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Co-delivery of thioridazine and doxorubicin using polymeric micelles for targeting both cancer cells and cancer stem cells

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ABSTRACT

In this study, thioridazine (THZ), which was reported to kill cancer stem cells, was used in a combination therapy with doxorubicin (DOX) to eradicate both cancer cells and DOX-resistant cancer stem cells to mitigate the reoccurrence of the disease. Both THZ and DOX were loaded into micelles with sizes below 100 nm, narrow size distribution and high drug content. The micelles were self-assembled from a mixture of acid-functionalized poly(carbonate) and poly(ethylene glycol) diblock copolymer (PEG-PAC) and urea-functionalized poly(carbonate) (PUC) and PEG diblock copolymer (PEG-PUC). The drug-loaded mixed micelles (MM) were used to target both cancer cells and stem cells via co-delivery. Cancer stem cells were sorted by a side population assay from BT-474 and MCF-7 human breast cancer cell lines, and identified by CD44+/CD24− phenotype. The cytotoxicity of various formulations was evaluated on the sorted cancer stem cells (side population SP cells), sorted non-stem-like cancer cells (non-side population NSP cells) and unsorted cancer cells. Antitumor activity was also evaluated on BT-474 xenografts in nude mice. As compared with NSP cells, DOX suppressed SP cell growth less effectively, while THZ and THZ-MM were more effective in the inhibition of SP cells. A stronger inhibitory effect was observed on SP cells with the co-delivery of free DOX and THZ or DOX-MM and THZ-MM as compared to free DOX or DOX-MM. THZ and THZ-MM were capable of lowering the population of SP cells in unsorted cells. In the BT-474 xenografts, the co-delivery of DOX-MM and THZ-MM produced the strongest antitumor efficacy, and both THZ and THZ-MM showed strong activity against cancer stem cells. This combination therapy may provide a promising strategy for breast cancer treatment by targeting both cancer cells and cancer stem cells.

1. Introduction

Breast cancer is the most common form of cancers diagnosed in women worldwide [1,2]. Despite recent advances in the treatments of breast cancer, about 40% of the patients treated for early-stage disease eventually develop recurrence with most of these recurrences being distant metastases [3]. One of the key reasons is the presence of cancer stem cells [4,5]. Cancer stem cells comprise a small sub-population within tumors with enhanced capacity for tumor generation, and possess several fundamental attributes similar to normal adult stem cells [6,7]. Studies showed that the existence of cancer stem cells increased resistance to conventional chemotherapy [6,8]. A number of genetic and cellular adaptations have been found to be related to the resistance, such as relative dormancy/slow cell cycle kinetics, efficient DNA repair, the expression of multidrug-resistance transporters, and resistance to apoptosis [9,10]. Although conventional chemotherapies kill the majority of cancer cells, the surviving cancer stem cells could re-initiate the tumor, leading to relapse and recurrence. Studies demonstrated that conventional chemotherapies caused the enrichment of cancer stem cells both in human and animals [11–13]. Clearly a combination therapy using a conventional chemotherapeutic drug with an agent that can target cancer stem cells may provide a good approach to eradicate both cancer cells and stem cells. Successful examples of synergistic combination therapies include using parthenolide-loaded liposomes/vinorelbine-

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loaded liposomes [14] and all-trans retinoic acid-loaded liposomes/vinorelbine-loaded liposomes [15] to eradicate cancer cells and cancer stem cells effectively both *in vitro* and *in vivo*.

Thioridazine (THZ), a phenothiazine derivative, is a piperidine antipsychotic drug. Recently, it was reported that THZ selectively targeted leukemic cancer stem cells, but it had no effect on normal blood stem cells [16]. The selectivity might be because THZ antagonized dopamine receptors that were over-expressed on leukemic cancer stem cells. In addition, THZ significantly augmented the antitumor activity of the antiproliferative agent cytarabine *in vitro* [16]. Normal mammary gland tissue displays low levels of dopamine receptors, while breast cancer stem cells (CD44+/CD24−) have a high expression of dopamine receptors [16,17]. We hypothesized that THZ could target breast cancer stem cells and achieve a synergistic effect with other antiproliferative drugs such as doxorubicin (DOX), which is a highly potent and widely used chemotherapeutic agent for the treatment of various types of cancers including breast cancer. THZ is well tolerated at low concentrations, but an overdose of THZ often leads to uncontrollable movement, severe dizziness or fainting, coma, blurred vision and rash, irregular heartbeats, and hyperthermic or hypothermic body temperatures [18,19]. Besides, the use of THZ may lead to cardiotoxicity [19]. Similarly, DOX causes serious cytotoxicity in normal tissues, such as the induction of myelosuppression and cardiotoxicity, hence limiting the maximum tolerated dose [20,21]. In addition, DOX can induce drug resistance in cancer cells [21]. Therefore, a drug delivery system is necessary to decrease the nonspecific toxicity of THZ and DOX for *in vivo* applications.

