Published in final edited form as:

Biomaterials. 2015 October ; 67: 104–114. doi:10.1016/j.biomaterials.2015.07.027.

Effective co-delivery of doxorubicin and dasatinib using a PEG-Fmoc nanocarrier for combination cancer chemotherapy

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Abstract

A simple PEGylated peptidic nanocarrier, PEG₅₀₀₀-lysyl-(α -Fmoc- ϵ -Cbz-lysine)₂ (PLFCL), was developed for effective co-delivery of doxorubicin (DOX) and dasatinib (DAS) for combination chemotherapy. Significant synergy of DOX and DAS in inhibition of cancer cell proliferation was demonstrated in various types of cancer cells, including breast, prostate, and colon cancers. Co-encapsulation of the two agents was facilitated by incorporation of 9-Fluorenylmethoxycarbonyl (Fmoc) and carboxybenzyl (Cbz) groups into a nanocarrier for effective carrier-drug interactions. Spherical nanomicelles with a small size of ~30 nm were self-assembled by PLFCL. Strong carrier/drug intermolecular π - π stacking was demonstrated in fluorescence quenching and UV absorption. Fluorescence study showed more effective accumulation of DOX in nuclei of cancer cells following treatment with DOX&DAS/PLFCL in comparison with cells treated with DOX/PLFCL. DOX&DAS/PLFCL micelles were also more effective than other treatments in inhibiting the proliferation and migration of cultured cancer cells. Finally, a superior anti-tumor activity was demonstrated with DOX&DAS/PLFCL. A tumor growth inhibition rate of 95% was achieved at a respective dose of 5 mg/kg for DOX and DAS in a murine breast cancer model. Our nanocarrier may represent a simple and effective system that could facilitate clinical translation of this promising multi-agent regimen in combination chemotherapy.

Keywords

combination chemotherapy; micelle; 9-Fluorenylmethoxycarbonyl; carrier-drug interaction; doxorubicin; dasatinib

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1. Introduction

Doxorubicin (DOX) is a first-line chemotherapeutic agent in the treatment of a broad range of cancers including breast, ovary, bladder, cervix, prostate and lung cancers, and leukemias. DOX potently inhibits proliferation of cancer cells through binding to topoisomerase enzyme II or directly intercalating with DNA base pairs to suppress DNA replication and induce cell apoptosis [1–3]. In clinic, DOX is widely used in multi-drug regimens with other anti-cancer agents, such as cyclophosphamide, 5-fluorouracil, docetaxel, vinblastine, and bleomycin [4–6]. Due to the complex nature of cancer, simultaneous administration of multiple drugs with different mechanism of actions usually represents an attractive strategy over single drug treatment in clinical cancer therapy. This combination therapy can synergistically inhibit the tumor growth via modulating different signaling pathways. Such strategy shall help to maximize the therapeutic response of chemotherapy while minimizing the occurrence of multi-drug resistance [7–10].

Recently, *in vitro* studies have clearly demonstrated significant synergistic effect between DOX and dasatinib (DAS) in inhibiting the proliferation, migration, and invasion of cancer cells [11]. DAS is a tyrosine kinase inhibitor that targets to various kinases such as Src, BCR-Abl, FAK, c-Kit, and others [12–17]. This distinct mechanism of action by DAS suggests its potential application in combination chemotherapy with other anti-cancer agents. For example, Pichot et al. demonstrated that treatment of breast cancer cells with a combination of DOX and DAS led to significantly enhanced growth inhibition over each individual treatment [11]. Although this strong synergy may indicate a potential multi-agent regimen to enhance clinical therapeutic outcome of DOX, so far studies of this drug combination are only limited to *in vitro* investigation. One of the possible reasons lies in the difficulty in efficient co-delivery of the two agents to tumors due to their dissimilar physiochemical properties and pharmacokinetic profiles.

One of the most favored features of using nano-sized drug delivery system is its capability to encapsulate multi-agents of diverse chemical structures to achieve simultaneous delivery to target site [10, 18]. Several nano-sized delivery systems have been developed in recent decades [19–23]. Among them, micelles are one of the most attractive systems due to the simplicity in manipulation, high efficiency in solubilization of hydrophobic drugs, and small particle size that permits specific accumulation in solid tumor. Currently, various micellar systems have been intensively studied for their potential in target delivery of anti-cancer agents to tumor sites [24–28].

Previously, we developed a new type of micelles equipped with fluorenylmethyloxycarbonyl (Fmoc) as a highly effective drug interactive motif to facilitate carrier-drug interactions [29–33]. Compared with conventional micelles that simply entrap drugs through hydrophobic interactions, our micelles exhibited significantly improved compatibility with payload drugs due to the additional mechanisms of carrier-drug interactions, such as π - π stacking and hydrogen bonding interaction. We have demonstrated that a simple PEG-Fmoc conjugate, PEG₅₀₀₀-lysyl-(α -Fmoc- ϵ -t-Boc-lysine)₂, exhibited excellent loading capacity and colloid stability in delivery of anti-cancer agent paclitaxel (PTX) [30]. However, this nanocarrier has limited utility for other hydrophobic agents. Subsequent studies on structure-activity

relationship demonstrated that this problem can be solved through modulating neighboring groups of Fmoc. By replacing t-Boc motif with Cbz, the resulting system showed broadened utility in formulating a variety of anti-cancer agents including PTX, DOX, docetaxol, and imatinib [34].

