Colloids and Surfaces B: Biointerfaces 148 (2016) 385–391

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Self-assemblies of 5′-cholesteryl-ethyl-phosphoryl zidovudine

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ARTICLE INFO

Article history:
Received 11 June 2016
Received in revised form 29 August 2016
Accepted 6 September 2016
Available online 8 September 2016

Keywords:
Anti-HIV
Langmuir monolayer
Macrophage
Phosphorylation
Prodrug
Self-assembly
Zidovudine

ABSTRACT

Anti-HIV prodrugs are recently focused on due to their ability of self-assembly, macrophage targeting, and enhanced antiviral effects. Here, an amphiphilic prodrug of zidovudine, an anti-HIV nucleoside analogue, 5′-cholesteryl-ethyl-phosphoryl zidovudine (CEPZ) was synthesized. CEPZ showed some unique physicochemical properties. The solubility of CEPZ in the noncompetitive solvents chloroform and tetrahydrofuran (THF) was very high based on the hydrogen bonds between zidovudine groups, though CEPZ was sparing soluble in alcohols and almost insoluble in water. The typical amphiphilic property of CEPZ was demonstrated according to the Langmuir monolayers at the air/water interface. The Log*P* of CEPZ was high to 13.78, indicating the high hydrophobicity of amphiphilic CEPZ similar to phospholipids. Homogenous and stable self-assemblies were formed with the mean size of 128.7 nm and the zeta potential of −35.4 mV after injecting the CEPZ-in-THF solution into water. Hydrophobic interaction between the cholesteryl moieties of CEPZ could drive molecular self-assembly and lead to the formation of spherical vesicles. CEPZ self-assemblies showed strong stability even under high temperature and gravity probably due to the high surface charge. CEPZ was very slowly degraded in neutral solutions (e.g., pH 7.4), but fast in acid solutions (e.g., pH 5.0) and some tissue homogenates. CEPZ was quickly eliminated from the circulation and distributed into the mononuclear phagocyte system (MPS) including the liver, spleen and lung after bolus intravenous administration of CEPZ self-assemblies to mice. The MPS targeting effect of CEPZ self-assemblies makes them become a promising self-assembled drug delivery system to eradicate the HIV hidden in the macrophages.

1. Introduction

Acquired immune deficiency syndrome (AIDS) is a severe disease that threatens the health of human beings, caused by the human immunodeficiency virus (HIV). The World Health Organization estimates that 340 million new cases of curable sexually transmitted infections occur annually. HIV-1 infection rates have reached pandemic levels with an estimated 34.2 million people living with HIV-1 in 2011 and 2.5 million people becoming newly infected with HIV in 2012 [1]. Two important reasons of AIDS prevalence are drug resistance and absence of macrophage targeting.

Antiviral nucleoside analogues have played a significant role in reducing the mortality and new infections from HIV/AIDS in recent years and are also predominant treatment. But their major drawback is that these drugs can induce the drug resistance of HIV and cause treatment failure finally. Upon entering virus-infected cells, these nucleosides are phosphorylated to their active triphosphates or diphosphates by nucleoside kinases. More importantly, the phosphorylation is a rate-limited process for exerting their anti-viral effect. At the same time, the kinase activity of HIV-infected patients may be only about 1/3 of the activity of healthy men. The activity decreases continuously following the long-term use of nucleosides, finally possibly leading to drug resistance. Therefore, the nucleoside phosphorylation strategy is regarded as an effective approach to overcome viral resistance bypassing the first kinase [2]. It is noteworthy that two antiviral nucleoside phosphonates are currently marketed, including adefovir dipivoxil for hepatitis B treatment and tenofovir disoproxil for HIV treatment. However, the bioavailability of the drugs is still low due to deficiency of macrophage targeting and high polarity.

The mononuclear phagocyte system (MPS) is defined as a population of cells derived from progenitor cells in the bone marrow, which differentiate to form blood monocytes, circulate in the blood, and then enter tissues to become resident tissue macrophages [3]. Macrophages are very efficient for internalizing particles. They participates in development, tissue remodeling, the immune response,

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http://dx.doi.org/10.1016/j.colsurfb.2016.09.009
0927-7765/

and inflammation [4]. Macrophages play a central role in inflammation and they act as reservoirs for microorganisms that are involved in deadly infectious diseases, so that macrophage targeting is important for antiviral therapy [5].

