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A novel injectable phospholipid gel co-loaded with doxorubicin and bromotetrandrine for resistant breast cancer treatment by intratumoral injection

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ABSTRACT

Systemically administered anticancer treatments were greatly limited by extensive side effects mainly due to nonspecific distributions *in vivo*, and multidrug resistance in various tumors. A phospholipids-based *in situ*-forming gel platform has been developed for the concurrent delivery of doxorubicin (DOX) and bromotetrandrin (W198). Phospholipid gel containing DOX and W198 remained in a solution (sol) state before injection and underwent rapid gelation after injection *in vivo*. The release of DOX and W198 from phospholipid gel (PG) was sustained *in vitro* for over 20 days (d). DOX and W198 from PG achieved prolonged release for over two weeks in rats *via* subcutaneous injection. Compared with repeated injections of free drug, eliminated cardiac toxicity and less bone marrow inhibition were observed for DOX and W198-loaded PG (DOX/W198-PG) in normal rats *via* subcutaneous injection. Also, a single intratumoral injection of DOX/W198-PG in the resistant MCF-7/Adr xenograft-bearing mice showed much better antitumor efficacy compared to other treatment groups. In sum, DOX/W198-PG was well demonstrated to achieve sustained drug release both *in vitro* and *in vivo* with eliminated toxicity and improved antitumor efficacy by reversing the multidrug resistance in breast cancers.

1. Introduction

As part of the clinical management of cancer, almost all patients receive administration of local radiotherapy or systemic chemotherapy [1,2]. However, systemic chemotherapy could cause severe side effects due to the toxicity of anticancer drugs on normal tissues [3]. Moreover, many anticancer drugs like paclitaxel [4] and doxorubicin (DOX) [5] always manifest the multidrug resistance (MDR) in many types of human tumors that have relapsed after an initial favorable response to anticancer agents. Due to these problems above, the effect of systemic chemotherapy is always unsatisfactory.

In recent decades, to reduce the toxicity and improve therapeutic effect of chemotherapy, intratumoral injection (i.t.) has appeared as an attractive alternative [6–8]. Especially, the *in situ*-forming gel is a good option for local administration of chemotherapeutics, which have been shown to achieve site-specific drug delivery, prolonged release profiles, and improved patient compliance [9,10]. In our previous study, it was found that the phospholipids-based phase separation gel (PG) was a high safe and efficient *in situ*-forming implant for sustained delivery of anticancer drugs [11,12]. However, it was proved that the chemotherapy drugs such as doxorubicin (DOX) could manifest MDR in various tumor cells such as human uterine sarcoma cell MES/SA, Colon-26 cancer cell and human breast cancer cell MCF-7 [13–16]. Thus the PG system could not be used in the cancer with MDR.

The enhanced removal of chemotherapy drugs from tumor cells is identified as a major mechanism for MDR which is attributed to the overexpression of the energy-dependent ATP-binding cassette (ABC) transporters, such as P-glycoprotein (P-gp) [17]. Therefore, the co-administration of P-gp inhibitors with chemotherapy drugs was developed to reverse MDR and improve therapeutic effect recently [18–20]. In our previous study, we also found that co-encapsulated in nanoparticles and co-administrated DOX with bromotetrandrin (W198), a brominated derivative of tetrandrine, could reverse the P-gp mediated multidrug resistance [21]. However, it showed significant cardiac toxicity and suppression of bone

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marrow. Moreover, P-gp inhibitors themselves could show poor specificity and toxicity [22] and most of P-gp inhibitors have time- and concentration-dependent manner. For example, it was proved that dramatic increases of DOX accumulation started to appear at 24 h after incubation with P-gp inhibitors (verapamil or inhibitors derived from cardiotonic steroids) in resistant cancer cells *in vitro*. These inhibitors exerted their P-glycoprotein-inhibitory effects only at higher concentration ($IC_{50} \times 5$) [23]. Current particle delivery systems *via* intravenous injection or oral delivery could hardly meet the need of maintaining a high P-gp concentration at specific site for more than 24 h [21,24]. Thus, to exert a good inhibition effect, the MDR inhibitors should keep a high concentration in tumor for a long time.

Taken together, there is a need for developing an alternative delivery system for reducing the toxicity of chemotherapy and overcoming the MDR. Therefore, the optimized PG was chose as a drug delivery system in this study. DOX, as a model drug, was co-encapsulated in PG with W198 (DOX/W198-PG). The *in vitro* stability, release kinetics, cellular uptake behaviors, pharmacokinetics, biodistribution, and *in vivo* toxicities and antitumor effect of DOX/W198-PG were further investigated.

2. Materials and methods

2.1. Preparation of DOX/W198-PG

The *in situ*-forming gel was prepared with phospholipid-E80 (Lipoid, Germany), medium chain triglyceride (MCT, Beiya Medical Oil Co., Ltd., China) and ethanol (containing 2% (v/v) oleic acid) at 70:10:20 (w/w/w). To increase the solubility of DOX and W198 in the lipid matrix, a small amount of oleic acid is added in the prescription. DOX (Huafenglianbo, China), W198 (kindly gifted from Dr. Fengpeng Wang at West China School of Pharmacy, Sichuan University, China) and gel-forming materials (1:1:167, mass ratio) were mixed and then stirred at room temperature for about half an hour.

