International Journal of Pharmaceutics 478 (2015) 124–130

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical nanotechnology

Self-assembled drug delivery systems. Part 8: *In vitro*/*in vivo* studies of the nanoassemblies of cholesteryl-phosphonyl gemcitabine

Miao Li [a], Shuo Qi [a,b], Yiguang Jin [a,*], Junxing Dong [a,b]

[a] Department of Pharmaceutical Sciences, Beijing Institute of Radiation Medicine, 27 Taiping Road, Beijing 100850, China
[b] Beijing University of Technology, Beijing 100022, China

ARTICLE INFO

Article history:
Received 10 September 2014
Received in revised form 29 October 2014
Accepted 14 November 2014
Available online 15 November 2014

PubChem:
Gemcitabine (PubChem CID: 60750)

Keywords:
Gemcitabine
Molecular self-assembly
Nanoassemblies
Phosphonate
Prodrug

ABSTRACT

A lipid derivative of gemcitabine (Gem), cholesteryl-phosphonyl gemcitabine (CPNG) was synthesized in this study. The amphiphilicity of CPNG was confirmed using a Langmuir monolayer method. Nanoassemblies were formed when the mixture of CPNG and a long-circulating material, CHS-PEG$_{1500}$ (9:1, mol/mol) were injected into water. The nanoassemblies could be spherical vesicles according to the transmission electron microscopic images. Their mean size was 71.1 nm and the zeta potential was −17.6 mV. CPNG maintained stable in the weakly acidic and neutral environments although mouse plasma quickly degraded CPNG. The cytotoxicity of the nanoassemblies was 3–6 folds of Gem's cytotoxicity on five human cancer cell lines including 95C, 95D, A549, SW620, PANC-1 probably because of the phosphonyl substitution and amphiphilicity of CPNG. CPNG mainly distributed into the mononuclear macrophage system (including liver and spleen) after bolus intravenous administration of the nanoassemblies into mice though the expected significant long-circulating effect was not shown. The nanoassemblies with the high dose of CPNG showed the statistically higher *in vivo* anticancer effect than Gem. This study indicates that the *N*-substituted lipid derivative of Gem and the true long-circulating function are necessary for preparing a successful nanoassembly of Gem.

1. Introduction

Self-assembled drug delivery systems (SADDSs) have been developed in our lab for more than one decades, which are defined the nanoassemblies of amphiphilic prodrugs (Jin et al., 2006). SADDSs occupy some advantages over traditional drug nanocarriers, such as high stability, high drug loads and no drug leakage (Jin et al., 2009). Some SADDSs have progresses in the fields of antiviral and anticancer therapy (Du et al., 2014; Jin et al., 2012b, 2010, 2011, 2009, 2013). In our previous studies, the sizes of SADDSs are in the range of 50–200 nm so that SADDSs are suitable as tumor-targeted nanomedicines based on the enhanced permeability and retention (EPR) effect (Taurin et al., 2012).

Gemcitabine (2′-deoxy-2′,2′-difluorocytidine, Gem), an analog of deoxycytidine, is a potent anticancer agent for many solid tumors, such as non-small-cell lung carcinoma, pancreatic cancer and breast cancer. The cellular internalization of Gem depends on the nucleoside transporters on cellular membranes though the drug efflux proteins could pump them out. Gem is phosphorylated to its monophosphate (dFdCMP) in the cytoplasm, and sequentially phosphorylated to its diphosphate (dFdCDP) and triphosphate (dFdCTP) that inhibits the DNA synthesis of cancer cells. However, cancer cell resistance to Gem would be raised due to genetic mutation, involving down-regulation of nucleoside influx transporters and up-regulation of drug efflux proteins. Furthermore, the kinase activity would be weakened and the nucleoside activation process is hindered (Hung et al., 2012). Additionally, Gem is rapidly metabolized to 2′,2′-difluorodeoxyuridine by the deoxycytidine deaminase in the blood and then excreted by the kidney. In order to overcome Gem resistance and improve tumor targeting, the methods of lipophilic Gem prodrugs and Gem-loaded nanoparticles or liposomes were used (Paolino et al., 2010; Sloat et al., 2011; Zhang et al., 2013). The methods could overcome the nucleoside transporter barriers and the deaminase inactivation. However, the nucleoside activation problem induced by the absence of kinase remain.

