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A targeted nanocarrier based on polyspermine for the effective delivery of methotrexate in nasopharyngeal carcinoma

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ABSTRACT

Antitumor efficacy of methotrexate (MTX) is seriously limited due to its poor water solubility, nonspecific tumor distribution and low bioavailability. To overcome these obstacles, polyspermine (PSP) conjugated with MTX and folic acid (FA) as a novel targeted prodrug was designed and has been successfully synthesized using the amidation reaction. The strong hydrophilic properties of PSP made MTX well dispersed in water and the cellular uptake study indicated that the presence of FA enhanced uptake of the FA-PSP-MTX in folate receptor (FR) overexpressing human nasopharyngeal carcinoma HNE-1 cells. ^{1}H NMR spectra and UV–Vis spectral analysis were carried out to confirm the MTX and FA content in FA-PSP-MTX, respectively. In CCK-8 assay and apoptosis analysis, the prodrug showed significantly enhanced anticancer efficacy than free MTX in HNE-1 cells. These results suggested that the prodrug has the potential for targeted delivery of MTX into cancer cells to improve its anti-tumor efficacy.

1. Introduction

Methotrexate (MTX) is an antitumor agent being used extensively in cancer chemotherapy since 1948 [1]. MTX is widely used in the treatment of malignancies including childhood acute lymphocytic leukemia, osteosarcoma, non-Hodgkin's lymphoma, Hodgkin's disease, head and neck cancer, lung cancer, breast cancer, and nasopharyngeal carcinoma [2]. The drug hinders the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF) by the inhibition dihydrofolate reductase which ultimately inhibits the synthesis of purines and pyrimidines and transmethylation of DNA and RNA. The resulting cellular deficit of thymidylate causes the potent cytotoxic effects in cancer cells [3]. However, the potent anticancer effect of MTX is hindered due to its limited water solubility, non-specific distribution and low bioavailability. The high toxic effect against normal cells resulted in acute and chronic toxicity, and eventually limited its clinical application [4,5]. Therefore, efforts must be made to improve the physicochemical property as well anticancer efficacy of MTX by designing intelligent drug delivery systems (DDS) [6].

Some DDS such as polymeric nanoparticles [7,8], phospholipid nanoparticles [9], inorganic nanoparticles [10], graphene oxide [11], micellar nanosystems [12,13], hydrogels [14–18], nanofibers [19] and liposomes [20] have been developed. However, these DDS have many obvious shortcomings. For instance, graphene oxide is difficult to metabolize from the body due to non-degradable [21], and the micelles encapsulation by non-covalent interactions has a low drug loading and leads to drug loading leakage. It was reported that MTX was chemically conjugated to poly (ethylene glycol)-poly (2-hydroxyethyl aspartamide) (PEG-b-PHEA) to form self-assembled micelles with a mean diameter of 14 nm wherein MTX residues served as hydrophobic part in the inner core [22]. Compared to simple physical entrapment, chemical conjugation will be more stable and load more drugs [23,24]. Researchers have designed polyamidoamine (PAMAM) as MTX delivery systems [25–27]. The abundant surface functional groups of PAMAM could be functionalized with small molecules and targeting moieties. But, the degradation of the PAMAM produces the monomers, which are detrimental to the body during metabolic processes. PSP has a large number of amine groups, which could conjugate MTX and targeting molecules. PSP showed favorable degradability and the degradation of the resulting PSP produce the spermine monomers in their original endogenous states. Spermine, which is involved in cellular metabolism of all eukaryotic cells, is safe and naturally present in body tissues [28].