Macrophages are also demonstrated as a major reservoir of HIV [6,7]. Macrophages are not sensitive to hydrophilic cytotoxic drugs after infection [8], and macrophages could generate new HIV further. Therefore, macrophages become the host of HIV and carry them to many important organs, such as brain [9]. Current anti-HIV agents have little effects on macrophages resulting from the following reasons [10]. First, the anti-HIV drugs (mainly nucleoside reverse transcriptase inhibitors) are mostly of high polarity and distribute little into the cells. Second, the kinase activity in the macrophages is little and disable for the phosphorylation of nucleotides. Macrophages primarily remain in a resting, G1 state and undergo limited DNA synthesis. Therefore, it follows that cellular dNTP levels are significantly lower in macrophages compared to an activated and dividing cell. The last but not the most important, they lack macrophage targeting. Therefore, the modifications include hydrophobic modification, phosphorylation, and macrophage targeting. Nanoscale particles are preferred as the macrophage-targeted system due to the macrophage-specific recognition following opsonization in the circulation [11,12], so that anti-HIV agent-loaded nanoparticulate systems may be used for macrophage targeting. Antiviral drugs-loaded nanoparticles have been investigated, including dendrimers [13,14], solid lipid nanoparticles [15], nanosuspensions [16,17], nanoemulsions [18], ethosomes [19,20], and lipid nanocapsules [21].

Zidovudine (3′-azido-2′,3′-dideoxythymidine, AZT) is a potent inhibitor of HIV replication and the first clinically approved drug for AIDS therapy [22]. The major limitations of AZT chemotherapy are clinical toxicity that includes dose-related bone marrow suppression manifested as severe anemia and leucopenia, hepatic abnormalities, myopathy, limited brain uptake, a short half-life ($t_{1/2}$, ~1 h) in plasma and the rapid development of drug resistance, resulting in administration of higher doses for maintaining therapeutic drug levels in plasma, thus further leading to severe bone marrow toxicity [23].

Self-assembled drug delivery systems (SADDS) have been explored in our lab since 2001, defined as the self-assemblies of amphiphilic prodrugs, wherein three technologies involving prodrug, molecular self-assembly and nanotechnology are integrated [24]. Compared to traditional drug carriers, SADDS can deliver themselves *in vivo* with the unique advantages of high drug loads, no drug leakage, and controlled drug release at the targets. Some anti-HIV SADDSs has been prepared in our lab, including cholesteryl-succinyl didanosine (CSD), cholesteryl-adipoyl didanosine (CAD), cholesteryl-succinyl zidovudine (CSZ), cholesteryl-phosphonyl zidovudine (CPNZ), pentadecanedioyl dizidovudine (PDDZ), zidovudinephosphoryl-deoxycholyl didanosine (ZPDD) [25–28]. They likely self-assembled to nanoassemblies with different morphologies in water [29]. Some of the nanoassemblies (SADDSs) showed high in vitro anti-HIV effect and accumulation in the MPS (mainly including liver, lung, spleen) [26–28].

Based on the above analysis, an amphiphilic phosphorylated prodrug of AZT, 5′-cholesteryl-ethyl-phosphoryl zidovudine (CEPZ) with the cholesteryl moiety as the lipid tail and zidovudine as the polar head, was designed and synthesized for preparation of SADDS. It could self-assemble to nanoassemblies and naturally target macrophages. Upon getting into the cells, CEPZ would degrade into phosphoryl AZT and exert anti-HIV effect directly. In this paper, molecular self-assembly of the prodrug was explored and the formed self-assemblies were investigated on the *in vitro*/*in vivo* behavior, including characteristics, degradation, pharmacokinetics, tissue distribution, and macrophage targeting.

