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Polyphosphazene vesicles for co-delivery of doxorubicin and chloroquine with enhanced anticancer efficacy by drug resistance reversal

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ABSTRACT

The conventional chemotherapeutic agent doxorubicin hydrochloride (DOX·HCl) is often accompanied by drug resistance which has severely hindered its clinical application. By taking advantage of the self-assembled behavior of amphiphilic polyphosphazenes, we constructed novel polymersomes loading DOX·HCl or desalted DOX with chloroquine phosphate (CQ) as a drug resistance-reversal agent at 1:1 or 2:1 weight ratios via a one-step common dialysis method. The cytotoxicity evaluation of this dual drug-loaded polymersome was performed on DOX-resistant MCF-7/Adr breast cancer and HL60/Adr leukemia cells. Simultaneously, to simulate *in vivo* cancerous tissue, 3D tumor spheroid was constructed for tissue penetration and anti-cancer effect evaluation. As a result, PEP-DHC-1 containing DOX·HCl and CQ at 1:1 weight ratio exhibited the strongest toxicity. Furthermore, the *in vivo* tumor inhibition study carried out on a zebrafish xenograft model also validated that PEP-DHC-1 made the outstanding contribution to improve the sensitivity of MCF-7/Adr breast cancer to chemotherapeutics. These findings suggest that this DOX·HCl and CQ co-delivery system based on PEP polymersomes might be promising for drug resistance reversal of cancer therapy and consequently enhanced anti-cancer efficacy.

1. Introduction

Polymersomes are a class of artificial vesicles self-assembled by amphiphilic copolymers. Since Discher et al., (1999) reported on poly(ethylene glycol)-b-polylactide vesicles for the first time in 1999, polymersomes have drawn more and more attention as promising drug delivery systems (DDS). They have a similar architecture to liposomes, but possess superior tunable physico-chemical properties, improved pharmacokinetics behavior, and enhanced membrane stability that prevents drug leakage (Lee and Feijen, 2012; Meng et al., 2009). Therefore, polymersomes have been designed and fabricated as a progressive alternative to liposomes. Recently, liposomes have played notable roles in dual drug co-delivery for combination cancer therapy (Li et al., 2014; Xu and Qiu, 2015). However, only a few papers have examined polymersomes containing two drugs (Ahmed et al., 2006; Liu et al., 2014), which all chose hydrophilic anticancer drug doxorubicin hydrochloride (DOX·HCl) and hydrophobic anticancer drug paclitaxel (PTX) for synergetic cancer therapy. In the preparation procedure, DOX·HCl had to be loaded into the lumen of polymersomes by a pH-gradient method after PTX-loaded polymersomes were formed. Otherwise, the encapsulation efficiency (EE) of DOX·HCl was very low (<40%) (Ahmed et al., 2006; Chen et al., 2010; Colley et al., 2014).

Multidrug resistance (MDR) has become a major obstacle in cancer treatment, causing 90% of failures in patients with metastatic cancer (Wilson et al., 2009). Several mechanisms have involved with MDR including intracellular drug accumulation reduction resulted by increased drug efflux and/or decreased uptake of the cytotoxic drug, metabolism disturbance, altered drug targets, increased repair of drug-damaged DNA and apoptosis modulation (Filipits, 2004). Resistance to DOX·HCl, a popular anticancer agent, is closely associated with overexpression of the ATP-binding cassette super-family of drug transporters such as P-glycoprotein (P-gp) and multidrug resistance-associated proteins (MRPs) (Vezmar and Georges, 2000). A number of strategies have been developed to overcome MDR including: (1) modification of chemotherapeutics (Teodori et al., 2002); (2) use of transporter modulators to inhibit drug efflux (Arora et al., 2012); (3) MDR gene expression blockage/inhibition (Li et al., 2015); (4) use of novel

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DDS such as liposomes and nanoparticles (Barraud et al., 2005). Autophagy is a natural, destructive mechanism that disassembles unnecessary or dysfunctional cellular components through a regulated process. Recently, it has been proposed that autophagy serves as a protective mechanism for tumors against chemotherapeutics, which also contributes to MDR (Kumar et al., 2012). For example, Ji et al., (2014) found that autophagy was induced in crizotinib-resistant lung cancer cells and that the degree of crizotinib resistance was correlated with autophagic activity. In reality, improved efficacy of chemotherapeutics has been observed in preclinical trials when autophagy regresses (Xi et al., 2011). Although chloroquine phosphate (CQ) is a traditional drug to treat or prevent malaria, recent studies have shown that CQ can inhibit P-gp-mediated transport of taxol in a concentration-dependent manner and also can act as an autophagy inhibitor (Amaravadi et al., 2011; Hayeshi et al., 2006; Katayama et al., 2007; Solomon and Lee, 2009; Wu et al., 2010). Therefore, combined administration of CQ and doxorubicin via nanoparticles may provide a potential better solution to overcome MDR and improve treatment efficacy. Indeed, we previously prepared CQ and DOX·HCl co-loaded liposomes and found CQ displayed distinct capability to reverse the resistance of MCF-7/Adr cells to DOX·HCl *in vivo* (Qiu et al., 2012a,b). However, *in vitro* investigation was not performed. Furthermore, in order to achieve high encapsulation efficiency and controlled loading ratio of DOX·HCl and CQ in dual drug liposomes, pH-gradient method had to be employed, which resulted in complicated working process and low drug loading content due to quite a few buffer salts.

