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Synthesis and properties of a new micellar polyphosphazene–platinum(II) conjugate drug

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ABSTRACT

Aiming at tumor targeting delivery of oxaliplatin using polymer therapy, a new monomeric platinum(II) complex (dach)Pt[HEDM] (dach: *trans*-(±)-1,2-diaminocyclohexane; HEDM: 2-hydroxyethoxydiethylmalate) was designed to include the antitumor moiety (dach)Pt and HEDM as a linker to the polyphosphazene backbone. This monomeric Pt-complex could easily be grafted to the PEGylated polyphosphazene backbone to prepare a novel polyphosphazene–Pt conjugate, $[NP(MPEG550)(dach)Pt(EM)]_n$ [MPEG550: methoxy poly(ethylene glycol) with an average molecular weight of 550; EM: ethoxymalate]. This amphiphilic polyphosphazene–Pt conjugate was found to self-assemble into stable polymeric micelles of a mean diameter of 130 nm, which is suitable for passive tumor targeting by enhanced permeability and retention (EPR) effect. Pharmacokinetic study of this polymer conjugate exhibited long blood circulation as expected and longer half-life ($t_{1/2}\beta = 9.52$ h) compared with oxaliplatin (3.47 h), and much larger AUC (area under the curve) value (25,831 ng·h/mL) compared with oxaliplatin (1194 ng·h/mL). Biodistribution study of the polymer conjugate has shown excellent tumor selectivity with the tumor to tissue ratio of 3.84 at 2 h post injection and 11.7 at 24 h post injection probably due to the EPR effect of the polymer conjugate while no tumor selectivity was observed for monomeric oxaliplatin. Furthermore, accumulation of this polymer conjugate in kidney was much lower compared with oxaliplatin. Also the nude mouse xenograft trial of the polymer conjugate has shown higher antitumor efficacy compared with oxaliplatin.

1. Introduction

Platinum based cancer chemotherapeutic agents like cisplatin, carboplatin and oxaliplatin have been widely used for treatment of testicular, ovarian, lung, head and neck, and colorectal cancers [1,2]. However, their usefulness is limited due to their significant toxic side effects such as nephrotoxicity, neurotoxicity, ototoxicity, nausea, and vomiting [3,4]. In order to reduce such toxicities along with improved efficacy of the platinum drugs, polymer therapy has widely been attempted particularly for the last decade by encapsulation or conjugation of the platinum drugs using a variety of polymic drug delivery systems [5,6]. Such polymer therapy has shown great improvements in pharmacokinetics and pharmacology of the platinum drugs by prolonging their blood circulation time and by suppressing the reaction of the platinum drugs with protein and glutathione in blood.

Polymer therapy of platinum drugs has been attempted in many different ways using different polymer carriers with a variety of morphology in aqueous solution. For example, platinum drugs were physically encapsulated by liposomes [7,8], nanocapsules [9,10], or polymeric micelles [11], and alternatively conjugated to linear polymers [12–14] or dendrimers [15,16] by covalent bonding. Among these various drug delivery systems, several platinum drugs formulated using liposome and conjugated to amphiphilic polymers are promising and entered clinical studies [17]. In particular, Kataoka and coworkers have successfully prepared a series of polymeric platinum drugs by incorporating cisplatin into the polymeric micelles of biodegradable PEG-b-poly(glutamic acid) [PEG = poly(ethylene glycol)] block copolymer [18] and by conjugation of the oxaliplatin parent complex moiety (dach)Pt (dach: *trans*-(±)-1,2-diaminocyclohexane) to the same block copolymer [19]. These polymeric drugs of cisplatin (NC-6004) and oxaliplatin (NC-4016) are under clinical studies.

Recently, we have prepared PEG grafted polyphosphazene–platinum(II) conjugates with a dipeptide glycyl-L-glutamate as a spacer

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and studied their tumor selectivity and antitumor activity [20,21]. Preparation and cytotoxicity of cyclotriphosphazene-platinum(II) conjugates with an oligopeptide as a spacer were also reported [22,23]. All the above-mentioned polymeric platinum(II) complexes exhibit aggregated morphology such as polymeric micelles or polymersomes with large nanoparticle sizes, which are suitable for passive targeting and exhibit good tumor targeting properties by EPR (enhanced permeability and retention) effect [24]. However, a critical problem encountered for clinical applications of the polymeric platinum(II) complexes employing amino acid as a spacer group for platination is the instability of the Pt to dicarboxylate bonding mode, in which (O,O) chelation mode slowly changes to inactive (O,N) chelation in aqueous solution [5,25].