To further enhance their recognition and internalization by the target tissues, tumor cell targeting molecules such as antibodies, peptides, nucleic acids, polysaccharides, or folic acid (FA) could be attached to the surface of the nanocarriers [29]. The administration of multivalent, folate-targeted dendrimer-methotrexate conjugates resulted in significantly lower toxicity and a 10-fold enhancement in efficacy compared to free methotrexate at an equal cumulative dose [30–32]. Folic acid belongs to the vitamin B family. It is important in

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cell division because it participates in the biosynthesis of nucleotide bases. There are two membrane-bound folic acid receptors (FR), FR-α and FR-β. Both of the 38 kDa FR isoforms bind folic acid with a high affinity [33]. The folate receptors are overexpressed in many cancer cells, including breast, ovary, endometrium, kidney, lung, head and neck, nasopharyngeal carcinoma, and myeloid cancers [34,35], and is internalized into cells after ligand binding [36]. Due to absence or low expression of these receptors on normal tissues, folate-linked carrier system does not normally accumulate in healthy tissues. The advantages of FA over other ligands are high affinity and specificity towards receptors, good conjugating property, low cost, easy availability and non-immunogenicity [37].

In the present investigation, we designed and synthesized a targeted DDS for MTX delivery in which MTX was loaded by chemical conjugation. Both FA and MTX were covalently conjugated to the PSP through the degradable amide bonds. The strong hydrophilic properties of PSP made MTX well dispersed in water [38]. The covalent conjugation of FA could provide active targeting and enhance uptake of the nano-conjugates, further increased tumor specificity and improved drug efficacy. FA-PSP-MTX, as an amphiphilic polymer, could self-assemble into nano-sized micelles. The nanoscaled micelles would help to improve the bioavailability of MTX, prolong it in vivo residence time while increasing its tumor accumulation [39]. ^{1}H NMR spectra and UV–Vis spectral analysis were carried out to confirm the MTX and FA content in FA-PSP-MTX, respectively. The physico-chemical properties of the developed formulations were investigated, namely particle size, zeta potential. Then, the conjugates were subjected to biological evaluation against HNE-1 cancer cells. The cellular uptake assay, CCK-8 assay and apoptosis analysis were performed to evaluate performances of FA-PSP-MTX conjugates. Finally, the biocompatibility including blood compatibility and in vivo toxicity of FA-PSP-MTX is also assayed to confirm its safety.

2. Materials and methods

2.1. Materials

Spermine, ethyl trifluoroacetate (99%), succinyl chloride, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDC·HCl), folic acid (FA), fluorescein isothiocyanate (FITC) and methotrexate (MTX) were purchased from Aladdin-reagent Company (Shanghai, China) and used directly. Triethylamine and chloroform were purified by refluxing over CaH_2 for 48 h, followed by distillation before use. Methanol and ammonium water (20%) were purchased from Guangzhou Chemical Reagent (China) and used without further purification. RPMI 1640 medium, fetal bovine serum (FBS) and Dulbecco's phosphate buffered saline (PBS) were purchased from Life Technologies Corporation. Ultrapure water was obtained from Millipore RIOs. Cell counting kit-8 (CCK-8) and Annexin V-FITC Apoptosis Detection Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

2.2. Cell culture

The human nasopharyngeal carcinoma HNE-1 cells were obtained from Southern Medical University. HNE-1 cells were cultured in complete RPMI 1640 medium (with 10% FBS, 100 U/mL penicillin G sodium and 0.1 mg/mL streptomycin sulfate). Cell cultures were maintained at 37 °C in a humid atmosphere containing 5% CO_2.

2.3. Synthesis of PSP

The synthetic routes of FA-PSP-MTX are showed in Scheme 1. For PSP synthesis, we first selectively protected the two primary amines of spermine with ethyl trifluoroacetate. Spermine (500 mg, 2.48 mmol) was dissolved in methanol (20 mL) by Schlenk technique under a dry nitrogen atmosphere. Then, ethyl trifluoroacetate (0.55 mL, 4.96 mmol) was added dropwise at − 78 °C and the solution was further magnetically stirred for 1 h at 0 °C after completion of the dropwise addition [40]. The solvent and unreacted trifluoroacetate were evaporated from the solution to give the protected spermine N^1, N^{14}-bis (trifluoroacetyl) spermine. The product was then recrystallized from ethyl acetate to 92% yield. Second, we added dropwise succinyl chloride (300 mg, 1.94 mmol) dissolved in chloroform (10 mL) to N^1, N^{14}-bis (trifluoroacetyl) spermine (764.87 mg, 1.94 mmol) dissolved in chloroform (15 mL) and triethylamine (5 mL) at 0 °C under a dry nitrogen atmosphere. The resultant solution was then continuously stirred at 25 °C for 12 h [41]. The solvent was evaporated to give a dark brown reaction product. To remove the trifluoroacetate ester protecting group, the reaction product was treated with a mixture of 20% ammonium water (3.5 mL) and methanol (12 mL) in a sealed vessel at 60 °C for 8 h [42]. The resultant polyspermine was dialyzed (MWCO = 1000, USA) for 3 days by changing distilled water at regular intervals.

