding properties, lysosome colocalization confocal images, intracellular quantification of amph-DOX in B16F10 cells, mitochondria accumulation in 4T1 tumor cells, intracellular quantification of amph-DOX in NCI/ADR-RES cells, uptake and intracellular distribution of DOX loaded DSPE-PEG_{2000} micells, uptake mechanisms of amph-DOX, effect of serum on amph-DOX uptake and subcellular location, intracellular distribution of amph-DOX with a pH sensitive hydrozone linkage, intracellular distribution of amph-Fluorescein, intracellular distribution of cholesterol-DOX, cytotoxic effect of DSPE-PEG_{2000}-NHS, photograph of tumor before and after isolation, H&E images of heart sections in mice treated with DOX or amph-DOX.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Table of Contents Graphic