We have found in recent years that a new monomeric platinum(II) complex, (dach)Pt[HEM] (HEM = 2-hydroxyethylmalate) exhibits much higher cytotoxicity than oxaliplatin, and furthermore, when this platinum(II) moiety bearing only (O,O) chelation mode is grafted to the polyphosphazene backbone along with poly(ethylene glycol), very stable polymeric micelles are formed with almost the same cytotoxicity as the monomeric Pt(II) complex based on the platinum content. Therefore, we have designed a novel polymeric platinum anticancer drug aiming to afford high tumor selectivity by EPR effect and controlled release of the active platinum moiety *in vivo* using the biodegradable nanosized polyphosphazene. Here we present the results of our studies on the synthesis, physicochemical properties, pharmacokinetics, biodistribution, and *in vitro* and *in vivo* antitumor activity of the our novel polyphosphazene–(dach)platinum conjugate drug.

2. Experimental

2.1. Materials

Hexachlorocyclotriphosphazene, $AlCl_3$, methoxy poly(ethylene glycol) with a molecular weight of 550 (MPEG550), barium hydroxide octahydrate and *trans*-(±)-1,2-diaminocyclohexane (dach) were purchased from Aldrich and used without further purification. Potassium tetrachloroplatinate(II) (Kojima), diethyl-L-(−)-malate and 2-bromoethanol (TCI) were also used without further purification but thoroughly vacuum dried. The (dach)Pt(II) sulfate was prepared by the literature method [26]. Tetrahydrofuran (THF) was dried by boiling at reflux over sodium metal and benzophenone and then distilled under argon atmosphere. Chloroform was dried by boiling at reflux over CaH_2 and then distilled under argon atmosphere.

2.2. Instruments and measurements

^1H and ^{13}C NMR spectra were recorded using Varian 500 MHz NMR spectrophotometer operating at 500 MHz in the Fourier transform mode. Proton decoupled ^{31}P NMR spectra were measured with Varian 500 MHz NMR spectrophotometer operating at 500 MHz using phosphoric acid as an internal standard. Elemental analysis was carried out with Carlo Ebra-EA1108. The particle size distributions of the polyphosphazene carrier and polyphosphazene–Pt(II) conjugate were measured for their 0.5% aqueous solution by dynamic light scattering (DLS) method using Malvern Zetasizer (Nano-ZS). We have measured their lower critical solution temperature (LCST) by cloud point method for 5% polymer conjugate in distilled water and PBS contained in a glass capillary immersed in an oil bath. We have also measured their critical micelle concentration (CMC) by the fluorescence probe technique using pyrine [27].

2.3. Synthesis of 2-hydroxyethoxydiethylmalate (HEDM)

To a suspension of NaH (0.38, 15.83 mmol) in dry THF (50 mL) was added slowly diethylmalate (2.61 mL, 15.83 mmol) at −20 °C. The reaction mixture was stirred for 1 h at the same temperature, and then to the reaction mixture was added 2-bromoethanol (1.69 mL, 23.66 mmol) and stirring was continued for 3 h at 0 °C. After the completion of reaction as monitored by TLC, THF was removed under reduced pressure. To the residue, water was added and extracted with ethyl acetate (5 × 40 mL). The combined washings were washed with brine, dried over anhydrous magnesium sulfate, filtered and solvent was removed under reduced pressure to give colorless liquid, which was further purified by silica column chromatography (EtOAc/n-Hexane 1:3). Yield: 2.5 g. R_f (EtOAc/n-Hexane 1:3): 0.16; Yield: 67%. Elemental analysis (%): Calcd for $C_{10}H_{18}O_6$; C, 51.28; H, 7.69. Found: C, 51.29; H, 7.71. ^1H NMR ($CDCl_3$, ppm): δ 1.26–1.35 (m, 6H, methyl(−CH_3)$_2$ protons of diethylmalate), 2.77–2.91 (m, 2H, malate methylene-(O═C−CH_2−C) protons), 3.49–3.58 (m, 2H, ethylmethylene-(O−CH_2−) protons), 3.80–3.92 (m, 2H, ethylmethylene-(−CH_2−O−) protons), 4.16–4.32 (m, 4H, methylene protons of diethylmalate-(CH_2)$_2$), 4.45–4.57 (m, 1H, malate-(O═C−CH−O−) proton). ^{13}C NMR ($CDCl_3$, ppm): δ 13.98 (methyl (CH_3)$_2$), 38.71 (CH_2), 60.86 (diethyl-(CH_2)$_2$), 61.79 (OH−CH_2), 62.62 (O−CH_2), 67.23 (−CH−), 170.49 (−C═O), 173.28 (−C═O).

