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Research Paper

Tumor targeting using polyamidoamine dendrimer–cisplatin nanoparticles functionalized with diglycolamic acid and herceptin

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ABSTRACT

Polymer mediated drug delivery system represents a novel promising platform for tumor-targeting with reduced systemic side effects and improved chemotherapeutical efficacy. In this study, we report the preparation and characterization of herceptin targeted, diglycolamic acid (DGA) functionalized polyamidoamine (PAMAM) dendrimer as a potent drug carrier for cisplatin. DGA dendrimers carrying cisplatin demonstrated enhanced anticancer activity when targeted with herceptin. *In vitro* cell line studies with herceptin-DGA-G4-cisplatin in HER-2 +ve and HER-2 −ve human ovarian cancer cell lines showed that these nanoparticles possessed remarkable features such as lower IC_{50} value, improved S-phase arrest, and enhanced apoptosis due to increased cellular uptake and accumulation than the untargeted DGA-G4-cisplatin and free cisplatin. Furthermore, *in vivo* results in SCID mice bearing SKOV-3 tumor xenografts, herceptin-DGA-G4-cisplatin, appeared to be more effective in inducing tumor regression as compared to free cisplatin. Collectively, these results indicate that herceptin targeted DGA functionalized PAMAM-cisplatin conjugates serve as better anti-tumor agents than individual therapeutic agents.

1. Introduction

Therapeutic efficacy of cisplatin, the key drug employed in the treatment of variety of solid tumors, is often limited by various side effects such as nephrotoxicity, neurotoxicity, ototoxicity, nausea and vomiting [1–3]. As a consequence, the quality of patient's life worsens and becomes even more threatening than the pre-existing conditions. Nanoparticle mediated drug delivery has shown promise in enhancing the therapeutic index of several drugs, including cisplatin, by circumventing several impediments encountered during conventional chemotherapy [4,5]. They are potential enough to carry large payloads of drug, protect them from physiological barriers and enable its sustained release within the desired site of action. These nanosystems passively provide better accumulation of drug in tumors through a mechanism popularly known as enhanced permeation and retention (EPR) effect [6]. Additionally, the tumor selectivity of nanoparticles can be further enhanced in an active manner by employing a targeting moiety [7]. The surface of nanoparticles can be functionalized with specific ligands that target corresponding cellular receptors over-expressed in cancer cells [8]. Among numerous cell surface receptors that are present on malignant cells, HER-2 a receptor tyrosine kinase presents itself as an interesting target for achieving tumor specific drug delivery. Being a member of epidermal growth factor (EGF) family, HER-2 is over-expressed in 30–40% of all breast and ovarian cancers and has been strongly associated with poor clinical outcomes [9,10]. Considering HER-2 as a potential candidate for targeted therapy, a recombinant humanized monoclonal antibody Trastuzumab (Herceptin) was developed and later approved by U.S. Food and drug administration (FDA) for the treatment of breast

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cancer [11]. Besides, being a therapeutic agent, herceptin is also widely employed as a targeting ligand in nanoparticle-drug conjugates, to specifically target HER-2 expressing tumors and maximize the intracellular concentration of chemotherapeutics, thereby preventing the side effects encountered due to non-specific drug interactions [12–14].

In this study, we have enhanced the targeting specificity of a pH sensitive diglycolamic acid functionalized PAMAM dendrimer with the aid of herceptin. DGA-PAMAM-cisplatin of generation DGA-G1-DGA-G3 has been previously reported to be a potent pH sensitive drug carrier in human breast and ovarian cancer cell lines [15]. Encouraged by the increased drug loading efficiency and enhanced *in vitro* toxicity of higher generation dendrimers, we selected DGA-G4-cisplatin in this study for targeting with herceptin. *In vitro* cell line studies with herceptin-DGA-G4-cisplatin in HER-2 +ve and HER-2 −ve human ovarian cancer cell lines showed that these nanoparticles possessed remarkable features such as lower IC_{50} value, improved S-phase arrest, and enhanced apoptosis due to increased cellular uptake and accumulation than the untargeted DGA-G4-cisplatin and free cisplatin. *In vivo* studies in human ovarian cancer xenograft model were done to further demonstrate the anti-tumor efficacy of this targeted dendrimer–drug conjugate.

2. Materials

Dry methanol, dry dimethyl sulfoxide (DMSO), methyl acrylate, silver nitrate and trifluoroacetic acid were purchased from Merck and used as received. EDA (Merck) was purified over CaH_2 prior to use. Diglycolic anhydride, cisplatin (II), o-phenylenediamine (o-PDA), triethylamine, dithiothreitol (DTT), N-ethylmaleimide (NEM) and methyl thiazolyl tetrazolium (MTT) powder procured from Sigma–aldrich were used in this study. Herceptin was purchased from Roche. Heterobifunctional crosslinkers succinimidyl 6-[3(2-pyridyldithio) propionamido] hexanoate (LC-SPDP) and sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC) were obtained from Pierce. Vivaspin TM columns (Sartorius), prepacked Sephadex G-25 PD-10 desalting columns (GE healthcare life sciences) and microcon YM100 (Sigma–aldrich) were used for the purification of dendrimer cisplatin conjugates.