An amphiphilic prodrug of Gem, *N*-octadecanoyl gemcitabine (NOG) was prepared in our lab. NOG formed the nanoscale self-assemblies that showed good anticancer effect (Jin et al., 2012b). Additionally, another amphiphilic prodrug of anti-HIV nucleoside analog zidovudine, cholesteryl-phosphonyl zidovudine (CPNZ) was prepared. The self-assemblies of CPNZ showed good anti-HIV effect

* Corresponding author. Tel.: +86 10 88215159; fax: +86 10 68214653.
E-mail address: jinyg@bmi.ac.cn (Y. Jin).

http://dx.doi.org/10.1016/j.ijpharm.2014.11.033
0378-5173/

due to the nanoscale property and the presence of phosphonyl group (Jin et al., 2009). In this study, an amphiphilic and phosphonyl prodrug of Gem, cholesteryl-phosphonyl gemcitabine (CPNG) was prepared. The prodrug was expected to have good self-assembling ability to form nanoassemblies and show high anticancer effect based on the EPR effect and the short phosphorylation process in cells. A poly(ethylene glycol) (PEG) derivative of cholesterol, cholesteryl succinyl poly(ethylene glycol) 1500 (CHS-PEG_{1500}), was mixed into the CPNG nanoassemblies to avoid the opsonization in the circulation and the sequential macrophage recognition. The amphiphilicity of CPNG and the pharmacokinetics and tissue distribution of the nanoassemblies were investigated. The *in vitro* and *in vivo* anticancer effects of the nanoassemblies were explored.

2. Materials and methods

2.1. Materials

Gem was purchased from Beijing Chemsynlab Pharmaceutical Science & Technology Co., Ltd. China. CHS-PEG_{1500} was synthesized in our lab according to the literature (Xu et al., 2008). Organic solvents were of analytical grade. The other chemicals were of reagent grade. Purified water was prepared with Heal Force Super NW Water System (Shanghai Canrex Analytic Instrument Co., Ltd., China) and always used unless otherwise indicated. Ultraviolet–visible (UV–Vis) spectra, infra-red (IR) spectra, ^{1}H nuclear magnetic resonance (NMR) (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Shimadzu UV-2501PC spectrophotometer, a Bio-Rad FTS-65A infrared ray (IR) spectrometer, and a JNM-ECA-400 NMR spectrometer, respectively. ESI-MS were recorded on a Thermo LCQ Advantage mass spectrometer.

2.2. Synthesis of CPNG

The synthesis of CPNG was referred to the literature (Xiao et al., 2003). One pot reaction was used (Fig. 1). Cholesterol (1.94 g, 5 mmol) was dissolved in anhydrous pyridine (10 ml). Diphenyl phosphonate (DPP, 1.2 ml, 5 mmol) and triethylamine (0.72 ml, 5 mmol) were added to the above solution and stirred for 12 h at 70 °C to obtain cholesteryl-phosphonyl benzene. Gem (1.32 g, 5 mmol) was dissolved in anhydrous pyridine (10 ml), and the above reaction solution was added and agitated for 6 h at room temperature. Most of the solvent was removed under vacuum to leave a viscous liquid. The liquid was purified on a silica gel column with an eluent of dichloromethane/methanol (20:1 to 10:1, v/v). The filtrate was evaporated under vacuum to obtain a white powder of CPNG ($C_{36}H_{56}F_2N_3O_6P$, MW = 695.8). TLC: dichloromethane/methanol, 10:1, v/v, R_f = 0.6. UV–Vis (tetrahydrofuran, THF): λ_{max} = 245 nm. ^{1}H NMR (DMSO-d_6, 400 MHz) δ (ppm): 0.646 (3H, C—CH_3), 0.83–0.99 (40H, —cholesterol), 1.35 (2H, CH—CH_2—C), 2.4 (2H, —CH_2—CH), 4.01 (1H, 3′—H), 5.318 (1H, CH_2—CH—C), 5.77 (1H, 3′—OH), 6.19 (1H, 1′—H), 6.46 (1H, P—H), 7.44–7.46 (2H, NH_2), 7.52 (1H, 5—H), 7.83 (1H, 6—H). ^{31}P NMR (DMSO-d_6, 100 MHz) δ (ppm): 8.089, 8.625. MS ESI-MS(+): 696.07 $(M+H)^+$.

