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Single Protein Encapsulated Doxorubicin as an Efficacious Anticancer Therapeutic

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Abstract

Small-molecule chemotherapeutics are potent and effective against a variety of malignancies, but common and severe side effects restrict their clinical applications. Nanomedicine approaches represent a major focus for improving chemotherapy, but have met limited success. To overcome the limitations of chemotherapy drugs, we have developed a novel Single Protein Encapsulation (SPE)-based drug formulation and delivery platform and tested its utility in improving doxorubicin (DOX) treatment. Using this methodology, a series of SPEDOX complexes were generated by encapsulating various numbers of DOX molecules into a single human serum albumin (HSA) molecule. UV/fluorescence spectroscopy, membrane dialysis, and dynamic light scattering techniques showed that SPEDOXs are stable and uniform as monomeric HSA and display unique properties distinct from those of DOX and DOX-HSA mixture. Furthermore, detailed procedures to precisely monitor and control both DOX payload and binding strength to HSA were established. Breast cancer xenograft tumor studies revealed that SPEDOX-6 treatment displays improved pharmacokinetic profiles, higher antitumor efficacy, and lower DOX accumulation in the heart

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author

Conflict of Interest

The author C. J. Yu is a named inventor for patent applications regarding "Single Protein-Encapsulated Pharmaceuticals for Enhancing Therapeutic Effects" in pending. The other authors declare no conflict of interest.

tissue compared with unformulated DOX. This SPE technology, which does not involve nanoparticle assembly and modifications to either small-molecule drugs or HSA, may open up a new avenue for developing new drug delivery systems to improve anticancer therapeutics.

Graphical Abstract

Keywords

SPEDOX; doxorubicin; human serum albumin; single protein encapsulation; chemotherapy

Doxorubicin (DOX) is a widely used anticancer therapeutic agent with a broad spectrum of antineoplastic activities against both solid and hematologic malignancies. However, as a small and hydrophobic molecule, DOX indiscriminately infiltrates all tissues and organs, leading to organ toxicities such as cardiomyopathy and myelosuppression. When DOX's cumulative dose reaches a certain level, the incidence of congestive heart failure increases sharply [1]. Thus, a cumulative limit of $< 450 \text{ mg/m}^2$ for DOX treatment is imposed. DOX-induced cardiomyopathy may involve oxidative stress, contractile protein downregulation, and p53-induced apoptosis [2]. Much effort has been undertaken to reduce DOX's toxicity by limiting its access to normal cells while increasing its delivery to tumors, which in principle may be achieved by a rationally designed strategy of binding or conjugating DOX with a macromolecular or assembled system with a molecular weight above the renal clearance limit of $\sim 50 \text{ kD}$. Associating DOX with a nano-sized moiety, such as liposomes, biopolymers/polymers, and nanoparticles (NPs) significantly improves the pharmacokinetics (PK) of DOX, increases its circulation lifetime, and enhances its delivery to tumor cells due to tumor's irregular neovasculature and poor lymphatic drainage leading to high permeability and retention of DOX [3–9]. However, current systems have demonstrated limited success [10, 11]. These non-natural systems may be recognized by the human body's sophisticated immune system, leading to a broad range of responses that may vary among different patients. In addition, it is not clear how the human body disposes synthetic polymers and NPs. Of note, different forms of human serum albumin (HSA) NPs [3, 12–19] have been prepared with distinct chemistry to yield varying sizes, but they all differ significantly from free HSA. Consequently, their PK, interaction with host cells, and potential elicitation of immune responses can be considerably different from those of free HSA.

As the most abundant serum protein [20], HSA possesses some unique properties [21] – 1) It is highly soluble and stable, 2) capable of binding a variety of both natural ligands (amino acids, fatty acids, steroids, vitamins, etc.) and synthetic drugs (Warfarin, Diazepam, Ibuprofen etc.) with different binding affinities [20], 3) endocytosed and transcytosed by the

cell via specific receptors, 4) displays a long half-life of 19 days due to effective endosome recycling by the neonatal Fc receptor (FcRn) [22–25] and rescue from renal clearance via Megalin/Cubilin-complexes, 5) able to accumulate at tumor tissues due to high permeability and retention effects of the tumor vasculature, and 6) preferentially taken up and metabolized by cancer cells to serve as nutrients. These properties, together with its ready availability, biodegradability, and lack of toxicity and immunogenicity, make HSA an ideal carrier to improve a drug's therapeutic efficacy and reduce its toxicity.

