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Role of generation on folic acid-modified poly(amidoamine) dendrimers for targeted delivery of baicalin to cancer cells

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ABSTRACT

Baicalin (BAI) has been reported to exert antitumor effects. However, BAI has limited water solubility, non-specific tumor targeting, and low bioavailability, which severely limited its clinical application. The aim of this study was to develop folic acid (FA) covalently conjugated-polyamidoamine (PAMAM) dendrimers (PAMAM-FA) as carrier systems for improvement of water solubility and tumor-specificity of BAI, and study the role of generation on the physiochemical properties and biological effects of PAMAM-FA/BAI complexes. In this work, four generations of PAMAM-FA were synthesized to entrap BAI. The average sizes of G3-FA/BAI, G4-FA/BAI, G5-FA/BAI, and G6-FA/BAI complexes were 174.4 nm, 184.5 nm, 258.8 nm, and 247.5 nm, respectively, and the zeta potentials of four PAMAM-FA/BAI complexes were −2.9 mV, −6.6 mV, −9.3 mV, −9.0 mV, respectively. The entrapment efficiencies of four PAMAM-FA/BAI complexes were 91.1%, 53.5%, 80.3%, and 91.9%, respectively, and the drug loading of PAMAM-FA/BAI complexes were about 22%. The formed PAMAM-FA/BAI complexes allowed sustained release of BAI in acidic PBS (pH 5.4). In cellular uptake assay, PAMAM-FA/BAI complexes demonstrated increased drug uptake level in folate receptor (FR)-positive Hela cancer cells than FR-negative A549 cells, and the cellular uptake efficiency of PAMAM-FA is closely related with the generation of PAMAM. The MTT assay results showed that PAMAM-FA/BAI complexes demonstrated enhanced toxicity against Hela cells than non-FA-modified PAMAM/BAI complexes, and the G6-FA/BAI demonstrated the best inhibition efficiency. The cell cycle and cell apoptosis analysis further demonstrated the tumor-specific therapeutic efficacy of PAMAM-FA/BAI. These results suggested that the PAMAM-FA have the potential for targeted delivery of BAI into cancer cells to enhance its anti-tumor efficacy.

1. Introduction

Baicalin (BAI), which is a flavonoid isolated from the root of *Scutellaria baicalensis* Georgi, has been used to prevent and treat various diseases including cardio vascular diseases, hypertension, allergy, bacterial infection, and inflammation [1,2]. Over the past decade, a considerable amount of research has demonstrated that BAI displayed potent anticancer effects on bladder, lung, breast, prostate, colorectal, ovarian and liver cancer cells [3]. It was also demonstrated that BAI has not only cytostatic but also cytotoxic efficacy against various human cancer cell lines *in vitro* and restrains tumor growth *in vivo* [4]. However, the limited water solubility and non-specific tumor targeting resulted in low bioavailability of BAI, which severely limits its clinical application [5].

Nanoparticles have been used as intelligent drug delivery systems (DDSs) to improve the bioavailability of poorly soluble drugs [6]. Through the enhanced permeability and retention (EPR) effect, nanocarriers preferentially extravasate and accumulate in tumor tissues, leading to tumor selective passively targeting [7]. In addition, nanocarriers could be modified with tumor cell targeting molecules to achieve tumor active targeting. Folate receptors (FR) which are frequently over-expressed in a wide range of tumor types, have been widely used as recognition receptors for a variety of folic acid (FA)-modified nanocarriers for diagnostic and therapeutic application in FR-positive tumor cells [8,9]. Till now, some DDSs such as nanoemulsions [10], solid lipid nanoparticles [11], solid nanocrystals [12] and liposomes [13] have been designed to improve the absorption and bioavailability of BAI. Besides, BAI loaded in folate modified liposomes [3] and BAI-loaded cationic solid lipid nanoparticles modified

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with OX26 antibody [14] have been reported to improve anti-tumor activity of BAI by active targeting.

Dendritic architecture is absolutely one of the most general topologies observed universally throughout biological systems. Comparing with the lipid-based DDSs, dendrimers as the targeted DDSs have some incomparable advantages. They are nanosized (1–100 nm) three-dimensional architecture consisting of three distinct components: a core, a hyperbranched mantle, and terminal functional groups [15]. The stable chemical structure of dendrimers made them have better physical stability than self-assembled lipid nanoparticles or liposomes. The relatively hydrophobic interior of the dendrimers could be served as a pocket for physical encapsulation or complexation of hydrophobic cancer drugs, which could significantly improve water solubility and reduce cytotoxicity [16]. Importantly, amine-terminated dendrimers could display pH-dependent drug release behavior [17]. Moreover, dendrimers inherently possess the abundant surface functional groups which could be modified more easily with small molecules and targeting moieties in comparison with lipid-based DDSs [18]. Till now, there is no report about using dendrimer as delivery systems for BAI.