2.3. Langmuir monolayers of CPNG

The surface pressure–molecular area (π–A) isotherm of CPNG was measured using a Minitrough film balance (KSV, Finland) equipped with the dual barriers and a Pt Wilhelmy plate-sensing device. The Teflon trough had a width of 75 mm and the area of 24,300 mm^2. The subphase was the phosphate buffered solutions (PBS, 150 mM) at the various pH (7.4, 6.8, 4.0). The experiment was performed at 25 °C. A CPNG solution (0.4 mg/ml, 25 μl) in chloroform was prepared and deposited onto the subphase using a microsyringe. Compression was initiated after a delay of 15 min to allow chloroform evaporation. The compression rate was 10 mm/min.

2.4. Preparation and characterization of nanoassemblies

CPNG alone or a mixture of CPNG/CHS-PEG_{1500} (9:1, mol/mol) was dissolved in THF to obtain the solutions containing 2 mg/ml CPNG. The solutions were slowly injected into the water under ultrasound with a microsyringe at room temperature until a slightly turbid suspension was obtained. The suspension was evaporated to remove THF and further obtain a concentrated suspension. In this study, the name of the nanoassemblies generally represented the CPNG/CHS-PEG_{1500} nanoassemblies unless especially indicated otherwise. When CPNG alone was used, the name of the CPNG alone nanoassemblies was used.

Nanoassemblies were observed on a Hitachi H-7650 transmission electron microscope (TEM). The samples were negatively stained with a sodium phosphotungstate solution (pH 6.5). Size distribution and zeta potentials of nanoassemblies were measured at 25 °C on Zetasizer Nano ZS (Malvern, UK) using the dynamic

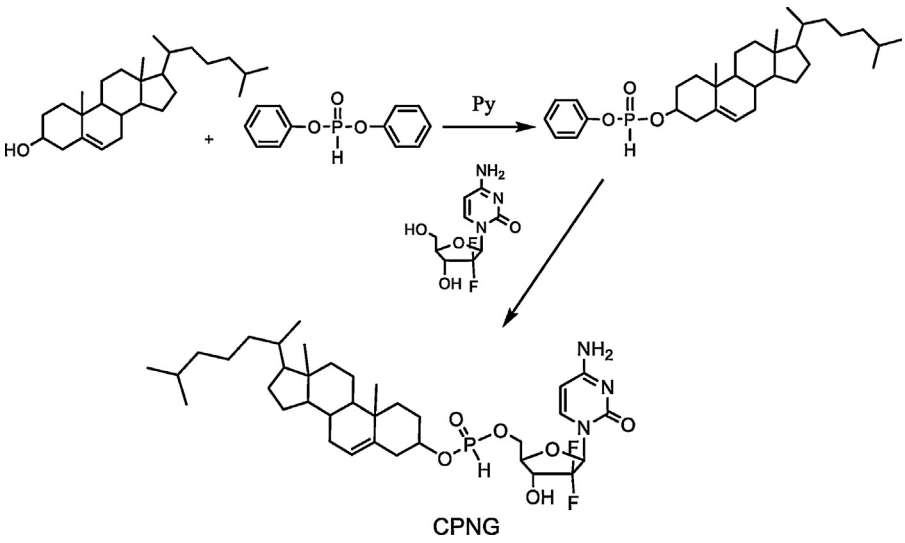

Fig. 1. Synthetic route of CPNG.