Capitalizing on these unique features of HSA, we have developed a Single Protein Encapsulation (SPE) technology to carry multiple DOX molecules by an unmodified monomeric HSA molecule, thereby avoiding potential issues associated with synthetic polymers, conjugated HSA, and HSA NPs. The novel HSA-DOX complex (SPEDOX) is highly stable and uniform in size. Importantly, the number (up to 9) of DOX molecules enclosed in each HSA can be accurately controlled (Figure 1). Furthermore, we have established procedures to monitor and precisely regulate DOX binding strength in SPEDOX. *In vivo* studies with mice demonstrated better PK, lower toxicity, and superior tumor-inhibitory activity of SPEDOX compared with DOX. To the best of our knowledge, our findings represent the first report that an unmodified monomeric protein molecule can be used as a delivery and formulation vehicle to encapsulate a defined number of anticancer molecules for reducing their systemic toxicity and enhancing their antitumor efficacy.

As shown in Figure 1A (see Supporting information for detailed methods), we have developed a methodology to encapsulate multiple DOX molecules by a single unmodified HSA molecule with multiple sequential preparation steps under defined conditions. We would like to emphasize the importance of the addition of the desired amounts of organic solvents and properly adjusting the pH during the process for the successful encapsulation of drug molecules. Among the established procedures, steps B and D are critical for controlling the DOX/HSA ratio and their binding strength. A series of SPEDOX complexes with distinct DOX/HSA ratios and binding strength were generated. The DOX/HSA ratio reflects the DOX payload per HSA molecule, and the binding strength dictates the dissociation/release of DOX from SPEDOX. While the DOX/HSA ratio can vary, multiple attempts failed to produce SPEDOX with DOX/HSA > 9.0, suggesting a maximum loading capacity of 9 DOX molecules by a single HSA molecule. During the encapsulation process, we observed systematic spectral changes (Figure 1B) of DOX in the visible region where HSA had no absorbance. The maximum absorbance at 481 nm (A_{481}) gradually decreased, while A_{547} remained unchanged. Although the meaning of the isosbestic point at 547 nm is not yet understood, it provides a consistent reference point for us to accurately determine the concentrations of DOX in all SPEDOX complexes. Notably, plotting A_{547}/A_{481} vs. encapsulation time yields a straight line ($R^2 = 0.9936$), indicating a linear correlation between A_{547}/A_{481} and encapsulation time (Supporting Information, Figure S1). Thus, the A_{547}/A_{481} ratio may serve as an excellent indicator of the encapsulation process and can be used to monitor SPEDOX production. In an attempt to understand the nature of the A_{547}/A_{481} ratio of SPEDOX, we determined the A_{547}/A_{481} ratio of free DOX in a MeOH/water mixed solvent system, whose polarity indexes are 10, 7.05, 6.27, and 5.1 for 100% water, 50/50% MeOH/water, 70/30% MeOH/water, and 100% MeOH, respectively [26]. While the A_{547}/A_{481} ratio of DOX does change with solvent polarity, the change is in the opposite

direction from what we observed in SPEDOX (data not shown), suggesting that the A_{547}/A_{481} ratio of SPEDOX does not simply reflect the less polar environment in HSA.

