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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, PEG_{5000}-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.

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

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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, PEG_{5000}-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, PEG_{5000}-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 PEG_{5000}, 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 PEG_{5000}-lysyl-(α-Fmoc-ε-Cbz-lysine)$_2$ (PLFCL)

PLFCL was synthesized through a simple three-step synthesis route. Monomethoxy PEG_{5000} (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 PEG_{5000}-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 PEG_{5000}-lysine-NH_2 (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 ^{1}H 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: H_2O (52.5%:47.5%) containing 2.5 mM NH_4Ac and 0.05% CH_3COOH (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 to 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