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A pH-sensitive doxorubicin prodrug based on folate-conjugated BSA for tumor-targeted drug delivery

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ABSTRACT

Doxorubicin (DOX) is one of the most effective anti-cancer drugs, but its therapeutic efficacy is greatly hampered by its non-specific delivery to the target tissue and the resultant cumulative cardiotoxicity and nephrotoxicity. In order to overcome this limitation, we prepared a folate-bovine serum albumin (BSA)-*cis*-aconitic anhydride-doxorubicin prodrug, denoted by FA-BSA-CAD. A tumor-targeting agent, folic acid, was linked to BSA to increase the selective targeting ability of the conjugate. BSA provided a large number of reactive sites for multivalent coupling of bioactive molecules and improved the water-solubility of the prodrug. DOX is attached to the BSA via a pH-sensitive linker, *cis*-aconitic anhydride, which hydrolyzes in the acidic lysosomal environment to allow pH-responsive release of DOX. The *in vitro* results demonstrate a pH-responsive drug release under different pH conditions. Furthermore, the targeting ability and therapeutic efficacy of the prodrug were assessed both *in vitro* and *in vivo*. The results demonstrate that FA-BSA-CAD prodrug selectively targeted tumor cells and tissue, with associated reduction in non-specific toxicity to the normal cells. More importantly, the therapeutic efficacy of the prodrug for FA-positive tumors increased compared to the non-conjuagted DOX.

1. Introduction

Doxorubicin (DOX) is one of the most effective and widely prescribed chemotherapeutic agents for the treatment of various malignancies, such as the cancers of the bladder, breast, bile duct, endometrial, esophagus, and liver, as well as osteosarcomas, soft tissue sarcomas, and Hodgkin's lymphoma [1]. However, the clinical use of DOX is restricted because of its diverse toxicities, including kidney, cardiac, hematological, and testicular toxicity [2–4], as well as severe cardiotoxicity and nephrotoxicity. To overcome these limitations, an efficient strategy is to conjugate DOX with targeting agents, which may improve selective cytotoxicity of the drug to targeted cells and reduce the systemic toxicity to normal cells.

Various targeting moieties or ligands for tumor cell-specific receptors have been conjugated to anti-cancer drugs for selective therapy via receptor-mediated endocytosis [5,6]. Among them, folic acid has been widely employed as a targeting moiety for various anti-cancer drugs [7,8]. The folate binding protein, a glycosylphosphatidylinositol (GPI) anchored cell surface receptor for folate [9], is over-expressed in many human tumors, including tumors of the ovary, uterus, endometrium, brain, kidney, head and neck, and mesothelium [10–12], with limited expression on the normal cells. For this reason, folic acid has been covalently conjugated to anti-cancer drugs for the purpose of selective targeting to tumors [13,14]. Some of the folate-drug compounds displayed better therapeutic effect, and six of them have been tested in the clinic to date [15–19]. But there are still some drawbacks. For instance, a paclitaxel-7-polyethylene glycol-folate conjugate was designed with a pH-sensitive ester connecting the taxane to the polyethylene glycol-folate in the hope that the low endosomal pH experienced during intracellular trafficking would release the paclitaxel [20]. Although the conjugate retained high affinity for the FR, the folate conjugate was found to be 50-fold less cytotoxic to folate receptor-positive (FR^+) KB cells than the free paclitaxel. This unexpected inactivity was probably caused by the rapid recycling of the monovalent folate-drug conjugates through endosomes without prolonged exposure to acidic intracellular compartments.

Clearly, acid-catalyzed drug release mechanisms can be enhanced through multivalent folate-targeted constructs [20]. To improve the multivalent compound, bovine serum albumin (BSA)

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containing about 30–50 amino groups could be used as a drug carrier and designed to provide numerous gaps in the mesh to contain and carry a large number of drugs. Furthermore, BSA is a natural polymer endowed with numerous advantages such as high safety, low immunogenicity, excellent biodegradability, good biocompatibility, facile modification, and availability at low cost [21–23]. In addition, BSA can offer the target agents and drugs with improved water solubility.

