International Journal of Pharmaceutics 550 (2018) 79–88

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Poly (L-glutamic acid)-*g*-methoxy poly (ethylene glycol)-gemcitabine conjugate improves the anticancer efficacy of gemcitabine

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ARTICLE INFO

Keywords:
Gemcitabine
Nanoparticle
Pharmacokinetics
Biodistribution
Glutamic acid

ABSTRACT

Gemcitabine is widely used for anticancer therapy. However, its short blood circulation time and poor stability greatly impair its application. To solve this problem, we prepared a poly (L-glutamic acid)-*g*-methoxy poly (ethylene glycol)-gemcitabine conjugate (L-Gem) with a 14.3 wt% drug-loading content. L-Gem showed concentration- and time-dependent cytotoxicity towards 4T1, LLC, MIA PaCa-2 and A2780 *in vitro*. Pharmacokinetic and biodistribution studies indicated that L-Gem had remarkably enhanced blood stability, prolonged blood circulation time and greatly improved selective tumor distribution compared with free gemcitabine. The area under the concentration–time curve from zero to infinity [$AUC_{(0-\infty)}$] of L-Gem in plasma was 43-fold higher than that of free gemcitabine. The $AUC_{(0-\infty)}$ of the inactive metabolite, 2′-deoxy-2′,2′-difluorouridine in the L-Gem group was ~20% of that observed in the free gemcitabine group. The drug tumor accumulation ratio in the L-Gem group relative to the free gemcitabine group was 9.9 at 36 h, while the tumor AUC ratio was 15.8. Testing on Balb/C mice bearing the 4T1 tumor further demonstrated that L-Gem had significantly higher anticancer efficacy than free gemcitabine *in vivo*. These findings indicated that L-Gem has great potential for cancer treatment.

1. Introduction

Gemcitabine (dFdC), a deoxycytidine antimetabolite (Hingorani et al., 2016), is used against a wide range of solid tumors including those involving the pancreas (Dimcevski et al., 2016; Hessmann et al., 2017; Manji et al., 2017), breast, lung (non-small cell) (Hirsch et al., 2016), ovary and bladder (Heinemann, 2001; Reid et al., 2004). However, the plasma half-life of gemcitabine following intravenous administration is very short, at 8–17 min in humans (Abbruzzese et al., 1991b; Reid et al., 2004) and 9 min in mice (Moog et al., 2002). In addition, gemcitabine is unstable in blood (Croissant et al., 2016), with more than 91% metabolized directly to inactive 2′-deoxy-2′,2′-difluorouridine (dFdU) by deoxycytidine kinase and cytidine deaminase (Bouffard et al., 1993). This rapid inactivation significantly impairs the anticancer efficacy of gemcitabine (Richards et al., 2017). Indeed, high doses of gemcitabine are necessary to obtain a desired therapeutic response (Bastiancich et al., 2017). However, this simultaneously results in a variety of serious side effects such as myelosuppression, vomiting and nausea, elevated transaminases, hair loss, and hematuria and proteinuria (Abbruzzese et al., 1991a; Reddy et al., 2008). Therefore, it is important to prolong the blood circulation time of gemcitabine and improve its stability in the blood circulation system (Han et al., 2016; Liu et al., 2016).

To overcome these deficiencies in gemcitabine pharmacokinetics, several delivery systems have been developed. Two research groups have described PEG–gemcitabine prodrugs with significantly prolonged blood circulation time (Pasut et al., 2008; Vandana and Sahoo, 2010). PEGylation markedly improved the cytotoxicity and apoptosis-inducing activity of gemcitabine against pancreatic cancer cell lines (MIA PaCa 2 and PANC 1) (Jaidev et al., 2017). However, the low drug loading content of the PEG–gemcitabine prodrugs (0.98–6.39 wt%) limited their clinical applicability. Kiew et al. (2012, 2010) described a poly-L-glutamic acid-gemcitabine conjugate with dose-dependent cytotoxicity in several cancer cell lines and remarkable antitumor efficacy (Kiew et al., 2012). However, the stability and long blood circulation time of this conjugate have not been confirmed by pharmacokinetic and tissue

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https://doi.org/10.1016/j.ijpharm.2018.08.037
Received 11 March 2018; Received in revised form 28 July 2018; Accepted 18 August 2018
Available online 20 August 2018

distribution studies *in vivo*. Another approach to improve the biopharmaceutical properties of gemcitabine is to covalently couple its 4-amino group to squalenoyl to produce gemcitabine-squalene (Réjiba et al., 2011). Squalene is abundant in nature and well-tolerated after intravenous and oral administration (Reddy and Couvreur, 2009). Conjugation with squalene could protect the nucleoside of gemcitabine from the deamination process (Castelli et al., 2006). The gemcitabine-squalene conjugate (SQdFdC) has been shown to have greater anticancer efficacy than gemcitabine, when administered in an identical dosing schedule (Fiorini et al., 2015). However, the plasma area under the concentration-time curve (AUC) of dFdU in SQdFdC-treated mice was 2-fold greater than in the free gemcitabine group (Reddy et al., 2008). This indicated that SQdFdC stability in blood could be improved further.

Recently, we developed poly (L-glutamic acid)-graft-methoxy poly (ethylene glycol) (PLG-*g*-mPEG) as a nanocarrier for delivery of cisplatin, patupilone and combretastatin A4 (Liu et al., 2017; Shi et al., 2015; Song et al., 2016a, 2016b; Yan et al., 2017; Yu et al., 2016, 2015). Considering that PLG-*g*-mPEG has poly (L-glutamic acid) and poly (ethylene glycol) segments, it is rational to make a gemcitabine-PLG-*g*-mPEG conjugate (L-Gem). The PEG segments could give the obtained conjugate superior water solubility and longevity in blood circulation. The poly (L-glutamic acid) segments could graft a large amount of gemcitabine by covalently coupling carboxyl to the 4-amino group of gemcitabine. This could enable the conjugate to have high drug loading content, improved blood stability and long blood circulation time (Garrido-Laguna and Hidalgo, 2015; Li et al., 2016).