To overcome these problems, we herein initially constructed polyphosphazene vesicles co-loading water-soluble CQ as a drug resistance-reversal agent with DOX·HCl to reverse drug resistance. Importantly, the drug loading with high EE and the self-assembly of amphiphilic polyphosphazenes to nano-vesicles was simultaneously accomplished by one-step dialysis method without any other additives. Based on the enhanced permeability and retention (EPR) effect of nano-scaled polymersome, CQ and DOX·HCl were supposed to accumulate at the tumor sites, followed by cellular uptake and collaboration to kill cancer cells. Therefore, after examining the drug-loading capability of polymersome, we conducted a detailed evaluation of the anti-cancer efficacy of this dual drug-loaded polymersome on DOX-resistant MCF-7/Adr breast cancer and HL60/Adr leukemia. The *in vitro* 2D cellular culture model, the intratumoral penetration and destructive efficacy of 3D tumor spheroids treated with combined drug formulations was investigated. Furthermore, an *in vivo* tumor inhibition study was carried out on a zebrafish xenograft model, which is considered a simple, rapid, and sensitive method for anti-cancer drug screening that has been approved by the U.S. Food and Drug Administration (Jung et al., 2012; Liu and Leach, 2011; Moshal et al., 2010). Meanwhile, the necessity of co-loading DOX·HCl and CQ in polymersomes for drug resistance reversal was emphasized in this study by compared with the mixture of two free drugs and the co-encapsulation of hydrophobic desalted DOX and CQ.

2. Materials and methods

2.1. Materials, cells and animals

Hexachlorocyclotriphosphazene was purchased from Acros Organics (Belgium) and sublimated at 80–90 °C before use (Zheng et al., 2009). Aluminum chloride (99%) purchased from Acros Organics (Belgium). Monomethoxy poly (ethylene glycol) (mPEG, molecular weight 2000) was purchased from Acros Organics (Belgium) and was azeotropically distilled with toluene before use. Amino-terminal PEG2k (PEG2k-NH_2) was prepared via a two-step protocol (Xu et al., 2014). Ethyl-p-aminobenzoate (EAB) was purchased from Sigma–Aldrich (USA). Doxorubicin hydrochloride (DOX·HCl) was purchased from HaiKou Manfangyuan Chemical Company (China). Chloroquine phosphate (CQ) (purity 99.6%) was purchased from Kaiyang Biotechnology Pharmaceutical (Shanghai, China). All other reagents were commercially available and used without further purification.

DOX resistant human breast cancer MCF-7/Adr cells and human acute myelocytic leukemia HL60/Adr cells were maintained in RPMI 1640 purchased from Gibco BRL (USA) containing 10% (v/v) fetal bovine serum (FBS) purchased from Sijiqing Biologic (China) and penicillin/streptomycin (100 units/ml, 100 units/ml) at 37 °C, 5% CO_2 and 95% humidity.

AB strain zebrafish with stable P-gp overexpression were used in the *in vivo* anti-cancer evaluation. The animal experiments were performed in accordance with the guidelines approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

2.2. Synthesis and characterization of amphiphilic graft polyphosphazene (PEP)

The amphiphilic graft polyphosphazene was synthesized by sequential substitution reaction of PEG2k-NH_2/EAB with chlorine atoms on poly (dichlorophosphazene) backbone (Xu et al., 2014). Briefly, 2 wt% of aluminum chloride was used as an initiator for thermal ring opening polymerization of hexachlorocyclotriphosphazene. The obtained poly (dichlorophosphazene) (0.50 g, 8.6 mmol P-Cl) bonds was reacted with a certain amount of PEG2k-NH_2 (1.25 g, 0.62 mmol) containing equal molar tri-ethylamine (TEA) in 20 mL dry tetrahydrofuran solutions for 24 h. Then, excessive amount of EAB (1.33 g, 8.1 mmol) was added to complete the substitution reaction for 72 h with TEA equaled to its theoretical mole. The unreacted EAB and PEG2k-NH_2 were eliminated by precipitation in diethyl ether. The obtained precipitate was dissolved in *N*,*N*-dimethylformamide (DMF) followed by dialysis (MWCO 14.0 kDa) against distilled water for 24 h with frequent exchange of water.

The final product was lyophilized and PEP was obtained. The chemical composition of PEP was confirmed by ^{1}H NMR spectrum using an AC-80 NMR spectrometer (Bruker Biospin, Germany). IR spectrum was measured by a FT/IR-4100 spectrometer (JASCO, Japan). The molecular weight of the copolymer was determined by PL-GPC 220 gel permeation chromatograph (GPC) purchased from Polymer Laboratories Ltd. (USA) with DMF as an eluent and monodisperse polystyrene as a calibration standard.

2.3. Preparation and characterization of blank and dual drug-loaded nanoparticles

Dialysis method was employed to prepare blank or dual drug-loaded PEP nanoparticles. PEP was dissolved in DMF at the concentration of 10 mg/mL and dripped by distilled water at 1:1 volume ratio. Then, the mixture was sealed in a dialysis bag (MWCO 14.0 kDa) and dialyzed against deionized water for 24 h with frequent change of water. After filtered by 0.45 μm filter and freeze–dried, blank PEP nanoparticles were obtained. The preparation of dual drug-loaded PEP nanoparticles was conducted based on the above process except that the drug and PEP was premixed as follows. 1 mg or 2 mg DOX·HCl was dissolved in 2 mL DMF. Hydrophobic desalted DOX was transferred from DOX·HCl by adding equal molar TEA. And 1 mg CQ was dissolved in 2 mL DMF with 1 wt% phosphoric acid. Then 40 mg PEP was dissolved in the mixure of DOX·HCl/DOX and CQ solution. The obtained DOX·HCl/CQ-loaded PEP nanoparticles were named as PEP-DHC. Likewise, DOX/CQ-loaded PEP was represented as PEP-DC.