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Intracellular Activation of a Prostate Specific Antigen-Cleavable Doxorubicin Prodrug: A Key Feature Toward Prodrug-Nanomedicine Design

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S *Supporting Information*

ABSTRACT: L-377,202 prodrug (Dox-PSA) was in phase I clinical trials for patients with metastatic castration-resistant prostate cancer (mCRPC). It consists of doxorubicin (Dox) conjugated to a prostate specific antigen (PSA)-cleavable peptide that can be selectively activated by secreted PSA at the tumor site. However, despite the initial promising results, further clinical testing with Dox-PSA was halted due to toxicity concerns emerging from non-PSA-specific cleavage, following systemic administration. In the present study, we have reported, for the first time, the intracellular activation of Dox-PSA, where Dox nuclear uptake was specific to C4-2B (PSA-expressing) cells, which agreed with the cytotoxicity studies. This finding was confirmed by encapsulating Dox-PSA prodrug into pH-sensitive liposomes to enable prodrug intracellular release, followed by its enzymatic activation. Interestingly, our results demonstrated that Dox-PSA loaded into pH-responsive nanoparticles exhibited cytotoxicity comparable to free prodrug in C4-2B monolayers, with superior activity in tumor spheroids, due to deeper penetration within tumor spheroids. Our approach could open the doors for novel Dox-PSA nanomedicines with higher safety and efficacy to treat advanced and metastatic prostate cancer.

KEYWORDS: prostate cancer, prostate specific antigen-cleavable doxorubicin prodrug, pH-sensitive liposomes, intracellular activation

■ INTRODUCTION

Despite the recent advances in the treatment of metastatic castration-resistant prostate cancer (mCRPC), its clinical outcome is still poor as an effective therapy remains an unmet need.¹ mCRPC is initially managed with castration therapy, but inevitably, 18 to 36 months after therapy initiation, hormone- and chemoresistant cells emerge.² Bypassing chemoresistance and dose-limiting systemic toxicity has urged the development of alternative therapeutic modalities such as prodrugs. These pharmaceuticals are characterized by the covalent modification of the parental drug with a promoiety that renders the compound pharmacologically inactive until certain conditions are encountered.^{3,4} Once in the *in vivo* milieu, the prodrug undergoes enzymatic and/or chemical bioconversion steps that transform it into the biologically active form.⁵ Doxorubicin (Dox) is an anthracycline antibiotic that acts by intercalating with DNA, therefore inhibiting topoisomerase II and causing double-strand DNA breaks and cell death. Despite Dox wide spectrum of anticancer activity in both solid and liquid tumors, the emergence of drug resistance and cardiotoxicity limits its clinical use, factors that could be ameliorated through a

prodrug approach.^{6,7} In the past decade, Dox prodrugs have been designed to be specifically metabolized by prostate specific antigen (PSA) in prostate cancer (PC). PSA is a serine protease with a physiological function of liquefying the sperm coagulum by specifically cleaving its natural substrates semenogelins I and II.⁸ The relevance of PSA as a therapeutic target is based on the premise that the PSA found in the tumor microenvironment is catalytically active, while the PSA that leaks into the blood is inactivated by its covalent binding to serum protease inhibitors, primarily α 1-antichymotrypsin and α 2-macroglobulin.⁹ Several PSA-specific peptide sequences have been developed, including Ser-Lys-Leu-Gln, His-Ser-Ser-Lys-Leu-Gln, and Ser-Ser-Lys-Tyr-Gln.¹⁰ A common feature among all these sequences is the presence of a Gln residue at position P₁, imparting PSA-specificity as PSA is the only member of the kallikrein family involved in the proteolytic cleavage of substrates having a Gln residue in that position.¹¹

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Conjugation of the PSA substrates to a wide variety of cytotoxic drugs¹⁰ has been accomplished by using the dipeptide Ser-Leu or Leu as a linker to improve PSA-hydrolysis rate. Other PSA substrates were developed comprising non-natural amino acids in their peptide sequence in order to increase the chemical stability of the peptide–drug conjugate and mitigate hydrolysis by non-PSA proteases. One such example is the peptide–drug conjugate glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox (L-377,202- hereafter called Dox-PSA), which, despite having reached the clinical setting for mCRPC, did not advance beyond phase I trials due to toxicity concerns.^{12,13} Although in preclinical studies in mice, rats, dogs, and monkeys, Dox-PSA selectively accumulated in the tumor, and the concentrations of Dox in the cardiac tissue (site of clinical dose-limiting toxicity) were much lower than those achieved when Dox was administered alone; there was 30–40% of non-PSA-specific metabolism of Dox-PSA.¹⁴ Moreover, while in the preclinical setting administration of the prodrug into nude mice bearing LNCaP xenografts greatly improved the therapeutic index, with 8- to 9-fold molar increase in the dose of Dox administered,¹⁵ the clinical scenario was largely dissimilar, where only 1.5-fold more molar equivalent of Dox could be administered. Additionally, 1 of 6 patients treated with Dox-PSA at the efficacious dose of 225 mg/m² exhibited dose-limiting neutropenia.¹⁶ These results would likely limit the therapeutic efficacy of this prodrug, and therefore, new strategies urge development before Dox-PSA can be used as an effective therapy for mCRPC.