In this study, a new nanocarrier, PEG₅₀₀₀-lysyl-(α -Fmoc- ϵ -Cbz-lysine)₂ (PLFCL), was developed for effective co-delivery of DOX and DAS for combination chemotherapy. In addition to comprehensive characterization of biophysical properties of this co-delivered nanomicelle formulation, synergistic chemotherapeutic efficacy was systemically evaluated in both cultured cancer cells and tumor-bearing animal models.

2. Materials and methods

2.1. Reagents

DOX and DAS were purchased from LC Laboratories (MA, USA). α -Fmoc- ϵ -Cbz-lysine and di-Boc-lysine were purchased from GL Biochem Ltd. (Shanghai, China). N, N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), trifluoroacetic acid (TFA), and triethylamine (TEA) were obtained from Acros Organic (NJ, USA). Propylene glycol was purchased from MP Biomedicals (OH, USA). Monomethoxy PEG₅₀₀₀, 4-dimethylaminopyridine (DMAP), ninhydrin, and other unspecified chemicals were all purchased from Sigma-Aldrich (MO, USA). Dulbecco's phosphate buffered saline (DPBS), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), 100X penicillin-streptomycin solution were all purchased from Invitrogen (NY, USA). All solvents used in this study were HPLC grade.

2.2 Synthesis of PEG₅₀₀₀-lysyl-(α -Fmoc- ϵ -Cbz-lysine)₂ (PLFCL)

PLFCL was synthesized through a simple three-step synthesis route. Monomethoxy PEG₅₀₀₀ (1 equiv.) was dissolved in dried dichloromethane (DCM), and mixed with di-Boc-lysine (1.5 equiv.), DCC (1.8 equiv.) and DMAP (0.3 equiv.). The reaction was allowed at 37 °C for 48 h, followed by filtration twice to obtain clear filtrate. A large amount of white precipitate was obtained in cold ether, and washed twice with cold ethanol and ether. The obtained PEG₅₀₀₀-di-Boc-lysine was then treated with DCM/TFA (1:1, v/v) for 2 h at room temperature, followed by precipitation in ice-cold ether, and two washes with cold ethanol and ether.

In the last step, α -Fmoc- ϵ -Cbz-lysine (3 equiv.) was mixed with DCC (3.6 equiv.), NHS (3.6 equiv.), and DMAP (0.6 equiv.) in dried DCM, and allowed to react for 4 h at 37 °C. Then a mixture of PEG₅₀₀₀-lysine-NH₂ (1 equiv.) and TEA (3 equiv.) in DCM was added into the reaction. The conjugation was conducted at 37 °C till negative result was observed in ninhydrin test. Then the mixture was filtered twice, and precipitated in ice-cold ether, followed by two washes with cold ethanol and ether. The raw material was further purified by filtration through a 450 nm PVGF filter in distilled water, followed by lyophilization to yield white powder of PLFCL. The identity of obtained material was confirmed by ¹H NMR and MALDI-TOF.

2.3 Preparation and biophysical characterization of drug-free and drug-loaded PLFCL micelles

DOX&DAS co-encapsulated PLFCL micelles were prepared through a thin-film hydration method [30]. Briefly, designated molar ratios of DOX (5 mg/mL in 1:1 (v/v) of DCM/methanol), DAS (1 mg/mL in ethanol), and PLFCL (50 mg/mL in DCM) were mixed in a glass tube, and organic solvent was removed through a gentle stream of nitrogen, followed by drying in vacuum for overnight. The obtained thin-film of carrier/drug mixture was then hydrated in DPBS and suspended by vortex to obtain a clear solution of DOX&DAS/PLFCL micelles. DOX/PLFCL, DAS/PLFCL, and drug-free PLFCL micelles were prepared similarly. All drug-loaded micelles were filtered through a PVGF filter (450 nm) prior to characterization.

The critical micelle concentration of PLFCL was determined using pyrene as a fluorescence probe [31]. The size distribution and zeta potential of drug-free and drug-loaded micelles were detected through a Malvern Zeta Nanosizer. The morphology was observed by transmission electron microscopy (TEM) using negative staining method. The quantity of agents in micelles was detected through a Waters Alliance 2695 Separations Module with a Waters Symmetry RP-C18 column (250 mm × 4.6 mm) using acetonitrile: H₂O (52.5%: 47.5%) containing 2.5 mM NH₄Ac and 0.05% CH₃COOH (pH 3.55) at a rate of 1 mL/min. DOX concentration was detected by a Waters 2475 Fluorescence Detector with excitation at 490 nm and emission at 590 nm. DAS concentration was measured through a Waters 2998 DAD Detector at 319 nm.

