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A facile method for synthesizing water-soluble and superior sustained release anti-HIV prodrug SCs–d4T

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ABSTRACT

To efficiently deliver stavudine (d4T) for AIDS therapy, chitosan–stavudine conjugate (Cs–d4T) was synthesized. However, its poor water-solubility limited its clinical application. In this study, a sulphonated chitosan–stavudine conjugate (SCs–d4T) was synthesized with a mild SO_3·Py complex sulphonation strategy. Chemical characteristics and morphology of Cs–d4T and SCs–d4T were performed by NMR, XRD, FTIR, ICP-AES and SEM. SCs–d4T demonstrated satisfactory solubility (106-bold of Cs–d4T solubility), good anti-HIV activity (6-fold of d4T anti-HIV activity), and well sustained release ability. The major release product *O*-isopropyl-5′-*H*-phosphonate of d4T (d4T-P-H) showed higher anti-HIV activity than d4T. For further evaluating the influence of linker and sulphonation strategy on anti-HIV activity, chitosan grafted with d4T by succinyl linker (Cs–sd4T) and SCs–d4T sulphonated by oleum were also prepared. The result showed that the *O*-isopropyl monophosphate linker of Cs–d4T and SO_3·Py complex sulphonation strategy revealed higher anti-HIV activity than succinyl linker of Cs–sd4T and oleum sulphonation strategy, respectively.

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1. Introduction

Over the last few decades, Acquired Immunodeficiency Syndrome (AIDS) has been one of the most devastating pandemic diseases human ever faced [1]. Nucleoside Reverse Transcriptase Inhibitors (NRTIs) such as zidovudine (AZT), didanosine (ddI), lamivudine (3TC) and stavudine (d4T) have been widely used in the AIDS therapy. However, these NRTIs are not ideal for recent clinical application because of some undesirable adverse effects such as neutropenia, peripheral neuropathy, drug resistance [2,3], limited stability, first pass metabolism and systemic toxicity [4].

To address these adverse effects and improve the compliance of NRTIs [4], a number of methods have been developed based on the consideration of bypassing the metabolic bottleneck, improving cellular uptake efficacy, targeting viral reservoirs, etc [5–7]. For instance, Sun et al. have reported the asymmetrical *O*-alkyl-*H*-phosphonates of d4T, in which *O*-isopropyl-5′-*H*-phosphonate of d4T (d4T-P-H) showed the favorable anti-HIV activity in clinical trials [8,9]. In addition, polymer–drug conjugates have attracted considerable attention in drug delivery due to their particular therapeutic properties, such as prolonged half-life, enhanced bioavailability, lower immunogenicity and antigenicity, and often targeting to specific cells, tissues or organs [10–12]. In particular, chitosan (Cs) has been extensively used in drug delivery due to its favorable biological properties such as low toxicity, biocompatibility and biodegradability [13]. In our previous reports [14,15], the synthesized chitosan-stavudine conjugate (Cs–d4T) demonstrated lower toxicity and higher selectivity index (SI) than the parent drug d4T. However, issues of intractability and insolubility [16] in organic solvents, neutral and alkaline pH aqueous solutions limited its clinical application. Therefore, it is essential to improve its solubility in aqueous solutions.

Many studies about chemical modification of Cs have been done to improve its solubility over a wide pH range [17]. Muzzarelli [18,19] has synthesized alkyl derivatives of Cs and quaternized Cs to improve its solubility over a wide pH range; however, the harsh reaction step may degrade the chitosan–drug conjugates. In addition, Cs grafted with poly (ethylene glycol) (PEG) was also available to improve solubility [20–23], but the harsh protecting step and several purification cycles limited its application [24]. Nishimura et al. [25] have reported a mild modification approach to improve the solubility and anti-HIV activity of the polymer by sulfonating polymers. Furthermore, Cs coupled with the sodium salt of 5-formyl-2-furansulfonic acid was also water-soluble [26]. The purpose of this study was to evaluate whether sulfonated Cs–d4T conjugate (SCs–d4T) was water-soluble, and select preferable linker as well as sulphonation strategy. To accomplish this goal, SCs–d4T was synthesized by sulfonating Cs–d4T with SO_3·Py complex or oleum and characterized. Its anti-HIV activity, cytotoxicity, water-solubility and in vitro release were evaluated. Cs–d4T with *O*-isopropyl monophosphate linker and chitosan-*O*-succinyl-5′-*O*-

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stavudine (Cs–sd4T) with succinyl linker were also synthesized and investigated the anti-HIV activity as well as cytotoxicity.