Aiming at improving the specificity of glutaryl-Hyp-Ala-Ser-Chg-Gln as a PSA-cleavable promoiety, Aloysius et al. have recently designed the sequences glutaryl-Ser-Ala-Ser-Chg-Gln and GABA-mGly-Ala-Ser-Chg-Gln. The authors have shown that, while substitutions between P₁ and P₄ would adversely impact PSA specificity, the given modifications at P₅ improved PSA-mediated hydrolysis rate and decreased non-PSA-specific cleavage.¹³ In the present work, we sought to explore a versatile approach where, rather than altering Dox-PSA prodrug design, we relied on its encapsulation inside a delivery system capable of improving Dox-PSA enzymatic stability. By revisiting Dox-PSA and transforming it into a macromolecular prodrug, we aim not only to alter the pharmacokinetics and pharmacodynamics of the prodrug but also to protect it from the premature proteolytic cleavage while in the bloodstream,^{17,18} which, overall, could contribute to an increased therapeutic efficacy. An additional benefit of this nanoparticle-based approach is the improved tumor targeting that might be achieved by exploring the enhanced permeability and retention (EPR) effect, therefore evading a potential non-PSA-specific prodrug activation in healthy tissues. Importantly, unlike small molecules that can freely diffuse across cellular membranes, nanotherapeutics can only enter the cell by endocytosis.¹⁹ A direct consequence of this is that our proposed approach would necessarily rely on Dox-PSA intracellular activation. However, to the best of our knowledge, previous studies with PSA-cleavable prodrugs have invariably grounded on the extracellular activation of the prodrug due to the secretory nature of PSA enzyme and the notion that high intracellular zinc concentrations inhibit PSA activity.^{12,13,20} Yet, the dysregulation of several zinc transporters has been associated with low intracellular zinc levels in malignant prostate epithelial cells.^{21,22} Therefore, the main goal of the present work is to investigate if Dox-PSA can be intracellularly activated and maintain its cytotoxic activity. If these premises

hold truth, such a provocative and sophisticated strategy could speed up the clinical translation of Dox-PSA. In a proof-of-concept study, and to eliminate the possibility of extracellular activation, Dox-PSA was encapsulated into pH-sensitive liposomes composed of phosphatidylethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS), which could destabilize the endosomal membrane and release their cargos to the cytosol, thereby bypassing lysosomal degradation.^{23–25} Interestingly, our results revealed for the first time that the intracellular delivery of Dox-PSA via DOPE:CHEMS liposomes resulted in high cytotoxicity in two- and three-dimensional PC models, confirming the intracellular activation of Dox-PSA. Our strategy holds great promise to improve the therapeutic efficacy of Dox-PSA and to avoid nonspecific Dox-PSA activation and consequent dose-limiting toxicity *in vivo* following systemic administration.

■ EXPERIMENTAL SECTION

Chemicals. PSA-cleavable peptide (glutaryl-4-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-OH) was purchased from Cellmano, China. (1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate (HATU), *N,N*-diisopropylethylamine (DIPEA), doxorubicin hydrochloride (Dox), acetonitrile, ammonium acetate, HEPES, sodium chloride, citric acid, poly-D-lysine, resazurin sodium salt, and cholesteryl hemisuccinate (CHEMS) were all acquired from Sigma-Aldrich, U.K. Dimethylformamide (DMF) was purchased from Fisher Scientific, UK. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1 (Δ^9 -Cis) PE, DOPE) was obtained from Avanti Polar Lipids (USA).

Synthesis of Glutaryl-Hyp-Ala-Ser-Chg-Gln-Ser-Leu-Dox (Dox-PSA). To a solution of PSA-cleavable heptapeptide (0.0368 mmol, 48.00 mg) in DMF (5 mL), HATU (0.0368 mmol, 13.99 mg) was added. The solution was stirred for 30 min at RT followed by the addition of doxorubicin hydrochloride (0.0735 mmol, 42.60 mg). The pH of the reaction was neutralized by the addition of anhydrous DIPEA (80 μ L, 0.459 mmol) by using pH paper. The reaction was stirred for 18 h at RT under nitrogen while covered in foil to prevent any photodegradation of doxorubicin. After completion, the reaction was concentrated under vacuum to dark red oil. The crude product was dissolved in 50 mL of a buffer consisting of 0.1% (w/v) ammonium acetate in water (80%, buffer A) and acetonitrile (20%, buffer B).

Purification of Dox-PSA. The solution of the crude Dox-PSA was passed through a 0.2 μ m PES filter (Millipore) before being purified by preparative RP-HPLC (Gilson) on a C18 150 $\times$ 4.6 mm column (Agilent). A step gradient consisting of buffers A and B was optimized as follows: 35% B in 2 min, 35–80% B in 4 min, 80% B in 1 min, and 80–35% B in 2 min. Injection volumes of 4 mL were performed per run. A flow rate of 20 mL/min was used to elute the product, which was detected using a UV detector at 214 nm. Pure fractions were pooled, and acetonitrile was removed prior to freeze-drying. The dried product was redissolved in 0.1% ammonium acetate, aliquoted, and freeze-dried again. The dried powder was stored at –80 °C. Mass spectrometry (MALDI-TOF) was performed to confirm the identity of the chromatographic peaks. Purity of the product (31 mg, 23% reaction yield) was checked by analytical HPLC and determined to be >98%.

Preparation of DOPE:CHEMS-Dox-PSA Liposomes. Phospholipids were dissolved in a chloroform/methanol (4:1 v/v) mixture. DOPE:CHEMS (3:2 molar ratio) liposomes

were prepared by the lipid film hydration method, as described previously.²⁶ Briefly, the organic solvents were evaporated under vacuum at 37 °C for 1 h using a rotary evaporator (BÜCHI, Labortechnik AG), then flushed with N₂ stream to remove any residual traces of organic solvent. Dox-PSA was loaded into DOPE:CHEMS by passive loading. Briefly, the dried lipid film was hydrated at room temperature (RT) with Dox-PSA in HEPES pH 8.0 by vigorous pipetting to ensure complete hydration to achieve a final lipid and Dox-PSA concentration of 5 and 0.25 mM, respectively. Next, liposomes were downsized by extrusion through 0.8 μ m (7 cycles), 0.2 μ m (17 cycles), and 0.1 μ m (13 cycles) polycarbonate filters using a mini-Extruder (Avanti Polar Lipids). Liposomes were then flushed with N₂ and allowed to anneal overnight at RT, to maximize prodrug encapsulation efficiency. Unencapsulated Dox-PSA was removed by passing the liposome suspension through a qEV size exclusion column (IZON Science Europe Ltd.). Dox-PSA encapsulation efficiency (EE, %) was determined by comparing prodrug fluorescence intensity before and after purification, following the equation below:

$$\text{Dox-PSA EE (\%)} = \frac{I(t)_{\text{after purification}}}{I(t)_{\text{before purification}}} \times 100$$

where $I(t)$ is the fluorescence intensity of liposome suspension after liposome lysis with 1% (w/v) Triton X-100. Liposomes were concentrated to the initial concentration.