More importantly, the prodrug can only have efficient therapeutic effect after the drug release from the carrier. The prodrug design requires not only that the bridging chemistry allow facile release of an unmodified drug following uptake by the target cell, but also that the folate-drug conjugate remain intact during its brief transit in the vasculature from its site of injection to the tumor mass [24]. Therefore, the cleavable linker connecting folate to the attached drug need be stable in the bloodstream circulation.

For anti-tumor drug, DOX, *cis*-aconityl bond is a promising linker for prodrug design [25]. It was hypothesized that doxorubicin conjugated by an acid cleavable linkage to the drug carrier could be readily delivered in cells by an endocytosis mechanism. Within the acidic endosomal compartment, DOX could be cleaved in an intact form from the prodrug [26,27]. Based on these reports, we designed and synthesized a prodrug, i.e., FA-BSA-CAD, by using folic acid as a targeting agent, BSA as a biocompatible, hydrophilic carrier, and *cis*-aconitic anhydride as a pH-sensitive linkage between the anticancer drug DOX and BSA. DOX release profile was examined as a function of pH, with emphasis on the acid cleavability. *In vitro* and *in vivo* tumor cell targeting and anti-tumor activity of the prodrug were also investigated and compared, systematically.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride was purchased from Beijing Huafenglianbo Technology Co. Ltd. (Beijing, China). Bovine serum albumin (BSA), folic acid (FA), cis-aconitic anhydride (CAA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), N-hydroxy-succinimide (NHS) and Sephadex G-25 were purchased from Sigma Chemical (St, Louis, MO). ICG-Der-02 was prepared in our laboratory. All other reagents and solvents were of analytical or HPLC grade and were used without further purification.

RPMI1640, 3-(4,5-dimethylthialzol-a-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), penicillin, streptomycin and trypsin-EDTA were purchased from commercial sources.

2.2. Synthesis of FA-BSA-CAD prodrug

2.2.1. Synthesis of BSA-CAD complex

The conjugation of DOX to BSA using a *cis*-aconityl bond was performed by modifying a literature method [28,29]. The synthetic scheme is shown in Fig. 1A. Briefly, doxorubicin hydrochloride (7 mg) was dissolved in distilled water (4 mL) and cooled on ice. *Cis*-aconitic anhydride (5 mg) dissolved in 1,4-dioxane (200 μL) was slowly added to the doxorubicin solution with stirring. The pH of the reaction mixture was immediately adjusted to 9.0 by carefully adding NaOH (0.5 M). The reaction was carried out for 20 min in ice bath. Then the pH was adjusted to 7.0 and the mixture was stirred for another 30 min. HCl (1 M) was added slowly to the mixture until a heavy precipitate (*cis*-aconitic anhydride-doxorubicin, CAD) was formed. After 15 min on ice, the precipitate was recovered by centrifugation (8000 rpm, 10 min). The structure of the product was confirmed by LC-MS. The pellet, EDCI (7 mg), and NHS (3 mg) were dissolved in distilled water (3 mL) and stirred at room temperature in the dark for 4 h. The solution was mixed with BSA (2 mL of 30 mg/mL in distilled water), and stirred for another 16 h at room temperature in the dark. After the reaction, the solution was purified and separated from free BSA and DOX by passage through Sephadex G-25.

2.2.2. Folate-modification of BSA-CAD complex

The conjugation of FA to bovine serum albumin-*cis*-aconitic anhydride-doxorubicin (BSA-CAD) was carried out according to the previously reported method [30,31]. Briefly, FA (5 mg) was dissolved in DMSO (1 mL), and then EDCI (3 mg) and NHS (2 mg) were added under stirring for 4 h at room temperature in the dark. The DMSO solution was added slowly to the BSA-CAD solution (3 mL of 20 mg/mL in distilled water) and stirred for 10 h at the room temperature in the dark. After the reaction, the mixture solution was purified and separated from the free FA using Sephadex G-25. The schematic diagram is presented in Fig. 1B.

2.2.3. The conjugation ratios of FA and DOX to BSA

The coupling ratios of the FA and CAD to the BSA were determined by following the recovery method ($n = 4$), respectively. The regression equations of standard curves were obtained by different concentration of DOX and FA solutions, respectively. The un-reacted FA and CAD were separated from the reaction mixture by gel filtration on a Sephadex G-25 column and measured by UV–vis spectrometry for calculation.