2.2. Viscosity measurements

The viscosity of DOX and W198-loaded phospholipids gel (DOX/W198-PG) was determined using a Brookfield DV-Rotational viscometer at the speed of 100 rpm under room temperature. A dialysis setup was used to help accomplish gel transition *in vitro*. DOX/W198-PG solution was placed in the dialysis bag (MWCO 3500–5000 Da) and immersed in pre-warmed phosphate-buffered saline (PBS) (0.01 M, pH 6.8). DOX/W198-PG was then obtained and subjected to viscosity test.

2.3. In vitro *drug release*

For release study, 150 μL of DOX/W198-PG (4 mg/mL) and 150 μL of DOX/W198-solution (4 mg/mL) were added into the dialysis bag and then incubated in 5 mL of different pre-warmed release media, including PBS (0.01 M, pH 6.8), 5%, 15%, 30% (v/v) ethanol of PBS (0.01 M, pH 6.8) solution at 37 °C under shaking at 100 rpm. At specified time, all release media were removed and replaced by 5 mL of pre-warmed fresh media. Samples were analyzed by LC–MS/MS (Agilent Technologies Co., Ltd., USA) and the concentrations of DOX and W198 were calculated by a standard calibration curve. The LC–MS/MS system consisted of an Agilent 1200 series, including an SL binary pump, degasser, autosampler and a triple quadruple MS. A Diamonsil ODS column (1.8 μm, 50 × 4.6 mm) with a corresponding guard column (ODS, 5 μm) was used for separation, and maintained at 30 °C. The mobile phase consisted of 70% deionized water (0.5% formic acid, v/v) and 30% acetonitrile (v/v) at a flow rate of 0.4 mL/min. Release profiles of DOX and W198 from DOX/W198-PG in different release media were analyzed using classical pharmaceutical release models such as zero-order, first order, Higuchi, Korsmeyer-Peppas, and Weibull equations.

2.4. Cytotoxicity

To evaluate the inhibitory effects of various DOX formulations against human cancer cells, MCF-7 or the doxorubicin-resistant breast cancer MCF-7/Adr cells (Cell Bank of Chinese Academy of Sciences, China) were seeded in a 24-well plate at a density of 4×10^4 cells per well (Nest Biotech Co., Ltd, China) and incubated in RPMI-1640 medium (Hyclone, USA) for 24 h at 37 °C in a humidified incubator containing 5% CO_2. To compare with clinical repeated injections of doxorubicin, the cells were then treated with saline, blank PG, free DOX (0.05, 0.075, 0.10 mg DOX/well, dosing twice, at 24 h and 72 h), free DOX/W198 (0.05, 0.075, 0.10 mg DOX/well, dosing twice, at 24 h and 72 h), DOX-PG (0.10, 0.15, 0.20 mg DOX/well) or DOX/W198-PG (0.10, 0.15, 0.20 mg DOX/well). After 96 h, the *in vitro* cytotoxicity of various formulations against MCF-7 or MCF-7/Adr cancer cells was compared using the WST-1 assay (Nanjing KeyGEN Biotechnology Co., Ltd., Nanjing, China) according to the manufacture's protocols.

2.5. Pharmacokinetics study in rats

Male Wistar rats (200 ± 20 g) were purchased from Dashuo Biotechnology (China) and maintained under standard housing conditions. All animal procedures were approved by the ethics committee of Sichuan University and conducted in accordance with institutional guidelines. To study pharmacokinetics in rats, two groups of rats were given DOX/W198-solution (5 mg/kg, on day 0, 4, 8, 12) or DOX/W198-PG (20 mg/kg, on day 0) *via* subcutaneous injection. The blood samples were collected at different time intervals and centrifuged at 5000 rpm for 10 min. Then, 300 μL of acetonitrile was added to 100 μL of plasma sample. The mixture was subjected to vortex-mixing for 10 min and centrifuged at 13,000 rpm for 10 min. The supernatant was taken for further analysis. The prepared samples were analyzed by LC–MS/MS system.

To observe the morphological changes of PG after injected *in vivo*, we anesthetized the rats and took out the injected PG at 0.5 h. These PG specimens were viewed under a scanning electron microscope (SEM, JSM-5600 LV, JEOL, Japan) at instrumental magnification of 100×, 500×, 2000× and 10,000×, respectively.

2.6. In vivo *imaging in tumor-bearing mice*

To observe the real-time distribution and tumor accumulation ability of DOX in mice bearing MCF-7/Adr xenografts, whole body imaging system was utilized. Female BALB/c nude mice(16–18 g, Dashuo Biotechnology, China) were used for *in vivo* imaging. Briefly, approximately 1×10^7 MCF-7/Adr cells were suspended in 200 μL of serum-free media, and subcutaneously injected into the right flanks of the nude mice. When tumors reached approximately 100 mm^3 in volume, mice were administered with physiological saline (i.t.), a single injection of DOX/W198-PG (4 mg/kg, i.t.), and a single injection of DOX/W198-solution (4 mg/kg, i.t.). Mice were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg), and observed on day 1, 16 and 30 using a whole body imaging system (Lightools Research, USA). The scanning parameters included the following: excitation wavelength = 540 nm, emission wavelength = 600 nm, and luminescent exposure time = 2 s. After *in vivo* imaging, mice were sacrificed and the major organs including hearts, livers, spleens, lungs, kidneys and brains and tumors were collected and visualized. The

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