Dynamic Light Scattering (DLS). Measurement of the hydrodynamic diameter and zeta potential (ζ -potential) of the prepared formulations were performed using DLS with a Malvern Zetasizer Nano ZS (Malvern). Disposable polystyrene cells and disposable plain folded capillary Zeta cells (Malvern) were used. Liposome suspensions were diluted in 0.2 μ m filtered deionized water at ratios of 1:100 for size measurements and 1:10 for ZP measurements. All measurements were performed at 25 °C. The hydrodynamic size was presented as the average value of 15 runs with triplicate measurements within each run, while ζ -potential measurements were performed in quintuplicate.

Cryo-transmission Electron Microscopy (Cryo-TEM). Samples for cryo-electron microscopy were prepared by pipetting 3 μ L of solution onto a holey carbon coated grid (Quantifoil 3.5/1). The grid was blotted and vitrified in ethane in a Vitrobot (FEI, The Netherlands). Grids were observed in a FEI T20 electron microscope operating at 200 keV with a Gatan model 626 cryo-stage. Images were recorded under low-dose conditions with a slow scan CCD camera.

Freeze Fracture Electron Microscopy. For freeze-fracture electron microscopy, Dox-PSA-loaded and empty DOPE:CHEMS liposomes were initially cryoprotected by incubation in 30% glycerol in 0.1 M cacodylate buffer for 60 min at 4 °C. Subsequently, 1.5 μ L of the sample was placed on the copper holder, fast-frozen by immersion into freon, and cooled by liquid nitrogen (−196 °C). Frozen samples were immediately transferred to freeze-fracture device (BALZERS, BAF200), fractured (knife temperature was −150°C), shadowed with platinum (nominal angle 45°), and further strengthened by carbon (nominal angle 90°). Replicas were transferred to room temperature and cleaned in sodium hypochlorite solution. Cleaned replicas were picked on copper microscopic grids and were examined with a transmission electron microscope (Philips, CM100) running at 80 kV.

Cell Culture. LNCaP (CRL-1740) and PC3 (CRL-1435) cells were obtained from American Type Culture Collection (ATCC, USA). LNCaP-derived C4-2B cells were purchased from (MD Anderson Cancer Center, Texas, USA). PC cells were cultured in Advanced RPMI 1640 (Invitrogen Gibco Life Technologies), supplemented with 10% FBS, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 1% L-glutamine and maintained in a humidified chamber at 37 °C and 5% CO₂. C4-2B cells were routinely grown in 75 cm² canted-neck CellBIND tissue culture flasks (Corning) to improve cell adherence and passaged twice a week using 0.05% Trypsin/EDTA when reaching 80% confluence in order to maintain exponential growth.

Quantification of Free PSA. One $\times 10^5$ cells were seeded in six-well culture plates, and 24, 48, and 72 h after incubation at 37 °C/5% CO₂, cell lysates and supernatants were collected. The presence of free PSA in LNCaP and C4-2B cell lysates and supernatants was quantitatively measured using the Free PSA Human SimpleStep ELISA Kit (Abcam) according to the manufacturer's instructions.

Uptake Studies by Confocal Laser Scanning Microscopy (CLSM). *Monolayers.* C4-2B cells (6×10^4) were seeded onto 13 mm glass coverslips (Fisher Scientific) precoated with poly-D-lysine in 24-well plates (Sardstedt). After 48 h, cells were incubated in complete media containing 5 μ M free Dox-PSA or DOPE:CHEMS-Dox-PSA liposomes. At different time points, cells were washed twice with 500 μ L of PBS and fixed with 0.5 mL of 4% (v/v) paraformaldehyde in PBS (RT for 15 min in the dark). Cells were washed twice with PBS to remove fixative and then incubated with 1 μ g/mL of Hoechst 33342 (ThermoFisher) for 10 min to allow nuclei visualization. Following counterstaining, the coverslips were mounted on glass slides (Scientific Laboratory Supplies) using ProLong Gold Antifade mounting media (Invitrogen Life Technologies). Confocal images were acquired with a Leica SP8 microscope (Leica microsystems) using a 63 $\times$ oil immersion objective (numerical aperture 1.4) and 405 and 488 nm laser detectors in the hybrid (HyD) mode. Image analysis was performed using the LasX software (Leica microsystems).

Spheroids. Two hundred microliters of C4-2B cells (5×10^3) was seeded in 96-well clear round-bottom Ultra-Low Attachment plates (Corning). Media was partially replenished every 2 days by withdrawing 100 μ L of spent media and replacing with equivolume of fresh complete media. On day 3, 5 μ M free Dox-PSA or DOPE:CHEMS-Dox-PSA liposomes was diluted in 30 μ L of media and added to the cells. At 48 h postincubation, spheroids were thoroughly washed with phenol red-free media and immediately imaged with a Leica SP8 microscope (Leica microsystems) using a 10 $\times$ dry objective (numerical aperture 0.3) and 488 nm laser detector in the hybrid (HyD) mode. Sequential Z-stack images were acquired from the top to the center of the spheroids. Image analysis was performed using the LasX software (Leica microsystems).