2.4 Fluorescence quenching

DOX&DAS/PLFCL, DOX/PLFCL, DAS/PLFCL, and drug-free PLFCL micelles were prepared as described above. Carrier/drug molar ratio was kept at 2:1, and concentrations of PLFCL, DOX and DAS were kept constant in different groups. The samples were placed into a 96-well plate. The fluorescence intensity of Fmoc was detected at the excitation wavelength of 270 nm and emission wavelength of 300–450 nm, and fluorescence of DOX was measured using an excitation wavelength of 480 nm and emission wavelength from 510–650 nm by a Synergy H1 Hybrid Multi-Mode Microplate Reader.

2.5 UV absorbance spectroscopy

DOX&DAS/PLFCL, DOX/PLFCL, DAS/PLFCL, and drug-free PLFCL micelles were prepared as described above, and the UV absorption spectra were scanned by a Varian Cary 50 Bio UV-Visible Spectrophotometer at a wavelength between 286 and 590 nm. All the samples were prepared at a carrier/drug molar ratio of 2:1, and all concentrations were kept constant between different groups for comparison. Blank aqueous solvent was used as the reference.

2.6 In vitro drug release kinetics

The release kinetics of DOX and DAS from PLFCL micelles was conducted through dialysis method using DPBS (PH = 7.4) containing 0.5% (w/v) Tween 80 as release medium. Free DAS solubilized in 50% propylene glycol (v/v), and free DOX were used as controls. Two mL of sample solution was placed into a dialysis bag (MWCO 12 kDa,

Spectrum Laboratories), and incubated in a tank containing 200 mL of release medium under gentle shaking at 37 °C. At scheduled time points (0, 1, 2, 4, 8, 24, and 48 h), the concentration of DOX and DAS remaining in the dialysis bag was measured by HPLC as described above.

2.7 Cell culture

Mouse metastatic breast cancer cell line 4T1.2, human prostate cancer cell line PC 3, and human colonic carcinoma cell line HCT-116 were used for *in vitro* evaluation. These cell lines were all cultured at 37 °C in DMEM containing 10% FBS and 1% penicillin-streptomycin in a humidified environment with 5% CO₂.

2.8 Intracellular trafficking

4T1.2 cells were seeded in glass bottom dishes (In Vitro Scientific, USA) at a density of 1×10^5 cells/dish. Following culture for 24 h, cells were treated with DOX&DAS/PLFCL, DOX/PLFCL, or free DOX in Opti-MEM (Life Technologies, USA) for 0.5, 2, and 4 h, respectively. The concentration of DOX or DAS was kept at 10 µmol/L. Then cells were stained with Hoechst 33342 (1 µg/mL) for 30 min, and LysoTracker Green DND-26 (50 nM) (Invitrogen, USA) for 5 min, followed by two washes with cold DPBS. The intracellular distribution of DOX of various formulations was observed under a confocal laser scanning microscope (CLSM, FluoView 1000, Olympus, Japan).

2.9 In vitro cytotoxicity study

4T1.2, PC 3 and HCT-116 cancer cell lines were used in *in vitro* cytotoxicity study. Cells were seeded in 96-well plates at a density of 1000 (4T1.2), 2000 (HCT-116) and 3000 (PC 3) cells per well 24 h prior to treatment. Then cells were treated by various concentrations of DOX and/or DAS in different formulations. Drug-free PLFCL micelles were dosed at equivalent concentrations as in corresponding drug-loaded groups. Cells were incubated for 72 h, and the cell viability was measured through MTT assay as reported before [30]. Untreated cells were included as a control.

2.10 Cell migration inhibition

Cell migration inhibition was conducted using murine 4T1.2 cell line through a wound healing assay. Briefly, 4T1.2 cells were seeded into a 6-well plate, and allowed 24 h to attach and grow to a confluence of 80%. Then cells were streaked with a sterile pipette tip, treated with media containing various formulations at each drug concentration of 100 nmol/L, and allowed 16 h to recover. Wound width before and after treatment was observed and quantified under a Nikon eclipse TE 300 microscope.

2.11 Animals

Female BALB/c mice (6 to 8 weeks) were purchased from Charles River (Davis, CA). All animals were housed under pathogen-free conditions according to AAALAC guidelines. All animal-related experiments were performed in full compliance with institutional guidelines and approved by the Animal Use and Care Administrative Advisory Committee at the University of Pittsburgh.

2.12 In vivo therapeutic efficacy

Female BALB/c mice were s.c. inoculated with 4T1.2 cells (2×10^5 cells/mouse) at the right flank. When the tumor size reached $\sim 50 \text{ mm}^3$, mice were randomly divided into five groups ($n=5$) and administered with saline, DOX/PLFCL, DAS/PLFCL, DOX+DAS, and DOX&DAS/PLFCL, respectively, through tail vein on day 1, 4 and 7. The dosage of DOX or DAS was kept at 5 mg/kg. Tumor volumes were calculated according to the formula: $(L \times W^2)/2$, in which L is the longest and W is the shortest tumor diameter (mm), and presented as relative tumor volume (RTV, the tumor volume at a given time point divided by the tumor volume prior to first treatment). Mice were sacrificed and tumors were harvested at the completion of the experiment. Tumor weights were measured, and tumor growth inhibition ratio was calculated as: $1 - (\text{mean tumor weight of drug treated group} / \text{mean tumor weight of saline treated group}) \times 100\%$. Changes in body weights of all mice were also monitored during the entire course of treatment.