To test whether DOX molecules were indeed encapsulated by HSA in different SPEDOX complexes, two sets of dialysis kinetics assays were performed by using 2 series of SPEDOX complexes – series 1 (SPEDOX-1, SPEDOX-2, and SPEDOX-3 with a constant $A_{547}/A_{481} = 0.54$ but varying DOX loading DOX/HSA = 4.0, 7.0, and 9.0) and series 2 (SPEDOX-4, SPEDOX-5, SPEDOX-6 and SPEDOX-7 with a constant loading DOX/HSA = 9.0 but varying $A_{547}/A_{481} = 0.46, 0.49, 0.53$ and 0.56). The first set of dialysis kinetics used the series 1 SPEDOX complexes to test how different DOX loading at a defined binding strength ($A_{547}/A_{481} = 0.54$) affected DOX release rate. Dialysis in PBS buffer (pH = 7.4, mimicking blood plasma pH) revealed that the DOX release rates of SPEDOX-1 SPEDOX-2 and SPEDOX-3 were very similar, indicating % release of DOX was dictated by the A_{547}/A_{481} ratio regardless how many DOX molecules were carried by each HSA (Figure 1C). In addition, dialysis of SPEDOX-2 in acetate buffer (pH = 5.2, mimicking the pH in the endosome and the acidic tumor microenvironment) was substantially faster than in PBS buffer, suggesting weaker DOX binding in SPEDOX due to the change either in HSA conformation or the DOX protonation state or both under acidic conditions. The second set of dialysis experiments used the series 2 SPEDOX complexes to analyze how different binding strength at a defined DOX loading (DOX/HSA = 9.0) affected DOX release rate. As shown in Figure 1D, the DOX release rate in PBS varied within a broad range and followed the order of SPEDOX-4 > SPEDOX-5 > SPEDOX-6 > SPEDOX-7, suggesting the A_{547}/A_{481} ratio as an indicator of the encapsulation strength. Of note, dialysis of SPEDOX-6 was much faster in acetate buffer than in PBS, confirming weaker DOX binding under acidic conditions. This SPEDOX pH-responsive property may facilitate *in vivo* DOX release during SPEDOX uptake by tumor and endocytosis into cancer cells in the acidic tumor microenvironment and endosome lumen, leading to enhanced anticancer effects due to increased DOX concentration in cancer cells. Thus, the SPE methodology is able to encapsulate DOX in a tunable manner to yield series of SPEDOX complexes, in which both DOX loading quantity and binding strength can be modulated separately and precisely. We hypothesize that both DOX loading and DOX-HSA binding strength in SPEDOX are critically important to achieve optimal antitumor efficacy. The higher the loading, the more DOX can be carried by a defined amount of HSA. If the DOX-HSA binding is too weak, DOX would be released quickly, leading to nonspecific DOX uptake by all cells, tissues and organs and high systemic and cardiotoxicity. On the other hand, when HSA binds DOX too strongly, DOX release from SPEDOX would be too slow to achieve desired antitumor effects. SPEDOX-6 has the high DOX loading of 9 DOX molecules per HSA and a medium binding strength ($A_{547}/A_{481} = 0.53$). Therefore, we believed that SPEDOX-6 might represent the optimal DOX loading and binding strength, and thus SPEDOX-6 was chosen for *in vitro* and *in vivo* evaluations in the following sections.

To further demonstrate that SPEDOX is unique and distinctive from either free DOX or a DOX-HSA mixture, we conducted a series of comparison studies (Figure 2). First, dialysis of DOX, DOX-HSA mixture, and SPEDOX-6, revealed drastically different dialysis kinetics (Figure 2A). While the dialysis rate was slower for DOX-HSA mixture relative to DOX, suggesting some weak interactions between DOX and HSA, DOX release from SPEDOX-6

was significantly slower compared with DOX or DOX-HSA mixture, indicating much stronger DOX binding in SPEDOX-6. Second, their UV spectra revealed clear differences (Figure 2B). DOX-HSA mixture had a similar spectral shape and slightly higher absorbance relative to that of DOX. In contrast, SPEDOX-6 displayed a distinctive shape and substantially lower absorbance, suggesting a change in DOX's physicochemical environment upon encapsulation by HSA. Third, emission fluorescence and potassium iodide (KI)-quenching studies further supported DOX's encapsulation. While mixing HSA with DOX increased fluorescence, SPEDOX-6 exhibited significantly reduced fluorescence (Figure 2C). This again suggests a change in DOX's physicochemical environment, which was further supported by the low sensitivity of SPEDOX-6's fluorescence quenching by KI (Supporting Information, Figure S2) due to restricted access of KI to HSA-encapsulated DOX. Of note, DOX fluorescence quenching may be caused by different mechanisms, such as a potential shorter fluorescence lifetime of DOX in SPEDOX compared to that of unformulated DOX.

Finally, to confirm DOX encapsulation by a single monomeric HSA molecule, we measured the particle size distribution of HSA and SPEDOXs by the dynamic light scattering (DLS) technique. As seen in Figure 2D, their size distribution profiles were almost identical (only HSA and SPEDOX-6 are shown), providing the conclusive evidence that DOX encapsulation does not change either the size or the monomeric state of the HSA molecule in SPEDOX.