Cell Viability Studies in Prostate Cancer Monolayers. *Resazurin Assay.* C4-2B cells (1×10^4) were seeded in poly-D-lysine coated 96-well culture plates (Sardstedt) in complete media. After 48 h, different concentrations of free Dox-PSA or DOPE:CHEMS-Dox-PSA liposomes were diluted in 30 μ L of media and added to the cells. The medium was not replenished to ensure sufficient PSA concentrations. At 48, 72, and 96 h postincubation, a resazurin cell viability assay was performed, which is based on the mitochondrial metabolic activity of live

Figure 2. Dox-PSA can be efficiently encapsulated inside DOPE:CHEMS liposomes. (A) Scheme of the procedure used to prepare DOPE:CHEMS-Dox-PSA liposomes by passive loading of Dox-PSA. (B) Release mechanism of DOPE:CHEMS-Dox-PSA liposomes. Optimization of Dox-PSA loading into DOPE:CHEMS liposomes. (C) Dox-PSA loading methods. (D) Effect of Dox-PSA loading time on the encapsulation efficiency. (E) Effect of total lipid concentration on the encapsulation efficiency. Data are mean $\pm$ SD ($3 \leq n \leq 15$).

been previously shown in human PSA-expressing PC cell lines.¹⁵ These studies used LNCaP cells as the PSA-expressing cell line; however, these cells represent early stage PC as they display an androgen-sensitive growth and are poorly tumorigenic.^{29–31} In the present work, we used androgen-independent C4-2B (PSA-expressing) and PC3 (PSA-non-expressing) cells. C4-2B cell line was selected as it constitutes a better model for mCRPC.³² Furthermore, we quantified the intracellular (cell lysates) and extracellular (cell supernatants) levels of free PSA over 72 h of culture and showed that, although a great fraction of PSA was secreted to the extracellular media, fairly good levels of PSA could also be found intracellularly (Figure 1B). Moreover, C4-2B cells produced significantly higher amounts of free PSA compared to LNCaP cells, suggesting that C4-2B cells could potentially constitute a more predictive model to study PSA-cleavable prodrugs for mCRPC. Interestingly, similar levels of free PSA can be found in cell lysates and supernatants of C4-2B cells up to 48 h of culture. However, as the cells are allowed more time to adhere and grow, PSA secretion outweighs the amount of free PSA inside the cells. Nevertheless, after 72 h, the concentration of secreted free PSA from LNCaP cells was comparable to the intracellular concentration of free PSA in C4-2B cells. We next evaluated the selectivity of Dox-PSA by

treating androgen-independent C4-2B and PC3 (negative control) cells with the free prodrug. Interestingly, Dox-PSA was a potent cytotoxic agent toward C4-2B cells, while minimal toxicity was obtained in PC3 cells (Figure 1C). The calculated IC₅₀ (concentration of prodrug that results in 50% cell death) for C4-2B cells at 72 h of incubation was approximately 3.37 μ M, whereas the maximum concentration tested (10 μ M) was not sufficient to reach this parameter in PC3 cells. The IC₅₀ reported in the literature for LNCaP cells incubated for 72 h with Dox-PSA is 5 μ M,¹⁵ which is comparable to the one achieved here in C4-2B cells. Yet, the somewhat lower IC₅₀ obtained for C4-2B cells might be attributed to the increased amounts of free PSA in these cells as opposed to LNCaP cells. We have also investigated the intracellular fate of Dox-PSA following uptake by PSA-secreting and nonsecreting cells. To that end, cells were incubated with 5 μ M of Dox-PSA for 24 and 48 h, after which confocal laser scanning microscopy (CLSM) was performed. Dox-PSA intrinsic fluorescence was used to easily track the prodrug inside the cells. The concentration used was selected as it is near the IC₅₀ and as lower concentrations could not be used due to detection limits. In addition, this concentration was previously used elsewhere.³³ Strikingly, Dox fluorescence signals were detected in the nuclei of C4-2B cells at both time

points, whereas in PC3 cells, the prodrug was localized in the cytosol and perinuclear region, with no signal colocalization in the nuclei (Figure 1D). These results show, for the first time, Dox selective nuclear uptake in PSA-expressing cells, suggesting PSA-mediated proteolytic cleavage of Dox-PSA, which resulted in free Dox trafficking to the nucleus. Whether Dox-PSA activation was carried out by extracellularly secreted PSA, resulting in free Dox diffusion across the plasma membrane, or whether it was accomplished intracellularly remains unclear. In efforts to elucidate if Dox-PSA could be activated intracellularly by enzymatically active PSA present in C4-2B cells, we encapsulated it in pH-sensitive liposomes, as will be described below. By using this responsive nanocarrier we want to ensure that (1) Dox-PSA is “protected” and thus would not be hydrolyzed by extracellularly secreted PSA and that (2) the fusogenic nature of these liposomes would ensure a rapid and efficient prodrug release once inside the acidic endosomes,²³ thus making Dox-PSA available for activation by enzymatically active PSA present in C4-2B cells.

Dox-PSA Encapsulation and Release from pH-Sensitive Liposomes. Different drug loading methods were investigated in order to select the one that provided the highest encapsulation efficiency (Figure S2). Passive encapsulation (Figure 2A) rendered significantly higher encapsulation efficiency ($39.4 \pm 10.3\%$) than the one achieved with either freeze–thawing ($23.5 \pm 0.96\%$) or remote loading ($15.2 \pm 1.2\%$), and therefore, passive loading was used in further experiments (Figure 2C). Drug incubation time, i.e., the time frame between extrusion and purification was also tested to assess if longer periods could impact the encapsulation efficiency. Figure 2D shows that the incubation time remarkably influenced the amount of encapsulated Dox-PSA, which indicates that the mechanism behind Dox-PSA loading in DOPE:CHEMS liposomes is a combination of passive and remote loading. In an attempt to further maximize Dox-PSA loading, we varied the initial lipid concentration. We hypothesized that having more liposomes per unit of volume would increase Dox-PSA passive loading in the liposome core. Yet, the latter was not the case as doubling the total lipid concentration from 5 to 10 mM did not improve encapsulation efficiency (Figure 2E); therefore, all future experiments were performed using 5 mM of total lipid. Importantly, prodrug-loaded DOPE:CHEMS liposomes displayed sizes compatible with the EPR effect (157.8 ± 0.9 nm). Furthermore, Dox-PSA encapsulation did not result in an increase in liposome size nor did it alter the ζ -potential of these negatively charged liposomes (Table 1), likely indicating that the prodrug resides in the liposome core rather than being adsorbed to the liposome surface. Next, to elucidate liposome structure, the prodrug-loaded liposomes were examined using cryogenic-transmission electron microscopy (cryo-TEM) and freeze-fracture electron microscopy (FF-EM). Both techniques showed that indeed the size and morphology of Dox-PSA-

loaded liposomes resembled that of their empty counterparts (Figure 3). Furthermore, the appearance of large prodrug crystals inside the liposomes was not evident, suggesting the presence of the prodrug in its soluble form.