2. Materials and methods

2.1. Materials

The chemicals and their commercial sources were listed as follows: AZT, Shanghai Desano Science and Technology Co., Ltd., China; cholesterol and triethylamine, Beijing Chemical Reagent Company, China; phosphorus oxychloride, Beijing Coupling Reagent Company, China; fluorescein isothiocyanate (FITC), Yunhui Biotechnology Co., Ltd., Zibo, China; DMEM culture medium and fetal bovine serum, Hyclone, Thermo Scientific, Utah, USA. The other solvents were of analytical grade. All of the reactive solvents were dehydrated. Distilled water was always used otherwise specially indicated. UV spectra, NMR spectra, Infrared spectra, and ESI–MS were recorded on a Purkinje TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co., Ltd., Beijing, China), a JNM-ECA-400 NMR spectrometer (Japan), a Bio-Rad FTS-65A infrared ray (IR) spectrometer (USA), and a Finnigen LCQ Max mass spectrometer (USA), respectively.

2.2. Cells and animals

RAW264.7 cell line was as a gift from Beijing Normal University. Plasma from Kunming mice, Sprague-Dawley rats, and rhesus monkeys were prepared in our lab. Male Kunming mice were from the Laboratory Animal Center of Beijing Institute of Radiation Medicine (BIRM). Principles in good laboratory animal care were followed and animal experimentation was in compliance with the Guidelines for the Care and Use of Laboratory Animals in BIRM. The mice were sacrificed by euthanasia to remove tissues. Tissue homogenates of mice used in the experiments of chemical stability and tissue distribution were prepared in tissue/water (1:1, w/w).

2.3. Synthesis of CEPZ

5′-Cholesteryl-ethyl-phosphoryl zidovudine (CEPZ, $C_{39}H_{62}N_5O_7P$, MW 743.91) was synthesized as follows (Fig. 1). Phosphorus oxychloride (3 mmol) was dissolved in ethylether (29 ml) and then agitated for 30 min at 0 °C. Cholesterol (1 mmol) and triethylamine (TEA, 1.4 mmol) were dissolved in ethylether (10 ml) and then dropped into the above phosphorous oxychloride solution followed by agitation for 2 h. Solvents were removed under vacuum. The white powder of cholesteryl phosphorochloridate (CPC) was obtained. CPC (0.78 g, 1.5 mmol) and AZT (0.27 g, 1 mmol) were dissolved in dichloromethane (DCM, 10 ml) under dark. TEA (1.4 mmol) was dropped into the above solution and then agitated for 3 h. Most of the solvents were removed under vacuum. The residual liquid was thoroughly mixed with the saturated NaCl solution (200 ml). The organic phase was separated from the water phase after storage, and ethanol (20 ml) was added. The solution was slowly heated in an oil bath to reflux for about 30 min. The hot solution was filtrated and then ethanol was removed from the filtrate. The residue was purified on a silica gel column with the eluent of petroleum ether/acetone (1:0-3:1, v/v). A white-like solid of CEPZ was obtained. TLC: cyclohexane/acetone, 1.1:0.9, v/v, R_f = 0.70; UV (MeOH): λ_{max} = 266 nm; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.67 (6H, cholesteryl CH_3CHCH_3), 1.01-1.68 (32H, cholesteryl H), 1.95 (s, 3H, CH_3), 2.33-2.45 (m, 4H, 2′-CH), 4.03-4.36 (m, 6H, CH_3CH_2, 3′-CH, 5′-CH), 5.38 (1H, cholesteryl C—CH—CH_2), 6.13, 6.21 (t, 1H, 1′-CH, $J_{1',2'}$ = 6.4 Hz), 7.38 (d, 1H, 6-CH), 8.24 (s, 1H, 3-NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 12.45, 18.62-31.72, 35.59-42.19 (22C, cholesteryl), 37.37 (2′-C), 49.82 ($CCHCH_2CH_2$), 59.98 (3′-C), 64.28 (5′-C), 65.99 ($-OCH_2CH_3$), 77.66 (-$OCHCH_2CH_2$), 82.12, 82.27 (1′-C), 84.85 (4′-C), 111.46 (5-C), 123.39 (CH_2CCH), 135.29 (6-C), 138.43 (CH_2CCH), 150.29 (2-C), 163.68 (4-C); ^{31}P NMR (161.9 MHz, $CDCl_3$) δ ppm: 7.43 (1P, J_{P-H} = 702 Hz); IR (KBr):