2. Experimental

2.1. Materials

Cs (average molecular weight: 20 kDa, 88% deacetylated) and 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). d4T was obtained from Yingjie Biotechnology Co. Ltd (Jiangsu, China), and dried at elevated temperature in vacuum. SO_3·Py complex was procured from Aladdin reagent Co. (Shanghai, China). Oleum, triethylamine (TEA), tetrachloromethane (CCl_4), succinic anhydride, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDC), phosphorus trichloride (PCl_3), tripolyphosphate (TPP), isopropanol and formic acid were purchased from Beijing Chemical Reagents Co. (Beijing, China). MT4 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). All other solvents were commercially available reagents of analytical grade, dried and purified by distillation before using.

2.2. Preparation of Cs–sd4T, Cs–d4T, SCs–d4T and Cs–d4T/TPP nanoparticles

2.2.1. Synthesis of 5′-O-succinyl-stavudine (**4**)

Succinic anhydride (1.00 g, 10 mmol) and d4T (1.40 g, 6 mmol) were added to a 250 mL round-bottom flask. Dichloromethane (100 mL) and TEA (1 mL) were then added in succession. The reaction solution was refluxed for 5 h followed by concentration under reduced pressure. The crude residue was dissolved in distilled water (100 mL) and adjusted to pH 2 with 1 N HCl. Finally, the filtrate was collected by filtration and lyophilized to get **4** (1.85 g, 77%). ESI-MS (m/z): 325 $(M + H)^+$.

2.2.2. Synthesis of Cs-sd4T (**1**)

The synthesized **4** (1.62 g, 5 mmol) dissolved in methanol (20 mL) was added dropwise to a mixture solution of Cs (0.16 g, ~1 mmol of free NH_2), NHS (0.86 g, 6 mmol) and EDC (1.44 g, 7 mmol) dissolved in acetic acid solution (2%, 20 mL) at room temperature. And then the mixture was stirred for 24 h at room temperature for complete reaction. The reaction mixture was dialyzed using dialysis membrane (MWCO: 3.60 kDa) against distilled water for 72 h followed by freeze-drying to get **1** (0.10 g, 63%). ^{1}H NMR (293 K, 2% CD_3COOD/D_2O, 400 MHz) δ 1.82 (s, H_{15}), 2.00 (s, H-Ac), 2.61 (d, H_7, H_8), 3.11 (s, H_2), 3.52–3.84 (m, H_3, H_4, H_5, H_6), 4.30 (d, H_9), 4.83 (d, H_1), 5.10 (s, H_{10}), 5.95 (s, H_{11}), 6.39 (s, H_{12}), 6.82 (s, H_{13}), and 7.46 (s, H_{14}).

2.2.3. Synthesis of d4T-P-H (**5**)

A solution of PCl_3 (10 mL, 115 mmol) in dichloromethane (120 mL) at −35 °C was added to d4T (2.24 g, 10 mmol) during 0.5 h followed by stirring for 1 h. Then the mixture was warmed to room temperature, and stirred for 5 h followed by concentration under reduced pressure. The residue was dissolved in dichloromethane (100 mL), and cooled to 0 °C. The isopropanol (2 mL, 26 mmol) dissolved in dichloromethane (70 mL) was added dropwise to d4T solution followed by stirring for 0.5 h at 0 °C and 1 h at room temperature. TEA (3 mL, 14 mmol) was added to the solution for 15 min followed by concentration under reduced pressure. Finally, the crude residue was purified by gel silica column chromatography (trichloromethane/methanol = 30:1) to get **5** (2.31 g, 70%). ESI-MS (m/z): 331$(M + H)^+$.

2.2.4. Synthesis of Cs–d4T (**2**)

The Cs–d4T was prepared according to previous study [14]. All experiments involving water-sensitive compounds were conducted under dry conditions. A solution of **5** (1.32 g, 4 mmol) in dimethylacetamide (10 mL) was added dropwise to 6-*O*-trityl Cs (Cs-Tr, 0.16 g, ~1 mmol of free NH_2) in a mixture of dimethylacetamide (10 mL), TEA (2 mL, 13 mmol) and CCl_4 (2 mL, 21 mmol) at 0 °C. After stirring for 24 h, the solution was filtered. The filtrate was added to ethanol (200 mL) and the precipitate formed was collected by centrifugation to get **6**. Then product **6** was dissolved in formic acid (10 mL), and stirred for 50 min at 60 °C. After removing formic acid by rotary evaporation, the residue was dissolved in ethanol (200 mL) and the filtrate was dialyzed using dialysis membrane (MWCO: 3.60 kDa) against distilled water for 72 h followed by lyophilized to provide **2** (0.12 g, 75%). ^{1}H NMR (293 K, 2% CD_3COOD/D_2O, 400 MHz) δ 1.15 (d, H_8), 1.79 (s, H_{15}), 1.95 (s, H-Ac), 3.07 (s, $H_{2\text{-}n}$), 3.22 (s, $H_{2\text{-}S}$), 3.49–3.89 (m, H_3, H_4, H_5, H_6, H_7), 4.10 (br, H_9), 4.77 (br, H_1), 5.02 (s, H_{10}), 5.91 (s, H_{11}), 6.38 (s, H_{12}), 6.81 (s, H_{13}), and 7.39 (s, H_{14}).