In a separate experiment, groups of seven 4T1.2 tumor-bearing mice were similarly treated as described above to follow the animal survival. For humane reasons, the end point of survival study was set when the implanted tumor volume reached 1000 mm^3 . The survival rates are presented as Kaplan–Meier curves.

2.13 Histochemical staining

After completion of the *in vivo* therapy study, tumor tissues were excised and fixed in 4% formaldehyde in PBS, followed by embedment in paraffin. The paraffin-embedded tumor samples were sectioned into slices at $5 \mu\text{m}$ using a HM 325 Rotary Microtome. The slices were then stained with hematoxylin and eosin (H&E) for histopathological examination under a Zeiss Axiostar plus Microscope (PA, USA). In situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using an In Situ Cell Death Detection Kit (Roche, USA) according to the manufacturer's protocol. DAPI was used for nuclear staining. The stained tumor slides were observed by a Nikon eclipse TE 300 fluorescence microscope.

2.14 Statistical analysis

Statistical analysis was performed with two-tailed Student's t-test between two groups. $p < 0.05$ was considered statistically significant, and $p < 0.01$ was considered highly statistically significant. One-way ANOVA was conducted to assess significance among multiple groups, followed by Newman-Keuls test if $p < 0.05$.

3. Results

3.1 Combinational effect of DOX&DAS on cancer cell proliferation

The combinational effect of DOX and DAS on cancer cells was firstly tested in human breast cancer cell lines by Pichot et al [11]. To examine its potential broad application, we investigated the therapeutic efficacy of this drug combo in a variety of other cancer cell lines, including murine breast cancer cell line 4T1.2, human prostate cancer cell line PC 3, and human colon cancer cell line HCT-116. As shown in Fig. 1A, 4T1.2 breast cancer cells were well responsive to each single treatment by DOX or DAS with an IC_{50} of 24.6 and

94.2 nM, respectively. Nonetheless, combination of the two agents led to a significant improvement in the inhibition of tumor cell growth. The combination of the two agents was also more active than each treatment alone in inhibiting the proliferation of PC 3 (Fig. 1B) and HCT-116 (Fig. 1C) tumor cells although these two cell lines were relatively more resistant than 4T1.2 cells to either DOX or DAS. Fig. 1D summarizes the combination index from the above studies, in which $CI > 1$ indicates antagonism, $CI = 1$ indicates additivity, and $CI < 1$ indicates synergy. It is apparent that DOX and DAS show a significant synergy in inhibiting the growth of all 3 tumor cell lines tested.

3.2 Design, synthesis, and characterization of PLFCL conjugate

We have previously developed a simple and effective nanomicellar carrier, PEG₅₀₀₀-lysyl-(α -Fmoc- ϵ -t-Boc-lysine)₂, that is highly effective in delivery of PTX to tumor cells *in vitro* and *in vivo* [30]. Drug loading into this carrier is largely achieved via the strong carrier and drug interactions including π - π stacking and hydrogen bonding. However, this carrier showed limited efficacy in formulating several other anticancer agents tested. Subsequent SAR studies showed that the neighboring structures of Fmoc also contributed significantly to the overall carrier/drug interaction. These studies have led to the development of PEG₂₀₀₀- α -Fmoc- ϵ -Cbz-lysine that was highly effective in formulating various therapeutic agents of diverse structures including PTX, docetaxel, DOX, and imatinib [34]. This nanocarrier is a simple conjugate in which Boc was replaced by Cbz. It can be readily obtained by a one-step synthesis. Interestingly, despite the success of PEG₂₀₀₀- α -Fmoc- ϵ -Cbz-lysine in formulating various anticancer drugs, DOX and DAS could not be effectively co-encapsulated into this single chain nanocarrier even at high carrier/drug molar ratios above 5/1. This might be due to the limited size of drug-interacting pocket formed by PEG₂₀₀₀- α -Fmoc- ϵ -Cbz-lysine.

PLFCL is a PEG-peptidic conjugate that has two Fmoc/Cbz domains. We hypothesized that PLFCL will be more effective than PEG₂₀₀₀- α -Fmoc- ϵ -Cbz-lysine in co-delivery of DOX and DAS due to its increased number of drug-interactive motifs and its branched topology. The synthesis route of PLFCL conjugate was depicted in Fig. 2. This simple nanocarrier was prepared through a three-step route with DCC/DMAP as coupling reagents that are routinely used in peptide synthesis. A short peptide consisted of three lysines was conjugated to the terminal of PEG chain to introduce a branched structure decorated with two Fmoc/Cbz domains. As shown in the ¹H NMR spectrum (Fig. 3A), the proton signals of PEG backbone were located at 3.3–3.8 ppm with methylene protons at 3.26 ppm. The Fmoc and Cbz proton signals appeared at 7.2–7.8 ppm as multiple peaks and the protons at 1.4–1.8 ppm were attributed to lysine linker. The molecular weight of PLFCL detected by MALDI-TOF MS is close to the theoretical value (6098) (Fig. S1). Taken together, these data indicate the successful synthesis of PLFCL conjugate.