Figure 3. Structural elucidation of Dox-PSA-loaded DOPE:CHEMS liposomes. Images of empty and Dox-PSA-loaded DOPE:CHEMS as shown by cryo-TEM (top panel) and freeze-fracture EM (FF-EM) (middle and bottom panels). Scale bar cryo-TEM: 50 nm. Scale bar FF-EM: 100 nm.

Next, we evaluated the prodrug release from DOPE:CHEMS liposomes at 37 °C and pH 5.5 and 7.4. As expected, higher Dox-PSA was released under acidic pH than physiological pH (Figure S3A). It is important to highlight that the release studies were carried out in PBS and might not directly represent the “real” *in vitro* and *in vivo* situation, where longer incubation in a more complex medium and cellular uptake could result in higher release and cell toxicity.

Dox-PSA Can Be Released from pH-Sensitive Liposomes and Intracellularly Activated in C4-2B Cells. Following the successful encapsulation of Dox-PSA into DOPE:CHEMS liposomes, we then assessed the intracellular fate of either the free Dox-PSA or DOPE:CHEMS-Dox-PSA in C4-2B cells at an equivalent dose of 5 μ M Dox-PSA. Once again, the fluorescence properties of the prodrug and the fact that, unlike Dox, its fluorescence is not quenched when encapsulated in liposomes (data not shown), allowed us to easily track the subcellular localization of the prodrug/liposomes by CLSM. As depicted in Figure 4A, liposomes were more rapidly accumulated intracellularly compared to the free prodrug. These results are likely due to the fusogenic properties of DOPE lipid, which has a poorly hydrated polar headgroup, resulting in high affinity for the cellular membrane.^{34,35} Nevertheless, after 24 h of incubation at 37 °C, the liposomal Dox-PSA was still distributed throughout the cytosol of the cells, whereas Dox nuclear uptake was evident in the case of the free prodrug (Figure 4B). These observations are not surprising if we take into account that encapsulation of

Table 1. Physicochemical Characterization of the Optimized DOPE:CHEMS-Dox-PSA Liposomes^a

formulation	Z-ave (d-nm)	PdI	ζ -potential (mV)
empty	158.1 ± 1.3	0.073 ± 0.02	-54.9 ± 2.53
prodrug-loaded	157.8 ± 0.9	0.053 ± 0.02	-51.5 ± 3.51

^aZ-ave: hydrodynamic size. PdI: polydispersity index. ζ -potential: surface charge.

Figure 4. DOPE:CHEMS-Dox-PSA liposomes are taken up by C4-2B cells, where the prodrug is released and activated. (A) Representative images showing the intracellular localization of Dox-PSA and DOPE:CHEMS-Dox-PSA liposomes in C4-2B cells. Dox-PSA fluorescence (red) and the Hoechst 33342-stained nuclei of the cells (blue) were visualized by CLSM over 48 h. Scale bar: 20 μm . (B) Magnified images showing the nuclear uptake of cleaved Dox 24 and 48 h postincubation with free Dox-PSA and DOPE:CHEMS-Dox-PSA liposomes, respectively. Scale bar: 20 μm .

Dox-PSA inside a delivery system represents an additional barrier to PSA-mediated hydrolysis. Yet, longer incubation time with DOPE:CHEMS-Dox-PSA liposomes allowed the prodrug to be activated, and Dox translocated from the cytosol to the nuclei of those cells. It is noteworthy to highlight the relevance of these results as they clearly support the possibility of intracellular activation of Dox-PSA in C4-2B cells, following internalization, since intact Dox-PSA did not traffic to the nucleus (Figure 1D). Moreover, by encapsulating Dox-PSA inside DOPE:CHEMS liposomes we ruled out any potential prodrug cleavage in the extracellular space. Interestingly, Dox nuclear localization in cells incubated with the free and encapsulated Dox-PSA demonstrates that there were enough amounts of enzymatically active PSA inside C4-2B cells to cleave Dox from the PSA peptide. Furthermore, the presence of nuclear fluorescent signals shows that DOPE:CHEMS

liposomes successfully released the prodrug that was followed by proteolysis by intracellular PSA. These data are exciting as they open up the possibility of using a delivery system of Dox-PSA, which could protect the prodrug while in the systemic circulation, thus bypassing any potential nonspecific hydrolysis.

DOPE:CHEMS-Dox-PSA Liposomes Induce Toxicity in C4-2B Monolayers. To explore if the intracellular activation of Dox-PSA could result in significant cell toxicity, a resazurin assay was used to determine the viability of C4-2B cells treated with different doses of either free Dox-PSA or DOPE:CHEMS-Dox-PSA liposomes. Moreover, to ensure that any observed toxicities were a result of Dox-PSA release from the liposomes, followed by its enzymatic activation, and not due to the carrier itself, the toxicity of empty DOPE:CHEMS was assessed, where no toxicity was observed up to 96 h of incubation (Figure 5A). Conversely, dose- and time-dependent reductions