Given that SPEDOX complexes reformulate DOX, *in vivo* studies were then conducted to compare them with unformulated free DOX. To evaluate the PK properties of SPEDOX, we chose SPEDOX-6 for testing in BALB/c mice, considering the desired intermediate binding strength of DOX in this SPEDOX. Each of the collected blood samples was equally divided into two portions, yielding 2 sets of samples. From one set of samples, DOX was extracted and analyzed by HPLC/MS. Figure 3A shows the total mouse serum DOX concentration-time profile after SPEDOX-6 IV injection in comparison with that of unformulated DOX injection at the same dose [27]. From the PK profiles, the area under the time-concentration curve (AUC) values were calculated to yield 82.5 and 1.7 h* μ g/mL for SPEDOX-6 and unformulated DOX, respectively (Supporting information, Table S1), showing a significant 48-fold increase of DOX in circulation upon its encapsulation. Thus, SPEDOX is expected to effectively reduce the non-specific DOX uptake by tissues, organs, and cells, leading to its observed high AUC value.

Separately, to determine the amount of free DOX in circulation, the other set of the collected blood samples were first passed through desalting columns to remove free DOX, followed by the same DOX extraction and HPLC/MS analysis. The measured DOX represented the HSA-bound form, whereas the DOX differences between the 2 sets of the measurements at each time point reflected the amount of free DOX in blood samples. It was found that the percentage of the encapsulated DOX accounted for about 80% of the total DOX in the blood of SPEDOX-injected mice (Supporting information, Table S2). The remaining 20% was attributed to the released DOX from SPEDOX during circulation.

The dose-dependent cardiomyopathy from DOX treatment is a major side effect of the drug. To test whether SPEDOX decreases the accumulation of free DOX levels in the heart tissue, mice were sacrificed at 2 h and 24 h post IV injection of 12 mg/kg (DOX equivalent) SPEDOX-6 and heart tissues were harvested, weighed, and homogenized. Total DOX was then extracted and analyzed by HPLC/MS. The concentration of free DOX was calculated after 20% correction (based on the previous section) and compared to DOX's well-established results from unformulated DOX injection [27]. As shown in Figure 3B, over a period of 24 h post IV injection, free DOX concentration in the heart tissue was 4-8 times lower from SPEDOX than from unformulated DOX, suggesting that cardiotoxicity from SPEDOX may be substantially lower compared with DOX. Future studies on the immunohistochemistry-based cardiomyopathy evaluation of the mouse heart tissue are needed for further evaluating SPEDOX's effects on cardiotoxicity.

Given that DOX is used for treatment of triple-negative breast cancer, an aggressive subtype of breast cancer, we tested the effect of SPEDOX on breast tumor growth in a common xenograft triple-negative breast cancer model MDA-MB-231. It is known that MDA-MB-231 human breast cancer cells are relatively resistant to DOX compared with other cell lines [28, 29]. One of the major goals of developing SPEDOX is to deliver high doses of DOX *in vivo* to cancer cells in order to achieve significant, robust tumor growth inhibition (TGI), while limiting its toxicity. Therefore, our TGI experiments were designed to test the anti-tumor effect of HSA-encapsulated DOX at multi-fold maximum tolerated doses (MTDs) of unformulated DOX. As shown in Figure 4A, DOX at its established MTD dose (5 mg/kg) [5], as compared with the vehicle control (saline only), did not show a statistically significant TGI effect in the MDA-MB-231 model on Day 20 of treatment. In contrast, SPEDOX-6 at 2X of DOX's MTD dose (10 mg/kg DOX equivalent) significantly reduced xenograft tumor growth in comparison to the control group, achieving 55% TGI on Day 20. Of note, the normalized body weight (BW) change vs. time (Figure 4B) did not show significant differences between DOX or SPEDOX-6 and the control groups. These results indicate that SPEDOX-6 has a higher TGI activity without increasing toxicity.

In addition, higher doses of SPEDOX-6 at 20 and 30 mg/kg DOX equivalent, i.e., 4X and 6X of DOX's MTD, were tested for MTD assessment and antitumor efficacy. As shown in Figure 4C and 4D, TGI of SPEDOX-6 at 20 mg/kg DOX equivalent in comparison with the control reached 88% on Day 29 ($P = 0.0059$), which was much higher than that of SPEDOX-6 at 10 mg/kg DOX equivalent. Normalized body weight of the SPEDOX-6 group (20 mg/kg DOX equivalent) remained relatively healthy. Notably, 3 mice in the 30 mg/kg DOX equivalent SPEDOX-6 group died on Day 11, indicating that the SPEDOX-6 MTD had been exceeded. Thus, the MTD of SPEDOX-6 is estimated to be between 20 to 30 mg/kg DOX equivalent, i.e. 4-6 times higher than that of free DOX.