3.3 Fabrication and biophysical characterization of PLFCL micelles for co-encapsulation of DOX&DAS

A simple thin-film hydration method was used in fabrication of PLFCL micelles. Upon exposure to aqueous environment, PLFCL was self-assembled into nano-sized micelles (Fig. 2). The measured CMC of PLFCL was 2.6 μ M (Fig. S2). This low CMC would contribute to

a high stability of the micelle formulation upon dilution in blood stream after injection. The particle size of PLFCL micelles was ~30 nm, as determined by dynamic light scattering (DLS) (Table 1). TEM images using negative staining showed the spherical morphology of these self-assembled micelles (Fig. S3).

As an initial step to examine the potential of PLFCL in co-formulating DOX and DAS, a molar ratio of 1:1 between DOX and DAS was used. This ratio was chosen as DOX and DAS showed comparable cytotoxicity towards 4T1.2 cells and this cell line was used in the subsequent synergistic study both *in vitro* and *in vivo*. Fig. 3 shows the results of ^1H NMR study of drug-loaded PLFCL micelles. Since PLFCL is expected to be dispersed as monomers in organic solvent, the proton signals from PLFCL (Fig. 3A), DOX (Fig. 3B), and DAS (Fig. 3C) were all clearly observed as expected when they were mixed in DMSO- d_6 (Fig. 3D). However, the signals of Fmoc and Cbz were greatly suppressed in deuterated water (Fig. 3E), indicating the self-assembly of nanomicelles in aqueous solution and the embedding of Fmoc/Cbz into inner core of formed micelles. Also, significant shielding of DOX and DAS protons was observed (Fig. 3E). These data strongly suggest that both DOX and DAS were effectively loaded into the inner hydrophobic pocket formed by PLFCL.

3.4 Demonstration of carrier-drug intermolecular interaction

The effectiveness of PLFCL in co-encapsulation of DOX and DAS may largely attribute to the strong carrier-drug intermolecular interaction. We hypothesized that, in addition to the hydrophobic interaction, intermolecular π - π stacking may greatly contribute to the overall carrier-drug interaction. In order to verify this hypothesis, we used fluorescence and UV-absorbance studies to demonstrate π - π stacking interaction between carrier and drug molecules. As shown in Fig. 4A, drug-free PLFCL micelles showed a maximum fluorescence emission at 305 nm at an excitation wavelength of 270 nm. This fluorescence emission is attributed to Fmoc motifs in PLFCL conjugates, which is consistent with previous studies [30, 31]. However, the Fmoc fluorescence was substantially quenched in DOX/PLFCL and DAS/PLFCL micelles, and a further fluorescence quenching was observed when both of the agents were co-loaded. This decreased fluorescence intensity of Fmoc in mixed micelles is due to the strong π - π stacking interaction of Fmoc motifs with payload DOX or/and DAS, both of which bear multiple aromatic ring structures (Fig. S4). As controls, DOX and DAS alone did not show any fluorescence emission at this range of wavelength.

Fig. 4B shows the fluorescence spectrum of DOX. With an excitation at 480 nm, free DOX exhibited fluorescence maxima at 600 nm. Compared with free DOX, a dramatically decreased fluorescence intensity of DOX was observed when it was formulated in PLFCL micelles. In addition, a red shift of DOX fluorescence spectrum was recorded in DOX/PLFCL micelles in comparison to free DOX. These data again indicate the π - π stacking of DOX with Fmoc and Cbz domains in PLFCL micelles. Compared with DOX, which has conjugated aromatic ring structures, fluorescence emission of DAS was much weaker and partially overlapped with the strong Fmoc signals (data not shown).

The UV-absorbance spectra of DOX and DAS were also recorded as shown in Fig. S5. After mixed with PLFCL conjugates, dramatic decrease in UV-absorbance of both DOX and DAS

was observed compared with each free drug alone. Similarly, this is likely due to the π - π stacking of aromatic rings in payload drug molecules with Fmoc/Cbz in PLFCL carrier. Taken together, these evidences strongly supported the carrier-drug intermolecular π - π stacking interaction, which may significantly contribute to the overall carrier-drug interactions.