Figure 5. DOPE:CHEMS-Dox-PSA liposomes maintain cytotoxic activity in C4-2B monolayers. (A) Viability of C4-2B cells incubated with empty (prodrug-free) DOPE:CHEMS liposomes was assessed by resazurin assay up to 96 h. Data are expressed as mean $\pm$ SD ($n = 3$) of viable cells normalized to untreated cells. Statistical significance was determined using two-way analysis of variance (ANOVA) over untreated cells, with no statistically significant results found. C4-2B cells were treated with Dox-PSA (blue bars) or DOPE:CHEMS-Dox-PSA liposomes (red bars) for (B) 48 h and (C) 72 h. 10% (v/v) DMSO was used as a positive control for toxicity. Cell viability was quantified by resazurin assay and normalized to the vehicle-treated cells. Data are expressed as mean $\pm$ SEM ($n = 8$). Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Bonferroni posthoc test showing significant effects between cells treated with free Dox-PSA and cells treated with DOPE:CHEMS-Dox-PSA liposomes ($*p < 0.05$). Dose-response viability curves of C4-2B monolayers treated with Dox-PSA (blue circles) or DOPE:CHEMS-Dox-PSA liposomes (red circles) for (D) 24 h, (E) 48 h, and (F) 72 h. Impedance was continuously monitored using a Real-Time Cell Analyzer (RTCA). Cell viability was normalized to the vehicle-treated cells. Data are expressed as mean $\pm$ SD ($n = 2$). Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Bonferroni posthoc test showing significant effects between cells treated with Dox-PSA and cells treated with DOPE:CHEMS-Dox-PSA liposomes ($*p < 0.05$, $**p < 0.01$, $***p < 0.0001$).

Table 2. Dox-PSA and DOPE:CHEMS-Dox-PSA Potency and Efficacy in C4-2B Monolayers, As Determined by RTCA

	IC ₅₀ $\pm$ SD (μ M)			I _{max} $\pm$ SD (%)		
	24 h	48 h	72 h	24 h	48 h	72 h
Dox-PSA	3.91 $\pm$ 1.28	0.31 $\pm$ 0.08	0.14 $\pm$ 0.03	42.57 $\pm$ 1.28	0.00 $\pm$ 0.00	0.45 $\pm$ 0.63
DOPE:CHEMS-Dox-PSA	5.51 $\pm$ 0.20	1.45 $\pm$ 0.15 ^a	0.68 $\pm$ 0.06 ^b	28.52 $\pm$ 0.01 ^c	0.13 $\pm$ 0.18	0.00 $\pm$ 0.00

Statistical significance was determined using an unpaired *t* test, showing significant result between Dox-PSA and DOPE:CHEMS-Dox-PSA: ^a $*p < 0.05$. ^b $**p < 0.01$. ^c $***p < 0.01$.

in the viability of C4-2B cells treated with both the free prodrug and the pH-sensitive liposomes were observed. Furthermore, DOPE:CHEMS-Dox-PSA liposomes displayed toxicity levels similar to those achieved with the free Dox-PSA (Figure 5B,C), indicating successful liposomal release of Dox-PSA and enzymatic activation. More interestingly, C4-2B cells treated with conventional DPPC:Chol:DSPE-PEG₂₀₀₀-Dox-PSA liposomes showed no toxicity up to 96 h (Figure S3B), indicating no significant prodrug leakage from these liposomes. These findings suggest the need of a stimuli to trigger Dox-

PSA release and support the selective release from DOPE:CHEMS liposomes under acidic environment (Figure S3A).

It is worth mentioning that at some concentrations and time points, DOPE:CHEMS-Dox-PSA liposomes were slightly less toxic than the free Dox-PSA. Such observations could be explained by either an incomplete prodrug release from the liposomes at a certain time point, or by the added barrier to prodrug activation that the liposomes represent, which might delay the toxicity profile in comparison to the free prodrug. Furthermore, similar findings were obtained using the label-

Figure 6. DOPE:CHEMS-Dox-PSA show enhanced penetration and superior toxicity in C4-2B spheroids. C4-2B spheroids were treated with 2–10 μ M Dox-PSA (blue bars) or DOPE:CHEMS-Dox-PSA liposomes (red bars) for (A) 48 h, (B) 72 h, and (C) 96 h. 10% (v/v) DMSO was used as a positive control for toxicity. Cell viability was quantified by a modified resazurin assay (details in the [Experimental Section](#)) and normalized to the vehicle-treated spheroids. Data are expressed as mean $\pm$ SEM ($n = 4$). Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Bonferroni posthoc test showing significant effects between cells treated with free Dox-PSA and cells treated with DOPE:CHEMS-Dox-PSA liposomes (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (D) Bright field and fluorescent images depicting the penetration of Dox-PSA and DOPE:CHEMS-Dox-PSA over 48 h in C4-2B spheroids. Scale bar: 100 μ m.

free Real Time Cell Analysis (RTCA) (Figure 5D–F). Our RTCA results, showed that the IC_{50} values for the free Dox-PSA and DOPE:CHEMS-Dox-PSA liposomes were comparable at 24 h, but over time the free prodrug was slightly more toxic. Importantly, at 24 h of incubation, 10 μ M of Dox-PSA was more toxic in the liposomal form ($I_{max} = 28.52 \pm 0.01\%$) than as free prodrug ($I_{max} = 42.57 \pm 0.01\%$) (Table 2). Overall, the cell viability data obtained with the end point and real-time assays showed that Dox-PSA delivered by DOPE:CHEMS liposomes, although slightly less potent than the free Dox-PSA, preserves the cytotoxic activity.

DOPE:CHEMS-Dox-PSA Liposomes Are Therapeutically More Effective than the Free Dox-PSA in C4-2B Spheroids. Given the promising results obtained in C4-2B monolayers, we decided to assess the efficacy of prodrug-loaded liposomes in C4-2B spheroids, which more realistically replicate the *in vivo* environment. C4-2B cells growing on a nonadherent agarose matrix rapidly produced highly compact spheroids, which developed a visible hypoxic core. Tumor spheroids were treated with either free or encapsulated Dox-PSA for different time periods. Notably, treatment of C4-2B

spheroids with DOPE:CHEMS-Dox-PSA liposomes induced a very significant reduction in the viability in contrast to that observed for the free Dox-PSA (Figure 6A–C). These results suggest that Dox-PSA encapsulation in pH-sensitive liposomes contributes to its enhanced penetration across the spheroid cell layers. Furthermore, toxicity of free Dox-PSA was lower in spheroids compared to monolayers, which was not time- or concentration-dependent. Such observation could either indicate that spheroids do not produce sufficient amounts of enzymatically active PSA or that the free Dox-PSA does not penetrate deeply through the inner layers of the spheroids, as suggested by the absence of toxicity at 48 h postincubation (Figure 6A). Interestingly, when Dox-PSA was encapsulated in pH-sensitive liposomes, toxicity levels closely resembled those achieved in monolayers, which excluded the possibility of spheroids not producing enough enzymatically active PSA. Furthermore, the enhanced penetration of the prodrug using the fusogenic, pH-sensitive DOPE-CHEMS liposomes was confirmed using CLSM (Figure 6D). It was clear that free Dox-PSA was located mainly at the peripheral cell layers of the tumor spheroid, which might justify the minimal toxicity

obtained with free Dox-PSA at 48 h. Conversely, the liposomal Dox-PSA penetrated deeper within the spheroids, resulting in higher cell toxicity.