2.2.5. Synthesis of SCs–d4T (**3**)

The synthesis process of SCs–d4T was similar to that of Cs–d4T except sulphonation before cleavage of trimethylphenyl from Cs-Tr. After the Atherton–Todd reaction, the precipitate dissolved in dry N, N-dimethylformamide (DMF, 10 mL) was to added SO_3·Py complex (1.50 g), and heated to 80 °C under the nitrogen atmosphere [25]. After 2 h thermostatic reaction, the solution was poured into ethanol (200 mL) and the precipitate was adjusted to pH 9 with 1 N NaOH followed by drying. The product (0.10 g) was dissolved in formic acid (20 mL) at 60 °C for 50 min. After removing formic acid by rotary evaporation, the residue was dissolved in ethanol (200 mL) and the filtrate was dialyzed using dialysis membrane (MWCO: 3.60 kDa) against distilled water for 72 h followed by lyophilized to afford target product **3** (0.11 g, 69%). ^{1}H NMR (293 K, 2% CD_3COOD/D_2O, 400 MHz) δ 1.21 (d, H_8), 1.86 (s, H_{15}), 2.04 (s, H-Ac), 2.67 (s, $H_{2\text{-}n}$), 3.15, 3.27 (s, $H_{2\text{-}S}$), 3.81–4.39 (m, H_3, H_4, H_5, H_6, H_7, H_9), 4.93, 4.97 (br, H_1), 5.10 (s, H_{10}), 5.95 (s, H_{11}), 6.47 (s, H_{12}), 6.87 (s, H_{13}), and 7.50 (s, H_{14}); ^{13}C NMR (293 K, DCOOD, 150 MHz) δ 22.44, 23.29, 30.48, 56.35, 62.38, 70.59, 72.21, 76.61, 78.11, 80.37, 97.81, 101.54, 114.79, 116.69, 126.61, 128.32, 129.66, 160.39, 163.45, and 176.00.

2.2.6. Preparation of Cs–d4T/TPP nanoparticles

Cs–d4T/TPP nanoparticles were prepared based on the ionic gelation of Cs with TPP polyanions according to Calvo et al. [27,28]. Briefly, the Cs–d4T dissolved in 2% acetic aqueous solution was added dropwise by TPP dissolved in deionized water. After the continuous stirring for 1 h at room temperature, the suspension was dialyzed and passed through a syringe filter (pore size: 0.45 μm) prior to lyophilization to provide the target product, Cs–d4T/TPP nanoparticles.

2.3. Polymers characterization

The chemical structures and crystallinity of the synthesized SCs–d4T, Cs–d4T and Cs–sd4T were analyzed. ^{1}H NMR spectra of three polymers were carried out on a Bruker 400 MHz spectrometer (Bruker Co. USA) at 20 °C in D_2O and ^{13}C NMR spectrum of SCs–d4T was examined using a Bruker 150 MHz spectrometer at 20 °C in DCOOD.

FTIR spectra were recorded on a Nicolet FTIR spectrometer (Nicolet 5700, USA). A total of 2% (*w*/*w*) of sample was mixed with dry potassium bromide (KBr) and the spectra were recorded from 400 to 4000 cm^{-1}.

XRD spectra were performed on a Shimadzu X-ray diffractometer (Shimadzu XRD-6000, Japan). The angular range was recorded from 5° (2θ) to 40° with a scan speed of 5°/min and a step size of 0.02°.

The morphology of three polymers was examined on a field emission scanning electron microscope (SEM, Hitachi S4700, Japan) at an accelerating voltage of 20 kV. Prior to examination, a thin layer of gold was sputtered under vacuum onto the samples. Morphological characteristics of the Cs–d4T/TPP nanoparticles were observed by Transmission Electron Microscope (TEM, Hitachi, H-800, Japan) at an accelerating voltage of 200 kV. One drop of freshly made nanoparticles solution was placed on 300 mesh copper grids coated with carbon film, allowing to sitting until dried.

The sulfur content was detected on inductively-coupled plasma atomic emission spectroscopy (ICP-AES, JY ULTIMA, France). For the

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