Polymeric micelles are promising delivery carriers for anticancer drugs [22,23]. Polymeric micelles with particle size from 10 nm to 200 nm have been reported to enhance drug accumulation within tumors due to the enhanced permeability and retention (EPR) effect of leaky tumor tissues [24,25]. Recently, we reported the synthesis of diblock copolymers of poly(ethylene glycol) (PEG) and functional polycarbonates with well-controlled molecular weight via metal-free organocatalytic ring-opening polymerization of carbonate monomers using PEG as a macroinitiator. The polymers were used to prepare micelles for anticancer drug delivery, where the use of non-covalent interactions within the micellar core increased drug loading level and enhanced kinetic stability. Specifically, mixed micelles, which self-assembled from a diblock copolymer of PEG and urea-functionalized polycarbonate (PEG-PUC) and another diblock copolymer of PEG and acid-functionalized polycarbonate (PEG-PAC) via hydrogen-bonding interaction, provided nanosize with narrow size distribution and high loading capacity for anticancer drugs that contain micellar core-interacting amine groups. A key example is DOX due to ionic interaction between the amine group in DOX and acid group in PEG-PAC [26–28]. DOX-loaded mixed micelles also showed higher antitumor efficacy and decreased toxicity than free DOX in *vivo* [27,28].

THZ also has an amine group and it is anticipated that the PEG-PUC/PEG-PAC mixed micelles would be a suitable delivery carrier. In this study, DOX and THZ were loaded into mixed micelles, and delivered simultaneously *in vitro* and *in vivo* to target both human breast cancer cells and cancer stem cells. DOX-loaded and THZ-loaded mixed micelles (DOX-MM and THZ-MM respectively) were characterized for particle size, drug loading and *in vitro* release profiles. Two human breast cancer cell lines BT-474 and MCF-7 were employed as models. Side population (SP) cells, rich in cancer stem cells, were sorted by flow cytometry from both cell lines. The antiproliferative effects of single formulation or the combination of DOX-MM and THZ-MM on sorted SP cells, non-side population (NSP) cells and unsorted cells were tested. Furthermore, the antitumor activity of the formulations was tested in nude mice bearing BT-474 cancer xenografts to evaluate the feasibility of such delivery system *in vivo*.

2. Materials and methods

2.1. Materials

The synthesis procedures for preparation of 5-methyl-5-benzylcarboxyl-1,3-dioxan-2-one (MTC-OBn) and 5-methyl-5-(phenylureaethyl)carboxyl-1,3-dioxan-2-one (MTC-urea) carbonate monomers followed the methods reported previously [26,27,29]. Doxorubicin-hydrochloride (DOX-HCl), thioridazine-hydrochloride (THZ-HCl), Hoechst 33342 and all other reagents were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.) unless otherwise specified. Anti-human CD44-FITC and CD24-PE monoclonal antibodies and their isotype controls were purchased from Abcam (Hong Kong). Matrigel was bought from Becton Dickinson (San Jose, CA, U.S.A.). 17β-Estradiol pellets were obtained from Innovative Research of American (Sarasota, FL, U.S.A.), and Collagenase III from i-DNA (Singapore). Human breast cancer cell lines BT-474 and MCF-7 were purchased from ATCC (Manassas, VA, U.S.A.). Cells were cultured in RPMI-1640 medium (Lonza, Singapore) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere.

2.2. Polymer synthesis

2.2.1. Synthesis of urea-functionalized (PEG-PUC) and benzyl-protected acid-functionalized (PEG-P(MTC-OBn)) block copolymers (Scheme 1)

The details for organocatalytic ROP of the respective monomers with PEG are provided below. All polymerizations were carried out in a glove-box under nitrogen atmosphere.