■ DISCUSSION

Dox-PSA was developed to improve the therapeutic index of Dox by exploiting PSA relative specificity to the prostate tissue. Previous studies with this prodrug have shown that it was a selective anticancer agent against PSA-secreting cells. In addition, Dox-PSA was approximately 15-fold more effective in reducing tumor weight compared to Dox in nude mice bearing LNCaP tumors.¹⁵ Furthermore, selectivity studies with Dox-PSA-treated mice have shown that 2.5-fold more Dox molar equivalents accumulated in tumor, whereas substantially lower concentrations were found in heart tissue in comparison with the situation where Dox was administered alone.¹⁴ Despite the initially promising results, Dox-PSA did not advance beyond phase I clinical trials. The reasons behind this were likely toxicity concerns as it was shown that in four different species of laboratory animals there was approximately 30–40% non-PSA-mediated prodrug conversion into the active metabolite Dox.^{13,14} Aiming at increasing PSA specificity, Aloysius et al. modified the initial peptide sequence, glutaryl-Hyp-Ala-Ser-Chg-Gln, by introducing modifications at position P₅, which improved PSA hydrolysis rate and decreased non-PSA-specific cleavage.¹³ However, a safer therapeutic approach based on Dox-PSA encapsulation in a nanocarrier could potentially circumvent the nonspecific activation as the prodrug would be delivered to the tumor cells inside a “shield”. Several studies have used a wide range of liposomes to efficiently deliver a variety of prodrugs, including a number of anticancer agents,^{36,37} phospholipid prodrugs,^{38,39} anti-inflammatory prodrugs,⁴⁰ and β -blockers prodrugs.⁴¹ Interestingly, previous reports with PSA-cleavable prodrugs have relied on the enzymatically active extracellular PSA to activate the prodrug.^{13,15,20,42,43} As a proof-of-concept, in this work we used pH-sensitive liposomes composed of DOPE:CHEMS to ensure a fast and efficient Dox-PSA release inside the acidified endosomes, which is essential for prodrug activation. Interestingly, and for the first time, we have provided evidence for the intracellular activation of Dox-PSA prodrug following its encapsulation in DOPE:CHEMS liposomes, which did not hinder its cytotoxic activity but, indeed, improved its activity in 3D PC models.

Here, C4-2B cells were used as a model for mCRPC, and we have shown by ELISA that these cells secrete high levels of free PSA in a time-dependent fashion and that, more importantly, higher intra- and extracellular concentrations of free PSA were found in C4-2B cells in comparison to the LNCaP counterparts (Figure 1B), which have been consistently used to assess the selectivity of PSA-cleavable prodrugs.^{12,15,44} These observations are relevant as they show that C4-2B cells produce high amounts of free PSA, which is not exclusively secreted, but also present inside the cells. The reliance on extracellular cleavage in previous studies with PSA-cleavable prodrugs is based on several factors. First, it is known that divalent ions, specifically zinc, inhibit PSA proteolytic activity.^{20,45} However, malignant human prostate epithelial cells contain decreased levels of zinc in comparison with their normal counterparts as a consequence of dysregulation of a number of zinc transporters in PC.^{21,22,46} Second, it was reported that PSA enzyme is converted into the enzymatically active form upon extracellular removal of a seven amino acid

pro-sequence,⁴⁷ and thus, any PSA present intracellularly would be catalytically inactive. However, a previous study has shown that PSA is imported to the nuclei of both LNCaP and C4-2B cells and proposed two models to justify this finding: (1) full length PSA is extracellularly activated and then imported into the nucleus, or (2) PSA is cleaved intracellularly by unknown proteases and then translocated to the nucleus.⁴⁸ Independently of whether PSA is converted into the catalytically active form outside the cells and then imported back in, or whether its activation can also be accomplished intracellularly, we believe that C4-2B cells might produce enzymatically active PSA that could be explored for the intracellular activation of a Dox-PSA nanomedicine. We have shown that the in-house synthesized Dox-PSA prodrug is selectively toxic toward PSA-expressing C4-2B cells, whereas PSA-nonexpressing PC3 cells displayed only minor toxicity (Figure 1C). These data correlate well with the toxicity results reported in the literature using the same prodrug in LNCaP and PC3 cells.¹⁵ Our selectivity results were further supported by confocal imaging, where cleaved Dox was localized in the nuclei of C4-2B cells at 24 and 48 h of incubation, but not in the nuclei of PC3 cells, as Dox-PSA fluorescent signal was restricted to the cytosol and perinuclear region (Figure 1D). This intracellular activation of Dox-PSA was further confirmed by encapsulating Dox-PSA inside DOPE:CHEMS liposomes to eliminate any extracellular activation. Thus, any nuclear signals would come from Dox-PSA cleavage by intracellular enzymatically active PSA.