2.4. Synthesis of (dach)Pt[HEM] monomer (HEM: 2-hydroxyethoxymalate)

HEDM (1 g, 4.27 mmol) was hydrolyzed by $Ba(OH)_2 \cdot 8H_2O$ (1.35 g, 4.27 mmol) in methanol (50 mL) by stirring for 4 h at room temperature. The reaction mixture was concentrated under reduced pressure and excess diethyl ether was added to induce precipitation. The precipitate was filtered, washed with diethyl ether and dried under vacuum and the residue was dissolved in distilled water (10 mL). To this solution was added slowly an aqueous solution of (dach)Pt(SO_4) (0.65 g, 1.60 mmol) until neutral pH with constant stirring at room temperature for 3 h. The reaction solution was filtered off to remove barium sulfate and the filtrate was freeze-dried to obtain the (dach)Pt[HEM] monomer as a final product. Yield: 0.63 g (92%). ^1H NMR (D_2O, ppm): δ 1.03–1.19 (brm, 4H, cyclohexane-C-4, C-5 protons), 1.46 (brs, 2H, cyclohexane-C-3 protons), 1.93 (brs, 2H, cyclohexane-C-6 protons), 2.29–2.70 (brm, 4H, malate methylene-(O═C−CH_2−), cyclohexane-C-1, C-2 protons), 3.20–3.23 (m, 2H, ethyl-(−O−CH_2−) protons), 3.59–3.68 (m, 2H, ethyl-(−CH_2−O) protons), 4.20–4.22 (brm, 1H, malate-(O−CH−C═O) proton).

2.5. Synthesis of polyphosphazene carrier, [NP(MPEG550)(EDM)]$_n$ (EDM: ethoxydiethyl malate)

Poly(dichlorophosphazene) was prepared from hexachlorocyclotriphosphazene [$NPCl_2$]$_3$, (2.0 g, 5.72 mmol) in the presence of $AlCl_3$ (5.0 wt.%) according to the published method [28]. A typical synthetic procedure is as follows. The sodium salt of methoxypoly(ethylene glycol) (MPEG550) was prepared by reaction of MPEG550 (9.48 g, 17.3 mmol) with an excess amount of sodium metal (0.59 g 25.65 mmol) in THF at refluxing temperature for 1 day. After the resultant solution was filtered to remove excess of sodium metal, the filtrate was dropped slowly to the solution of poly(dichlorophosphazene) (2 g, 17.3 mmol) dissolved in THF (100 mL). The reaction mixture was stirred for 12 h at −50 °C to afford PEGylated polyphosphazene. Meanwhile, the sodium salt of HEDM prepared by reaction of HEDM (5.51 g, 21.50 mmol) with sodium hydride (0.620 g, 25.83 mmol) in dry THF (50 mL) was stirred from 0 °C to room temperature for 12 h to obtain yellow colored solid product, which was filtered and washed with diethyl ether, and then the product was dissolved in DMSO (80 mL) and added to the PEGylated solution. Finally the mixed solution was stirred at 50 °C for 48 h. The reaction mixture was filtered to remove sodium chloride byproduct and the filtrate was concentrated under reduced pressure. The product was dialyzed for 1 day in methanol and 1 day in distilled water using regenerated cellulose membranes with molecular weight cut-off (MWCO) at 4000. The dialyzed solution was freeze-dried to obtain the pure product. Yield: 11.54 g (72%). Elemental analysis (%): Calcd for $C_{35}H_{68}NO_{19}P \cdot 4H_2O$; C, 46.16; H, 8.35; N, 1.54.