2.1. Methods

2.1.1. Synthesis of partial DGA-functionalized G4 PAMAM dendrimer

Total number of amine groups in G4 PAMAM dendrimer was partially DGA functionalized leaving the remaining groups for LC-SPDP conjugation to enable herceptin targeting. Briefly, to a G4 dendrimer solution (0.05 g, 0.0035 mmol) in DMSO, diglycolic anhydride (0.019 g, 0.163 mmol) was added drop by drop and allowed to stir for 24 h at room temperature. At the end of the reaction, DMSO and excess diglycolic anhydride were removed from the product by dialysis against MilliQ water. Partially DGA functionalized G4 PAMAM dendrimer present in the aqueous retentate was obtained by vacuum drying.

2.1.2. Synthesis of herceptin-DGA-G4 PAMAM dendrimer

Partial DGA-G4 PAMAM dendrimers were employed for herceptin targeting studies following the previously reported protocol [16]. Briefly, the amine groups present in the partially DGA functionalized G4 PAMAM dendrimer were conjugated with LC-SPDP. The number of SPDP linked to the dendrimer was determined by pyridine-2-thione assay as per manufacturer's instruction. DGA-G4-LC-SPDP was then reduced with DTT to provide thiol group which readily reacts with the maleimide group introduced in herceptin. The final herceptin-DGA-G4 conjugate was purified by ultrafiltration and used for further drug conjugation studies.

2.1.3. Characterization of herceptin-DGA-G4 PAMAM dendrimers

Particle size and zeta potential of the herceptin targeted and untargeted DGA-G4 dendrimer (0.5 mg/ml) were analyzed using Malvern NanoZS particle size analyzer. Further, to ensure the absence of free herceptin in the final targeted conjugate, Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) was performed. Herceptin (100 μg/mL) and herceptin-DGA-G4 dendrimer (100, 200 μg/mL) were mixed with 5 μl of sample buffer (20% glycerol, 0.04% bromophenol blue in Tris–HCl pH 6.8 containing 2% SDS) and the samples were individually loaded into each well and separated on 12% resolving SDS–PAGE at 100 V for 1 h. Developed gels were stained with 0.025% Coomassie Blue R-250 overnight, in 40% methanol and 7% acetic acid aqueous solution. The gels were then destained in an aqueous solution containing 7% (v/v) acetic acid and 5% (v/v) methanol.

2.1.4. Conjugation of cisplatin with herceptin-DGA-G4 PAMAM dendrimer

Hydrolyzed cisplatin was prepared from cisplatin (1 mg/mL) using 2 mol equivalent $AgNO_3$ and its concentration was determined spectrophotometrically at 703 nm using o-phenylenediamine (o-PDA) [17]. The conjugation of herceptin-DGA-G4 dendrimer with hydrolyzed cisplatin was carried out overnight. Unconjugated cisplatin was then removed by ultracentrifugation using Vivaspin™ columns with a 2 kDa MWCO membrane. Drug loading efficiency (DLE) of herceptin-DGA-G4 dendrimer was then determined by analyzing the concentration of unbound hydrolyzed cisplatin in the filtrate by o-PDA assay.

2.1.5. Cell culture

Human ovarian cancer cell lines PA-1 and SKOV-3 were used in this study to check the efficacy of targeted dendrimer–drug conjugates. PA-1 procured from National Center for Cell Science (NCCS), Pune, India, was grown in Minimum Essential Medium (MEM) (pH 7.4). SKOV-3, cultured in Dulbecco's modified Eagle's medium (DMEM) (pH 7.4), was a kind gift from Dr. Subhash Chauhan, University of South Dakota, USA. The medium was supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity.

2.1.6. HER-2 expression analysis by flow cytometry and Western blot

HER-2 protein expression levels in human ovarian cancer cell lines SKOV-3 and PA-1 were evaluated by Western blot and flow cytometry.

For Western blot experiment, the cells (SKOV-3 and PA-1) were lysed and protein concentration was determined by Bio-Rad DC assay. Briefly, 100 μg of total protein was resolved on 7.5% polyacrylamide gel and then transferred to an Immobilon membrane (Millipore). Membranes were incubated overnight at 4 °C with anti-HER-2 rabbit monoclonal antibody (mAb). Blots were then washed with PBS containing 0.1% Tween 20 for 15 min and incubated with a secondary antibody conjugated with peroxidase. Signals were detected using the enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK).

For flow cytometry experiment, briefly 10^6 cells (SKOV-3 and PA-1) were suspended in 100 μl of PBS solution containing 10% FBS and exposed to the primary anti-HER-2 rabbit mAb for 30 min on ice. After washing three times, the cells were resuspended in 100 μl of PBS solution with 10% FBS containing alexa fluor 488 conjugated anti-rabbit IgG monoclonal secondary antibody. The samples were then analyzed for HER-2 expression levels