Liposomes are known to efficiently encapsulate a wide range of therapeutics.⁴⁹ In the present study, we were able to encapsulate approximately $39.4 \pm 10.3\%$ Dox-PSA in DOPE:CHEMS liposomes by means of passive encapsulation (Figure 2C), which was lower than a previous report, using the positively charged 25 natural amino acid peptide Print3G.⁵⁰ The higher entrapment efficiency attained with Print3G might be due to its higher hydrophilicity and its electrostatic interaction with negatively charged liposomes. Interestingly, our results confirmed that Dox-PSA was entrapped inside the liposomes since minimum changes in liposomes size, surface charge, and morphology were observed (Table 1 and Figure 3, respectively). Following a successful Dox-PSA encapsulation, cargo release and endosomal escape are of paramount importance to avoid prodrug degradation as a consequence of the low pH and enzymatic cocktail present in the endolysosomal system,^{51,52} and to ensure Dox-PSA delivery to the cytosol, where it can then be metabolized into Dox. Here resides the beauty of the pH-sensitive liposomes, which are tailor-made for endosomal escape.^{51,53} Using CLSM, we have shown that at early time points, despite their high negative surface charge, they accumulated faster inside C4-2B cells compared to the free Dox-PSA (Figure 4A). Although in the present work, the endocytic mechanisms were not investigated, we speculate that the difference in the extent of internalization between the free prodrug and the pH-sensitive liposomes could rely on the involvement of distinct uptake modalities and/or kinetics of intracellular processing. There is compelling evidence showing that liposome/nanoparticle uptake is influenced by a multitude of parameters, including nanocarrier size,^{52,54} surface charge,^{55,56} and hydrophobicity,^{56,57} lipid composition,^{58,59} shape,^{53,56} and cell type.^{53,54} Based on the available literature, we hypothesize that both the free and encapsulated Dox-PSA are taken up by PC cells via caveolae-mediated endocytosis (CME), in contrast to free

Dox, which is known to freely diffuse across the plasma membrane and readily localize in the nucleus.⁶⁰ Indeed, this endocytic pathway had previously been reported for pH-sensitive liposomes.^{23,61} Furthermore, the observation that Dox-PSA fluorescence was initially localized in the cytosol of C4-2B cells and gradually appeared in the nuclei in a time-dependent manner indicates that the prodrug does not enter the nucleus until it is cleaved into free Dox⁶² since the latter was not observed in PSA-negative cells (Figure 1D). As expected, Dox-PSA encapsulation in DOPE:CHEMS liposomes resulted in a delayed nuclear uptake since the prodrug must first be released from the carrier⁵⁸ prior its activation. Remarkably, longer incubation time enabled released Dox to travel to the nuclei of C4-2B cells (Figure 4A,B), which is an indication of efficient prodrug release and subsequent activation by enzymatically active PSA present in these cells. These results were further confirmed since relatively comparable cytotoxicity of free and encapsulated Dox-PSA in C4-2B was observed. It is worth mentioning that some differences in the IC₅₀ values were observed between resazurin and RTCA; however, such discrepancies were reported previously.⁶³ Lower viabilities were reported using RTCA compared to resazurin since the former measures cell impedance, which is highly sensitive to any stress that can potentially cause an alteration in the cellular morphology and/or adherence strength, which enabled us to detect changes, 24 h earlier than resazurin assay (48 h). Furthermore, metabolism-based assays such as resazurin assay have been reported to overestimate cell viability in comparison with nuclei-counting approach.⁶³ This could explain the differences in the IC₅₀ of Dox-PSA determined by resazurin (Figure 5B,C) and RTCA (Figure 5D–F). Nevertheless, despite these differences, our findings consistently showed that DOPE:CHEMS-Dox-PSA showed high cytotoxicity, further supporting Dox-PSA release from the liposomes and its delivery into the cytoplasm.

Although 2D models constitute an easy and practical solution in the preclinical validation of drugs, they are far too simplistic to replicate the complexity of the pathophysiology of tumors. To overcome the bottlenecks associated with cell monolayers, 3D models are being increasingly used as they more realistically recapitulate the *in vivo* tumor biology, with respect to cell–cell interactions, the presence of extracellular matrix and cancer stem cells (CSC) properties, oxygen, pH and nutrient gradients, hypoxic regions and necrotic core, and gene expression profiles.^{64,65} It has been reported that PC spheres are enriched in CSC in comparison with adherent cells. This is relevant as CSC are implicated in tumor initiation and progression, metastatic disease, and chemotherapy resistance.⁶⁶ In an attempt to evaluate the relevance of the DOPE:CHEMS-Dox-PSA liposomes in the therapy of advanced PC, we then assessed their toxicity in C4-2B spheroids. Unlike the results obtained in monolayers, incubation of C4-2B spheroids with the pH-sensitive liposomes resulted in significantly augmented toxicity in comparison to the free prodrug (Figure 6A–C). Such discrepancies could be justified by an enhanced penetration of these fusogenic liposomes⁶⁷ over the free prodrug, as confirmed by confocal imaging, where high fluorescent signals were detected at deeper levels in the treated spheroids (Figure 6D).

All in all, we have successfully demonstrated that Dox-PSA prodrug can be intracellularly activated in C4-2B cells. Furthermore, uptake and cytotoxicity studies showed that

(1) DOPE:CHEMS liposomes can efficiently deliver Dox-PSA to C4-2B cells, (2) Dox-PSA can be efficiently released from these pH-sensitive liposomes, (3) the released prodrug can be activated into Dox, followed by translocation to the nuclei of PSA-expressing cells, and (4) DOPE:CHEMS-Dox-PSA liposomes were able to retain prodrug activity in 2D cultures, while they were more toxic than the free Dox-PSA in 3D PC models. Overall, we propose that revisiting this “forgotten” prodrug and combining it with a nanomedicine approach can be a smart strategy to overcome “off-target” prodrug activation. Further *in vivo* studies are needed to validate the use of such a nanomedicine in the treatment of mCRPC; however, these nanomedicines should be engineered to exhibit relatively long blood circulation to passively target tumors while promptly releasing the encapsulated prodrug in response to external stimuli (e.g., pH or temperature), thus enabling its intracellular activation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.8b01257.

MALDI-TOF spectra of Dox-PSA, RP-HPLC chromatogram of Dox-PSA, schemes of Dox-PSA loading techniques, Dox-PSA release, and cytotoxicity of conventional liposomes (PDF)

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Notes

The authors declare no competing financial interest.

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