3.5 Release kinetics of DOX & DAS from PLFCL micelles

Release kinetics of DOX and DAS from DOX&DAS/PLFCL micelles was evaluated through a dialysis method using DPBS (pH 7.4) as releasing medium at 37 °C. DOX or DAS loaded micelles and free drug were used as comparison. As depicted in Fig. 5, free DOX and DAS showed a burst release with more than 80% of drugs released in the first 2 h. When encapsulated into PLFCL micelles, both agents exhibited significantly sustained release over free drugs. DOX release from DOX&DAS/PLFCL micelles was similar to that from DOX/PLFCL, while DAS release from DOX&DAS/PLFCL was slightly faster compared to the single agent formulation. This sustained drug release is likely due to the strong carrier-drug interaction between DOX/DAS and PLFCL conjugates. Importantly, DOX and DAS showed comparable release kinetics from DOX&DAS/PLFCL micelles. This release profile of DOX&DAS/PLFCL micelles shall favor the simultaneous delivery of these two agents to target site for combinational treatment.

3.6 Intracellular trafficking

Intracellular distribution of DOX&DAS/PLFCL micelles was followed by confocal laser scanning microscopy (CLSM) using DOX as a fluorescence probe. 4T1.2 cells were incubated with free DOX, DOX/PLFCL micelles, and DOX&DAS/PLFCL micelles for 0.5, 2, and 4 h, respectively. At 0.5 h, DOX was found to be distributed largely in perinuclear punctate pattern (Fig. 6A). After 2 h, increasing amounts of signals appeared in nuclei (Fig. 6B&C). This intracellular distribution suggests a cellular uptake largely through endocytosis, and a partial release of entrapped DOX from endo/lysosomes to cytoplasm. Of note, compared with DOX delivered by PLFCL micelles, significantly greater amounts of DOX were found in the nuclei of cells treated with DOX&DAS/PLFCL as evidenced by co-localization of DOX and Hoechst (Fig. 6B&C), suggesting a DAS-facilitated escape of DOX from endo/lysosomes inside cancer cells.

3.7 Inhibition of cancer cell proliferation and migration by DOX&DAS/PLFCL micelles

The effect of DOX&DAS/PLFCL micelles on cancer cell growth was tested with several cancer cell lines, including 4T1.2, PC 3, and HCT-116 cells. The cell survival was measured by MTT assay after a treatment of 72 h. As shown in Fig. 7A–C, drug-free PLFCL micelles did not show significant cytotoxic effect in all the tested cell lines. DOX/PLFCL or DAS/PLFCL showed a level of cytotoxicity that was comparable to that of free drug. In contrast, DOX&DAS/PLFCL exhibited dramatically enhanced inhibition on proliferation of all the tested cancer cells. This improved efficacy of DOX&DAS/PLFCL micelles was likely due to a strong synergy of DOX&DAS as demonstrated in Fig. 1. The DAS-facilitated drug release may also contribute to the enhanced combination effect.

Following the demonstration of the enhanced inhibitory effect of DOX&DAS/PLFCL on the proliferation of cancer cells, a wound-healing assay was utilized to further evaluate its effect on cancer cell migration. A monolayer of 4T1.2, a highly aggressive cancer cell line, was intendedly streaked to generate a wound in cell culture. The ability of cell repopulation to the denuded area was measured after 16 h. As shown in Fig. 7D, the wounded area was substantially repopulated by 4T1.2 cancer cells in the group that either received no treatment or was treated with the carrier alone. DAS was more effective than DOX in inhibiting the migration on cancer cells. Nonetheless, the combination group was most effective in inhibiting the migration of cancer cells; the denuded area was well retained with only 12.9% of repopulated cells (Fig. 7E). Taken together, DOX&DAS/PLFCL micelles exhibited synergistic effect in inhibition of both proliferation and migration of cultured cancer cells.

3.8 In vivo therapeutic efficacy and histochemical staining

In vivo therapeutic efficacy of DOX&DAS/PLFCL micelles was evaluated in BALB/c mice bearing 4T1.2 tumor. 4T1.2 is an aggressive cancer cell line with high proliferation rate, and saline-treated mice displayed a rapid increase in tumor size during the experiment (Fig. 8A). DAS/PLFCL showed a modest anti-cancer activity with a tumor growth inhibition rate at 33% (Fig. 8B), while a stronger therapeutic response was observed in DOX/PLFCL treated mice. Among all the groups, DOX&DAS/PLFCL exhibited the most potent anti-tumor activity. The tumor growth was almost completely inhibited (Fig. 8A&C) with a tumor growth inhibition rate as high as 95% (Fig. 8B). This high therapeutic efficacy is consistent with the strong synergy between DOX and DAS. The facilitated co-delivery of DOX and DAS by PLFCL also plays an important role as DOX&DAS/PLFCL was much more active than the free drug combination (DOX+DAS) in antitumor activity. All of the micellar formulations were well tolerated. In contrast, a decrease of body weight (Fig. 8D) was noticed in free drug combo treated mice, which is likely due to the toxicity caused by free DOX and DAS distributed to normal organs.

We further conducted a similar study to follow the survival times of the mice that received different treatments. As shown in Fig. 8E, mice treated with saline had a short survival time of around 15 days. Prolongation in survival times was achieved in mice treated with DAS/PLFCL, DOX+DAS, and DOX/PLFCL with a median survival time of 18, 20, and 27 days, respectively. The longest survival time (36 days) was observed in mice treated with DOX&DAS/PLFCL.