2.2.4. The pH sensitivity of the prodrug

To evaluate the pH sensitivity of the prodrug, *in vitro* release profiles of DOX from the FA-BSA-CAD with different drug graft ratios were examined at 37 °C in saline-sodium citrate (SSC) medium under different pH conditions (pH 3.5, 5.5, 7.2 and 9.0). FA-BSA-CAD (1 mL) containing 100 μg DOX was added into a dialysis tube, and then placed in a plastic tube containing 10 mL of SSC solution. The tests were conducted in incubator shaker, which was maintained at 37 °C and shaken horizontally at 50 rpm. At predetermined time intervals, the whole media were removed and replaced with fresh media. The amount of released drug was calculated by UV–vis spectrometry in comparison to a standard curve. All the experiments were performed in triplicate.

2.3. Characterization of FA-BSA-CAD prodrug

The absorption spectrum of the synthesized compound was measured by UV–vis spectrophotometer (JH 754 PC, Shanghai spectrum instrument Co. Ltd., China) to determine the components of BSA, CAD and FA in the FA-BAS-CAD prodrug. The structure of the FA-BSA-CAD prodrug was further confirmed by FTIR (Nicolet ECO 2000, USA). The size and morphology of FA-BSA-CAD and BSA were characterized by Mastersizer 2000 Laser Particle Size Analyzer (LPSA) and JEM-2100 transmittance electron microscope (JEOL, Japan). In addition, silica gel columns and a high-performance liquid chromatography (HPLC, Waters, USA) system were used to identify the products.

2.4. In vitro cell studies

2.4.1. Cell culture

The human breast carcinoma cell lines (MDA-MB-231, MCF-7), human hepatocellular cell line (Bel-7402) and human embryonic lung fibrablast cell line (HELF) were all purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were propagated in DMEM and RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL streptomycin at 37 °C in 5% CO_2 atmosphere.

2.4.2. In vitro anti-tumor activity study

MTT-based *in vitro* cytotoxicity assay was performed to compare anti-tumor effects of FA-BSA-CAD and free DOX against different cell lines according to the previously established method [32,33]. MDA-MB-231, MCF-7, BEL-7402 and HELF cell lines were maintained in RPMI1640 and DMEM medium without folic acid, and with 10% FBS and penicillin (100 U/mL) at 37 °C in 5% CO_2 atmosphere. Each cell lines harvested at the logarithmic growth phase was seeded on a 96 well plate at a cell density of 5×10^3 cells/mL. Every drug was tested in 6 wells. After incubating the cells in a logarithmic phase with free DOX, FA-BSA-CAD at different concentrations from 0.3125 to 10 μM for 48 h, MTT dye (20 μL of 5 mg/mL) was added to each well. After incubation for another 4 h, the percentage of cell viability was determined at 570 nm relative to non-treated cells.

2.4.3. In vitro targeting ability study

To investigate the targeting ability of the prodrug to tumor cells, MCF-7, MDA-MB-231 and BEL-7402 cancer cells with high FR expression were selected. The cells were washed with PBS, and then incubated at 37 °C in the presence of 40 μM of FA-BSA-CAD or BSA-CAD. After incubation for 1 h, cells were washed three times with PBS before conducting fluorescence microscopy (Laser scanning confocal microscope, Olympus FV1000, Japan).

To investigate further the competitive effect of folate on the uptake of the prodrug, MCF-7 cells (4×10^5) were incubated at 37 °C with RPMI1640 for 24 h, and then preincubated with the medium containing FA-BSA-CAD prodrug (40 μM) for 30 min. Next, FA was added to the final concentrations of 20 and 40 μM, and the cells were incubated for another 30 min. After incubation, cells were washed three times with PBS before subjected to fluorescence microscopy imaging.

2.5. In vivo animal studies

2.5.1. Animal subject and tumor models

Normal (Kunming) mice were purchased from Charles River Laboratories (Shanghai, China) for acute toxicity study. Athymic nude mice were purchased from Charles River Laboratories (Shanghai, China) for prodrug anti-tumor activity investigation. All animal experiments were carried out in compliance with the