Polyamidoamine (PAMAM) is one of the most studied dendrimers as the DDS. In this present study, amine-terminated PAMAM dendrimers modified with FA (PAMAM-FA) were used as targeted delivery systems of BAI (Fig. 1). The excellent hydrophilic properties of PAMAM made BAI well dispersed in water. In addition, the generation of PAMAM was reported to have great effects on its physicochemical and biological properties [19]. However, few reports have actually study the role of generation on the properties of PAMAM-FA as targeted drug delivery systems. There was a report indicated that PAMAM (generations from 7 to 10) displayed rigid surface-scaffolding structures which had limited surface-permeability and destroyed the drug encapsulation efficiency. However, PAMAM (generations from 3 to 6) showed flexible scaffolding and semi-rigid container-type structures [20]. Therefore, four generations of PAMAM (G3, G4, G5, and G6) were employed to synthesize G3-FA/BAI, G4-FA/BAI, G5-FA/BAI, and G6-FA/BAI complexes and the substitution degrees of FA in PAMAM-FA and the complexation time of PAMAM-FA with BAI were optimized in this work. The release profiles of BAI were then studied with the optimized G3-FA/BAI, G4-FA/BAI, G5-FA/BAI, and G6-FA/BAI complexes. Finally, the complexes were undergone biological evaluation against the Hela human cervical cancer cells and A549 human lung carcinoma cells, which are FR-positive and FR-negative cells, respectively. The cellular uptake and MTT study were performed to compare the different performances of PAMAM-FA/fluorescent probe or PAMAM-FA/BAI complexes. Cell cycle analysis and cell apoptosis analysis further attested the biological effects of the complexes.

2. Materials and methods

2.1. Materials

Different generations of amine-terminated PAMAM (G3-NH_2, G4-NH_2, G5-NH_2 and G6-NH_2) were purchased from Weihai CY Dendrimer Technology Co., Ltd. (Shandong, China). Glycidol, 2-chloro-1-methylpyridinium iodide (CMPI), 4-(dimethylamino) pyridine (DMAP), BAI, and FA were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). Trypsin–EDTA and phosphate buffered solution (PBS) were obtained from Gibco-BRL (Burlington, ON, Canada). The RPMI 1640 medium, Dulbecco's modified eagle's medium (DMEM), antibiotics, and fetal bovine serum (FBS) were purchased from Life Technologies GmbH (Darmstadt, Germany). DNA-free RNaseA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fluorescein isothiocyanate (FITC), and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). Other chemicals were of analytical grade.

2.2. Cell culture

The Hela (human epithelial carcinoma cell line) and A549 (human lung carcinoma cell line) cells were obtained from the Cell Resource Center of Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). Hela cells were grown in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. A549 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin G and 0.1 mg/mL streptomycin). Cells were maintained at 37 °C in a humidified and 5% CO_2 incubator.

2.3. Synthesis of G3-FA, G4-FA, G5-FA, and G6-FA dendrimers

Amine-terminated G3-NH_2, G4-NH_2, G5-NH_2, and G6-NH_2 were first transformed to hydroxyl-terminated PAMAM (G3-OH, G4-OH, G5-OH, or G6-OH) using method which has been reported previously [21,22]. Briefly, G3-NH_2, G4-NH_2, G5-NH_2, or G6-NH_2 (100 mg, about 0.46 mmol free amino groups, 1 eq) was dissloved in methanol (10 mL) in a flask and glycidol (85 mg, 0.92 mmol, 2 eq) was added dropwise to the solution. After stirring at room temperature under nitrogen overnight, the mixture was dialyzed against water with a cellulose dialysis membrane (MWCO 2000, 3500 or 10,000 Da) for 48 h before lyophilization to yield G3-OH, G4-OH, G5-OH, or G6-OH as white solid.

FA dissolved in dimethyl sulfoxide (DMSO) was added to G3-OH, G4-OH, G5-OH, or G6-OH (100 mg, about 0.34 mmol free hydroxyl groups, 1 eq) dissolved in DMSO (5 mL), and the mixture was introduced into a small vial covered with a rubber cap. The vial was filled with nitrogen in vacuum. The solution of CMPI (87 mg, 0.34 mmol, 1 eq) and DMAP (83 mg, 0.34 mmol, 1 eq) dissolved in 0.5 mL DMSO, respectively, were added dropwise with a thin pinhead piercing through the rubber cap. After stirred for 48 h at room temperature, the mixture was poured into water. The product was purified using dialysis against PBS and then ultrapure water with a cellulose dialysis membrane (MWCO 2000, 3500 or 10,000 Da) over 48 h. The dialysate was centrifuged and the supernatant was dried by lyophilization to yield G3-FA, G4-FA, G5-FA, or G6-FA as a yellow solid.

2.4. Encapsulation of baicalin within G3-FA, G4-FA, G5-FA, or G6-FA

G3-FA, G4-FA, G5-FA, or G6-FA dendrimers (15 mg) were dissolved in 5 mL methanol, and BAI (5 mg) dissolved in 5 mL methanol was added dropwise to the solution. After stirred for 8 h at room

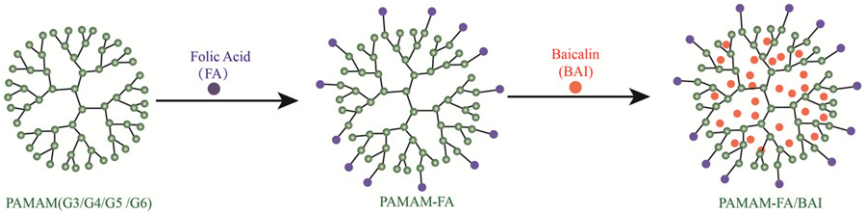

Fig. 1. Synthesis procedures of PAMAM-FA/BAI complexes.