The data on the tumor growth in this survival study were also collected and shown in Fig. S6. They are consistent with the results shown in Fig. 8A, further confirming the improved therapeutic efficacy of DOX&DAS/PLFCL micelles over other formulations.

Histological analysis was conducted to further evaluate the therapeutic efficacy of DOX&DAS/PLFCL micelles. Slices of fixed tumor tissues were stained with hematoxylin and eosin (H&E). As indicated in Fig. 8F, saline-treated tumors showed typical appearance of cancer cells with large nuclei due to high proliferation rate. In contrast, tumors that received other treatments showed altered morphology with shrunk nuclei and decreased tumor cell density. These changes in cell morphology suggested inhibition in proliferation of these treated cancer cells at tumor tissue. In addition, apoptotic cells were detected in tumor

tissues by TUNEL assay (Fig. 8F). Among all the treatments, DOX&DAS/PLFCL micelles showed the most potent efficacy.

4. Discussion

Combination chemotherapy represents one of the effective strategies in clinical cancer treatment. Simultaneous administration of multiple chemotherapeutics targeting to different signaling pathways in cancer cells shall markedly maximize the anti-cancer activity whereas reducing the occurrence of MDR [7–10]. As a potent anti-cancer agent, DOX has been used in a variety of multi-drug regimens with other chemotherapeutics. Among them, DOX/DAS combination represents a unique regimen for targeted cancer therapy as DAS specifically acts on molecular targets that are overexpressed in various types of cancers. The effectiveness of this drug combo was firstly demonstrated by Pichot et al in breast cancer cells [11]. Our data further showed the synergistic action of DOX/DAS in several cancer cell lines (Fig. 1). One unique aspect of codelivery of DAS with other chemotherapeutics via a nanocarrier is the DAS-facilitated drug release from endosomes following endocytosis (Fig. 6). DAS bears two tertiary amines that can be protonated in acidic environment (Fig. S4); therefore, it may act as “proton sponge” to induce rupture of endo/lysosomes to facilitate the escape of entrapped drugs to cytoplasm [35, 36]. The facilitated drug release from endosomes is likely to contribute to the overall antitumor effect both *in vitro* and *in vivo*.

Various carrier systems have been employed for co-delivery of DOX with other anti-cancer agents such as liposomes, double emulsions, and triblock copolymers [37–41]. The uniqueness of our PLFCL nanocarrier lies in its simplicity in both synthesis and fabrication. PLFCL can be readily synthesized through a simple three-step synthetic route, and self-assembled into nanomicelles with small particle size (~ 30 nm) via a simple thin film hydration method. These properties may greatly facilitate its scale-up production and rapid translation to clinical application. In addition to simplicity, our nanocarrier demonstrated high effectiveness in formulating anti-cancer agents with diverse physiochemical properties including DOX, DAS, paclitaxel, docetaxel, imatinib, gefitinib, etoposide, and curcumin (Tables 1 & S1). This is due to the effective carrier-drug interactions that are facilitated by π - π stacking, hydrogen bonding as well as hydrophobic interaction (Figs. 4 & S5). Although the application of PLFCL nanomicelles in codelivery of two drugs was demonstrated with DOX/DAS combo as a model system, its broad utility suggests it could serve as a platform that can be readily applied to other targeted combination therapies.

Synergistic action of DOX&DAS/PLFCL micelles was systemically assessed in cultured cancer cells and in a tumor-bearing animal model. Our data demonstrated an enhancement of DOX&DAS/PLFCL micelles in inhibition of proliferation and migration of cultured cancer cells over other treatments. *In vivo*, both DOX/PLFCL and DAS/PLFCL showed significant therapeutic effect against 4T1.2, a highly aggressive breast cancer model. This is likely due to the effective delivery of DOX or DAS to tumor by PLFCL via EPR effect. More importantly, DOX&DAS/PLFCL micelles demonstrated further improvement in anti-tumor activity with regard to both tumor growth inhibition and animal survival.

5. Conclusions

In summary, a PEG-peptidic conjugate incorporated with Fmoc/Cbz domains was developed for effective co-delivery of DOX&DAS, two chemotherapeutic agents exhibiting potent synergistic anti-cancer activity. This PLFCL nanocarrier can be readily synthesized through a three-step route. DOX and DAS were efficiently co-loaded into PLFCL micelles with a small particle size. DOX&DAS/PLFCL nanomicelles demonstrated strong synergistic anti-cancer activity both *in vitro* and *in vivo*. Our nanocarrier may represent a simple and effective co-delivery system for improved combination chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants R01CA174305, R01GM102989, R01HL083365, and R21CA173887. We would like to thank Dr. Lisa Rohan for her help in UV absorbance spectroscopy study.

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Fig. 1. Proliferation inhibition of 4T1-2 mouse metastatic breast cancer cell line (A), PC 3 androgen-independent human prostate cancer cell line (B), and HCT-116 human colonic carcinoma cell line (C) treated with combination of DOX and DAS for 72 h. Synergy of DOX with DAS was indicated by combination index (CI) (D).