PEG-PUC: To a mixture of MTC-urea (242 mg, 0.75 mmol), PEG (500 mg, 0.05 mmol, Mn 10,000 g/mol, PDI 1.04) and thiourea catalyst (TU) (18.5 mg, 0.05 mmol) dissolved in dichloromethane (5 mL) was added sparteine (11.5 μL, 0.05 mmol) to initiate the ROP. The homogeneous solution was stirred for 18 h at ambient temperature. Benzoic acid (25 mg; ~4 eqv.) was added to quench the polymerization, dropwise and the crude polymer precipitated in 50 mL diethyl ether. The white precipitate was collected after centrifugation, dried briefly and re-dissolved in dichloromethane. The crude polymer was re-precipitated four times to ensure complete removal of impurities and dried under vacuo to give a white solid (85%). PDI 1.12. ^{1}H NMR (400 MHz, DMSO-*d6*, 22 °C): δ 8.53 (s, br, 13H, PhNH-), 7.36 (m, 26H, PhH), 7.18 (m, 26H, PhH), 6.86 (m, 13H, PhH), 6.20 (s, br, 13H, $-CH_2NH-$), 4.05–4.25 (overlapping peaks, 78H, MTC$-CH_2-$ and $-COOCH_2-$), 3.50 (s, 906H, H of MPEG), 3.33 (s, 26H, $-CH_2NHCO-$), 1.13 (s, 39H, $-CH_3$).

PEG-P(MTC-OBn): In a similar manner, DBU (7.5 μL, 0.05 mmol) was added to a mixture of MTC-OBn (188 mg, 0.75 mmol) and PEG (500 mg, 0.05 mmol, Mn 10,000 g/mol, PDI 1.04) dissolved in dichloromethane (5 mL) to start the ROP. The mixture was stirred for 3 h at ambient temperature and subsequently quenched by the addition of benzoic acid (25 mg; ~4 eqv.). Identical purification protocol was employed as for PEG-PUC polymer. Yield, 80%; PDI 1.10. ^{1}H NMR (400 MHz, $CDCl_3$, 22 °C): δ 7.31 (m, 60H, PhH), 5.13 (m, 24H, $PhCH_2-$), 4.27 (m, 48H, MTC$-CH_2-$), 3.64 (s, 906H, H of PEG), 1.21 (m, 36H, $-CH_3$).

2.2.2. Deprotection of benzyl groups in PEG-P(MTC-OBn)

A mixture of PEG-P(MTC-OBn) (0.5 g) and Pd–C (10% w/w, 0.2 g) in THF/methanol (7.5 mL each) was stirred under hydrogen (7 atm) overnight. The reaction mixture was then filtered through Celite wetted with THF after removal of hydrogen. Additional THF and methanol washings were performed to ensure complete transfer of the deprotected polymer. The filtrate was collected, and the solvents were evaporated. The resultant residue was re-dissolved in THF and precipitated with diethyl ether before being freeze-dried under vacuo. The PEG-acid diblock copolymer PEG-PAC (or PEG-P(MTC-OH)) was obtained as a powdery white solid (yield: >90%).

2.3. Preparation and characterization of micelles (Scheme 1)

DOX-loaded micelles were prepared by a membrane dialysis method as described in our previous report [26]. Briefly, DOX-HCl (5 mg) was dissolved in N, N-dimethylacetamide (DMAc, 1.5 mL) containing 3 mole equivalents of triethylamine (3.84 μL). To this solution was added PEG-PAC (4.55 mg) and PEG-PUC (5.45 mg) in 0.5 mL of DMAc (PEG-PAC/PEG-PUC: 1:1 molar ratio). The resulting solution was added dropwise to 10 mL of DI water while being sonicated for 2 min (130 W) using a probe-based sonicator (Vibra Cell VCX 130). The free DOX was removed by dialysis against DI water for 48 h using a dialysis bag with molecular weight cut-off (MWCO) of 1000 Da (Spectra/Por 7, Spectrum Laboratories Inc.), and water was changed 3 times at 3, 6 and 24 h. The solution inside the dialysis bag was then collected and lyophilized to obtain the DOX-loaded mixed micelles (DOX-MM).

THZ-loaded micelles were prepared through a thin film hydration method. Briefly, thioridazine-HCl (3 mg) was dissolved in acetonitrile (1 mL) containing 3 mole equivalents of triethylamine (3.28 μL). To this solution was added PEG-PAC (4.55 mg) and PEG-PUC (5.45 mg) in 1 mL of acetonitrile (PEG-PAC/PEG-PUC: 1:1 molar ratio). The acetonitrile was evaporated by using a rotary vacuum evaporator at 50 °C for 30 min. The lipid film was hydrated with phosphate-buffered saline (PBS,