In this study, we have developed a new anticancer drug formulation technique to enhance antitumor efficacy and reduce toxicity. Unlike existing methods using conjugated polymers and NPs as drug carriers [3–9], the SPE system utilizes commercially available HSA that has been used for human clinical applications and possesses an intrinsic capacity to bind a variety of both natural ligands and synthetic drugs [20]. While simply mixing DOX with HSA leads to some weak interactions (Figure 2), DOX encapsulation by HSA can be

achieved under defined specific conditions to yield SPEDOX complexes. Although we do not yet fully understand the mechanism of the encapsulation process, we hypothesize that our unique procedures promote transient access of DOX to HSA's internal binding sites and enable multiple weak interactions between DOX to HSA. Of note, even after the conditions are removed, the state of DOX-HSA interactions in SPEDOX is maintained, allowing us to produce SPEDOX complex series with distinctive variables – DOX payload quantity and binding strength. UV spectroscopy, fluorescence spectroscopy, and dialysis studies provide indisputable evidence that SPEDOX is a novel form that is distinct from either DOX or DOX-HSA mixture. In addition, SPEDOXs, regardless of DOX loading amount and binding strength, are formed by single monomeric HSA molecule, since they have virtually identical size to the unmodified native HSA molecule. Therefore, SPEDOXs are uniform and contain no chemical conjugation or multimeric aggregates. To gain insight into SPEDOX formation, we conducted computational DOX docking onto HSA by using UCSF Chimera [30] and AutoDock Vina [31]. The computational docking assumes rigid structures for both HSA and DOX. The results can be summarized as: 1) multiple DOX molecules can fit into HSA at non-overlapping locations, 2) the binding energy (ΔG°) of top 9 DOX molecules ranges from -9.1 , -8.7 , -8.6 , -8.0 , -7.3 , -7.2 , -7.2 , -7.1 to -7.0 kcal/mol, corresponding to Kd values of 0.2 , 0.5 , 0.5 , 1.0 , 2.0 , 5.0 , 6.0 , 7.0 , and 8.0 μM , 3) the 10th DOX's docking ΔG° and Kd are -6.5 kcal/mol and 18.0 μM , representing a steep drop in binding affinity (Figure S3). While the validity of the computational docking results needs to be verified experimentally through structure determination by either crystallography or CryoEM, the results suggest that 1) HSA has multiple binding sites for DOX molecules with binding affinities (Kd) ranging from submicro- to micromolar. 2) Instead of rigid structures used in the computational docking, DOX-HSA interaction may actually change the structures of both DOX and HSA (induced fit), which may takes time to proceed and result in better DOX-HSA interaction. This consideration may explain the defined multiple-step SPEDOX preparation processes (Figure 1A & Figure S1) and why simply mixing DOX with HSA will not yield DOX-HSA complexes similar to SPEDOX. 3) The sharp drop of the 10th DOX's docking onto HAS in ΔG° and Kd may explain the maximum loading capacity of 9 DOX molecules per HSA.

Our *in vivo* studies demonstrate that HSA-encapsulated DOX displays improved PK, higher MTD, better antitumor efficacy, and lower free DOX accumulation in the heart tissue compared with unformulated DOX. In addition, since SPEDOXs are formed by unmodified monomeric HSA molecule, there is a low probability of activating immune responses in humans, which is in stark contrast to many synthetic polymer- and NP-based systems. Our future studies will further test this notion. Therefore, SPE may serve as a drug delivery approach to improve the therapeutic index of DOX.

The long half-life of HSA in humans is due to hFcRn-mediated rescue and recycling from its endocytotic pathways to lysosomes [22–25]. While mice have a similar mFcRn to maintain mouse serum albumin (MSA)'s long half-life, mFcRn has a low binding affinity for HSA. As a result of weak binding to mFcRn and competition from MSA, HSA has a much shorter half-life in mice [32]. Therefore, mice are not ideal animal models for *in vivo* evaluation of SPEDOX. While there are genetically modified mouse models expressing hFcRn [33, 34], we chose to use unmodified mice to conduct exploratory studies as a proof of principal test.

We expect SPEDOX to display better PK and efficacy in hFcRn-expressing mice or humans. An additional advantage of SPEDOX is cancer targeting, based on the hFcRn-mediated HSA recycling mechanism [21, 35, 36]. If a cancer cell expresses less hFcRn than its normal counterpart, SPEDOX recycling would be less efficient, leading to increased endocytosis of SPEDOX into the cancer cell. DOX may be released from SPEDOX by different mechanisms, such as dissociation and HSA degradation, leading to higher free DOX concentration in the cancer cells. Using the GEPIA web server [37], we found 31 different cancers with FcRn expression levels. Half of them express less FcRn than their normal tissue counterparts, while a quarter express more FcRn, with the remaining having similar FcRn levels. Among the first group, 12 cancers with the normal/tumor FcRn ratio > 1.6 (Supporting information, Table S3), including invasive breast cancer, lung cancer, and ovarian cancer, may be suitable targets for SPEDOX treatments.