Fig. 2.

Synthetic route of PLFCL and schematic representation of PLFCL-mediated combination cancer therapy. **(A)** Self-assembly of PLFCL into nano-sized micelles in aqueous environment and co-encapsulation of DOX&DAS into core area of formed micelles; **(B)** Internalization of DOX&DAS/PLFCL micelles by cancer cells through endocytosis, and induction of cancer cell death as a consequence of released DAS binding to tyrosine kinase and accumulation of DOX inside nucleus.

Fig. 3. ^1H NMR spectra of PLFCL (A), DOX (B), DAS (C), DOX&DAS/PLFCL (D) in $\text{DMSO}-d_6$, and DOX&DAS/PLFCL in D_2O (E). The suppression of proton signals from Fmoc, Cbz, and both drug molecules in D_2O indicated the self-assembly of PLFCL micelles in aqueous medium and encapsulation of drug molecules into inner core of formed micelles.

Fig. 4.

Fluorescence emission of Fmoc at 300–450 nm was recorded with an excitation wavelength at 270 nm (**A**). Fluorescence of DOX was measured using an excitation wavelength of 480 nm and emission wavelength from 510–650 nm (**B**). Both Fmoc and DOX showed significant fluorescence quenching in mixed micelles.

Fig. 5.

The kinetics of DOX (**A**) and DAS (**B**) release from DOX&DAS/PLFCL micelles as examined through a dialysis method. DPBS containing 0.5% (w/v) Tween 80 was used as release medium. Drug concentration was analyzed by HPLC at 0, 1, 2, 4, 8, 24 and 48 h.

Fig. 6.

Intracellular trafficking of DOX in 4T1.2 cells. Cells were treated with DOX&DAS/PLFCL, DOX/PLFCL micelles, or free DOX at 10 $\mu\text{mol/L}$ for 0.5 h (**A**), 2 h (**B**), and 4 h (**C**). The nuclei were stained with Hoechst 33342, and endo/lysosomes were stained by LysoTracker DND-26. The intracellular distribution of DOX in various formulations was observed under a confocal laser scanning microscope (CLSM).

Fig. 7.

In vitro inhibition of cancer cell proliferation and migration. *In vitro* cytotoxicity was examined in 4T1-2 mouse metastatic breast cancer cell line (A), PC 3 androgen-independent human prostate cancer cell line (B), and HCT-116 human colonic carcinoma cell line (C) treated with different formulations for 72 h, and cancer cell inhibition was determined by MTT assay. Inhibition of cancer cell migration was evaluated via wound healing assay of 4T1-2 mouse metastatic breast cancer cells at 0 and 16 h following treatment with drug-free or drug-loaded PLFCL micelles at the DOX or DAS concentration of 100 nmol/L. Bright field images were obtained under microscope (D) and relative cell migration was quantified using cells without treatment as control (E).

Fig. 8.

In vivo therapeutic efficacy of DOX&DAS/PLFCL micelles. Groups of 5 BALB/c mice bearing 4T1.2 murine breast cancer grafts were injected with DOX&DAS/PLFCL, DOX/PLFCL, DAS/PLFCL micelles (5 mg each agent/kg body weight) and saline on days 0, 3, and 6, respectively. Free DOX+DAS combination dissolved in 50% propylene glycol was used for comparison. Tumor growth was monitored and plotted as relative tumor volume (**A**). * $P < 0.05$; ** $P < 0.01$ (DOX&DAS/PLFCL micelles vs other treatment). Tumor weights were measured after excision and % tumor growth inhibition was calculated as: $1 - (\text{mean tumor weight of drug treated group} / \text{mean tumor weight of saline treated group}) * 100\%$ (**B**). Significant difference in tumor size was observed between groups (**C**), and changes of body weight in mice was also monitored (**D**). Kaplan-Meier survival curves for BALB/c mice ($n=7$) bearing 4T1.2 murine breast cancer grafts (**E**). Histological analyses of tumor tissues using H&E staining and TUNEL assay (**F**).

Table 1

Biophysical characterization of drug-free and drug-loaded PLFCL micelles.

	Molar ratio	Particle size (d, nm)	^a PDI	Zeta potential (mV)	Drug content (w/w) %	^b DLE %	^c Stability (d)
PLFCL	-	29.1±0.6	0.433	-2.747±2.185	-	-	-
^d DOX/PLFCL	1:2	26.5±0.2	0.407	+1.061±0.387	4.27	95.34	>7
^d DAS/PLFCL	1:2	18.5±0.3	0.167	-1.802±1.236	3.85	98.60	3
^d DOX&DAS/PLFCL	1:1:2	25.4±0.8	0.351	+0.519±0.730	7.80	98.50	6

^a PDI, polydispersity index.^b DLE, drug loading efficiency.^c Stability indicates no noticeable drug precipitation or significant size change during the follow up time period.^d Drug concentrations were kept at 1 mmol/L each, and nanocarrier concentration was 2 mmol/L.