2.4. Synthesis of PSP-conjugating FA and MTX (FA-PSP-MTX)

FA-PSP-MTX has been synthesized using the two-step approach. For the synthesis of MTX-PSP, MTX (20 mg, 0.044 mmol) was dissolved in dimethyl sulfoxide (5 mL) (DMSO), and then EDC·HCl (33.75 mg, 0.176 mmol) and NHS (20.26 mg, 0.176 mmol) dissolved in H_2O (0.5 mL) were added for activating MTX [43]. The pH value of the mixture was adjusted to 4–6 by HCl. After reacted for 1 h at 25 °C, 1 mL H_2O containing 0.40 g PSP was added dropwise to the mixture with stirring. The mixture was stirred for 24 h at 25 °C in the dark. MTX-PAAs was obtained by dialysis (MWCO = 1000, USA) for 12 h and freeze drying with a yield of 64%. The synthesized MTX-PSP was stored at − 20 °C for subsequent reaction use.

FA-PSP-MTX was prepared by coupling FA onto MTX-PSP via EDC·HCl/NHS. Briefly, FA (10 mg, 0.023 mmol) was dissolved in DMSO (0.5 mL), and then activated by EDC·HCl (17.64 mg, 0.092 mmol) and NHS (10.59 mg, 0.092 mmol) [43]. After its pH value is adjusted to 4–6 by HCl, the mixture was stirred at 25 °C for 1 h. MTX-PSP was then added to the reaction mixture of activated FA. The reaction was continued at 25 °C for 24 h in the dark conditions. The resultant solution was kept in a pretreated dialysis bag (MWCO = 1000, USA) and dialyzed for 12 h by changing the media at regular intervals in dark to remove free FA. FA-PSP-MTX was got by freeze drying with a yield of 52% and stored at − 20 °C.

2.5. Structural characterizations of FA-PSP-MTX

^{1}H NMR spectra of N^1, N^{14}-bis (trifluoroacetyl) spermine, PSP and MTX-PSP were obtained from a Bruker AVANCE 300 spectrometer (300 MHz), using $CDCl_3$ as the deuterated solvents of N^1, N^{14}-bis (trifluoroacetyl) spermine and D_2O as the deuterated solvents of the other three samples, respectively. The weight-average molecular weight (Mw), number-average molecular weight (Mn) and polydispersity indexes (PDI: Mw/Mn) of the FA-PSP-MTX were measured by a gel permeation chromatography (GPC) system. The elution time of PSP, MTX-PSP and FA-PSP-MTX were determined by GPC. GPC measurements were carried out with a VE 1122 HPLC pump and a Model 270 RI detector using dextran as a standard at 35 °C. Aqueous sodium nitrate (0.8 mol/L) was used as the eluent (elution rate: 1.0 mL/min). UV–Visible spectrum of PSP, MTX-PSP and FA-PSP-MTX were recorded on a 1 cm quartz cuvette using a UV-2550 spectrophotometer (Shimadzu, Japan) at 25 °C. MTX, FA, MTX-PSP and FA-PSP-MTX were dissolved in sodium hydroxide solution (0.1 mol/L). The mean particle size, size distribution and zeta potential of FA-PSP-MTX at different concentrations were determined by Malvern Zetasizer Nano ZS (United Kingdom). Each sample was measured in triplicate.