Thus, we prepared L-Gem and evaluated its use for cancer treatment. The drug release profile, cytotoxicity, pharmacokinetics and biodistribution of L-Gem were assessed and compared with free gemcitabine. The metabolic kinetics to produce dFdU from L-Gem and free gemcitabine were also investigated and compared.

2. Materials and methods

2.1. Materials

PLG-*g*-mPEG, with an average of 160 L-glutamic acid repeating units and 8.3 mPEG5K chains, was synthesized as described previously (Yu et al., 2015). Gemcitabine was purchased from Yangzhou Huihong Chemical Co. Ltd., China. N,N′-dimethylformamide (DMF) was stored over CaH_2 for 3 days and distilled under vacuum prior to use. N-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3 ethylcarbodiimide hydrochloride (EDC·HCl) were supplied by Aladdin Reagent Co., Ltd., China. All other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd., China and used as received.

2.2. Characterizations

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on an AV-300 or AV-400 spectrometer (Bruker, Germany) in trifluoroacetic acid-d or a sodium deuteroxide/deuterium oxide solution at room temperature. Gel permeation chromatography (GPC) measurements were conducted on a water GPC system (Waters Ultrahydrogel Linear column, 1515 HPLC pump with 2414 Refractive Index detector) using phosphate buffer (0.2 M, pH 7.4) as eluent (flow rate: 1 mL/min, 25 °C, and polyethylene glycol as the standard). Dynamic laser scattering (DLS) measurements were performed on a Wyatt QELS instrument with a vertically polarized He–Ne laser (DAWN EOS, Wyatt Technology, USA). The scattering angle was fixed at 90°. High performance liquid chromatography-mass spectrometry (HPLC-MS) was performed on a Triple TOF 5600 mass spectrometer with electrospray ionization source, Analyst TF data processing software, Agilent 1100 liquid chromatography system, and Shimadzu UFLC SIL-20A XR column compartment.

2.3. Preparation of L-Gem

L-Gem was synthesized through amidation between PLG-*g*-mPEG and gemcitabine in the presence of NHS and EDC·HCl. In brief, 500 mg of PLG-*g*-mPEG was dissolved in 10 mL of DMF. The temperature was kept at 0 °C and 1.5 mmol (175 mg) of NHS was added to the solution. The mixture was gradually brought to room temperature and stirred overnight. EDC·HCl [1.5 mmol (291 mg) dissolved in 5.0 mL DMF], gemcitabine [1 mmol (263 mg)] and triethylamine [1.5 mmol (154 mg) dissolved in 5.0 mL DMF] were then added into the reaction mixture. The mixture was then stirred at 50 °C under nitrogen protection. After 72 h, the reaction mixture was precipitated into excess cold ether to give the crude product, which was dissolved in phosphate buffer solution (pH 6.8) and dialysed against deionized water for 72 h (MWCO = 7000 Da). L-Gem was obtained after freeze-drying.

For evaluating the loading content and efficiency of gemcitabine, 10 mg L-Gem was dissolved in 10 mL NaOH solution (4 mg/mL) for 4 h. The concentration of gemcitabine released was measured by HPLC at 273 nm. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following formulae:

$$\text{DLC (wt\%)} = (\text{weight of loaded gemcitabine}/\text{weight of L - Gem}) \times 100\%$$

$$\text{DLC (wt\%)} = (\text{weight of loaded gemcitabine} / \text{weight of feeding gemcitabine}) \times 100\%$$

2.4. Cell cultures

Murine breast cancer cells (4T1), Lewis lung cancer cells (LLC), human pancreatic carcinoma cells (MIA PaCa-2) and human ovarian carcinoma cells (A2780) were cultured at 37 °C in a 5% CO_2 atmosphere. Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum, penicillin (50 U mL^{-1}) and streptomycin (50 U mL^{-1}) was used as the culture medium.

2.5. Animals

Balb/C mice (female, average body weight 18 g, 6–8 weeks old) and Sprague Dawley (SD) rats (male, average body weight 250 g) were obtained from Beijing Huafukang Biological Technology Co. Ltd. (HFK Bioscience, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Animal Care and Use Committee of Jilin University.

2.6. Drug release in vitro

The release of gemcitabine from L-Gem in phosphate-buffered saline (PBS) (pH 7.4 or 5.5) was evaluated by dialysis. Typically, 5.0 mg of L-Gem in 5 mL of PBS (pH 7.4 or 5.5) was added to a dialysis tube (MWCO 7000 Da), which was then incubated in 40 mL PBS buffer (pH 7.4 or 5.5) at 37 °C with a shaking rate of 100 rpm. At selected time intervals, 2 mL of incubated solution was taken out and replaced with an equal volume of fresh media.

The release of gemcitabine from L-Gem in Chym opapain b was also evaluated by dialysis. L-Gem 5.0 mg in 5 mL of water containing 0.2 mg Chym opapain b (800 U) was added to a dialysis tube (MWCO 7000 Da) (Zhang et al., 2017), which was then incubated in 40 mL PBS (pH 7.4) at 37 °C with a shaking rate of 100 rpm. At selected time intervals, 2 mL of incubated solution was taken out and replaced with an equal volume of fresh media.

Gemcitabine content was determined by HPLC. The HPLC system consisted of a reverse-phase C-18 column (Symmetry), with a mobile phase of acetonitrile and water (80:20 v/v) pumped at a flow rate of 1.0 mL/min at 25 °C. The column effluent was detected at 273 nm with