In addition to DOX, the SPE technology can be applied to a broad spectrum of anticancer drugs. HSA-encapsulated epirubicin, daunorubicin, mitoxantrone, idarubicin, vincristine, vinblastine, and docetaxel have been prepared. All SPE-DRUGS have a similar size to that of unmodified native HSA and are highly water soluble to form clear and stable solutions. Alternatively, the single protein molecule carrier is not restricted to HSA. Other globular proteins such as immunoglobulin G (IgG) also have drug encapsulation capacity. Therefore, the SPE technology presents a new platform for drug formation and delivery. The system is highly versatile and may be precisely tuned to encapsulate a variety of anticancer drugs to achieve increased efficacy and reduced toxicity, leading to an improved therapeutic index.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(c)

(d)

Figure 1. Preparation and UV absorbance features of SPEDOX.

a) Schematic diagram of SPEDOX preparation process; **b)** DOX's UV spectral change in the visible region during its encapsulation process. The maximum absorbance at 481 nm (A_{481}) decreased with encapsulation time, while the absorbance at 547 nm (A_{547}) remained constant (isosbestic point); **c)** DOX release rate comparison of series 1 SPEDOX complexes with different DOX/HSA ratios but a constant $A_{547}/A_{481} = 0.54$ in PBS & acetate buffer (in triplicate); **d)** DOX release rate comparison of series 2 SPEDOX complexes with different A_{547}/A_{481} ratios but a constant DOX/HSA = 9.0 in PBS & acetate buffer (in triplicate).

(c)

(d)

Figure 2. Dialysis comparison and spectroscopic characterization of SPEDOX.

a) DOX release rate comparison (in triplicate); **b)** UV spectrum comparison; **c)** Fluorescence emission spectrum comparison of 3 different DOX forms; **d)** Particle size distribution profiles of SPEDOX-6 and HSA, determined by DLS. All samples contained the same amount of DOX equivalent.

(a) **(b)**

Figure 3. SPEDOX-6 PK study and DOX level measurement in mouse heart tissues.

a) Comparison of total mouse plasma DOX concentrations from SPEDOX-6 and DOX injection at the same dose; **b)** Comparison of free DOX concentrations in mouse heart tissues from SPEDOX-6 and DOX injection at 2 h and 24 h. Triplicate for each sampling point.

(a)

(b)

(C)

(d)

Figure 4. Tumor-inhibitory effects of SPEDOX in breast cancer xenograft models.

a) MDA-MB-231 xenograft tumor volume vs. day profiles in mice treated with vehicle, DOX (5 mg/kg, MTD), or SPEDOX-6 (10 mg/kg DOX equivalent, 2X of DOX's MTD). At Day 20, 34% and 54% TGI were achieved for DOX vs. vehicle (ns, $p > 0.05$) and SPEDOX-6 vs. vehicle (*, $p = 0.01$), respectively; **b)** Normalized mouse body weight changes during the treatment period. $p > 0.05$, DOX vs. vehicle and SPEDOX-6 vs. vehicle at Day 20. **c)** MDA-MB-231 xenograft-derived tumor volume vs. day profiles in mice treated with vehicle or SPEDOX-6 of 20 and 30 mg/kg DOX equivalent (4X and 6X of DOX's MTD). At Day 18 and 29, 63% and 88% TGI were achieved for 20 mg/kg DOX equivalent SPEDOX-6 vs. vehicle (**, $p = 0.0086$ and $p = 0.0059$, respectively). Three mice died on Day 11 of treatment with 30 mg/kg DOX equivalent SPEDOX-6, suggesting that this dose exceeded the MTD for SPEDOX-6. **d)** Normalized mouse body weight changes during the treatment with 20 and 30 mg/kg DOX equivalent SPEDOX-6. There is no substantial weight loss during SPEDOX treatment. $p = 0.0018$ and $p < 0.001$, 20 mg/kg DOX equivalent SPEDOX-6 vs. vehicle at Day 18 and 29. Fo4a) and 4b), 8 mice each group. For 4c) and 4d), 4 mice each group.