European Journal of Pharmaceutical Sciences 79 (2015) 20–26

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

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

Chlorambucil gemcitabine conjugate nanomedicine for cancer therapy

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ARTICLE INFO

Article history:
Received 9 June 2015
Received in revised form 16 August 2015
Accepted 25 August 2015
Available online 2 September 2015

Keywords:
Chlorambucil
Gemcitabine
Conjugate
Nanodrug
Nanoparticle

ABSTRACT

Self-assembly of anticancer small molecules into nanostructures may represent an attractive approach to improve the treatment of experimental solid tumors. As a proof of concept, we designed and synthesized the conjugate prodrug of hydrophilic gemcitabine by its covalent coupling to hydrophobic chlorambucil via a hydrolyzable ester linkage. The resulting amphiphilic conjugates self-assembled into nanoparticles in water and exhibited significant anticancer activity in vitro against a variety of human cancer cells. In vivo anticancer activity of these nanoparticles has been tested on subcutaneous grafted SMMC-7721 hepatocellular carcinoma model. Such chlorambucil gemcitabine conjugate nanomedicine should have potential applications in cancer therapy.

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1. Introduction

Nanocarriers have attracted extensive research interest over the last few decades due to their potential applications in drug delivery (Anselmo and Mitragotri, 2014; Brannon-Peppas and Blanchette, 2004; Dawidczyk et al., 2014). In an attempt to reduce side effects of anticancer drug and to improve therapeutic index, various nanocarriers have been developed, including liposomes (Hertlein et al., 2010; Roth et al., 2007), dendrimers (Wang et al., 2015; Wu et al., 2014), inorganic nanoparticles and polymer nanoparticles (Ashley et al., 2011; Chou et al., 2014; Hung et al., 2014; Wang et al., 2012). While these approaches can be effective, there are concerns regarding the biodegradability and potential long-term toxicity arising from the synthetic nanoscale carriers (Duncan, 2006; Nel et al., 2009).

In drug high-throughput virtual screening field, false positives plague screening libraries and hit lists partly due to promiscuous inhibition by colloid-like aggregates of the small molecule (Coan and Shoichet, 2008; McGovern et al., 2002; McGovern et al., 2003; Seidler et al., 2003). However, the nanostructure forming ability of these molecules also endow themselves application potential in nanomedicine field (Feng et al., 2008). Biocompatible nanostructures formed by self-assembling of amphiphilic anticancer molecules show remarkable therapeutic potential as they have a high drug loading capacity and minimized toxicity associated with using additional synthetic nanocarriers (Lock et al., 2013). Many systems have been developed during the last decade, including polyethylene glycol modification (Shen et al., 2010; Xu et al., 2014), polypeptide modification (Cheetham et al., 2013; MacKay et al., 2009), squalenoylation and amphiphilic drug–drug conjugate concept (Couvreur et al., 2006; Desmaele et al., 2012; Huang et al., 2014; Maksimenko et al., 2014b).

Gemcitabine (2′,2′-difluorodeoxycytidine) is a potent anticancer nucleoside analogue active against various solid tumors. Since this compound is too hydrophilic to passively cross the plasma membrane, its cellular internalization is strongly dependent on the nucleoside transporter hENT1, whose down regulation leads to drug resistance. Gemcitabine loaded nanoparticles, however, can partially bypass the hENT1 as they are internalized through endocytosis (Maksimenko et al., 2014a; Pili et al., 2010). Chlorambucil (4-[*bis*(2-chloroethyl) amino]benzenebutanoic acid) is a lipophilic DNA alkylating agent, its toxic side effect is a major limitation in achieving the optimum therapeutic performance. A promising approach to minimize the toxic effect is delivery of chlorambucil in tumor-targeted nanocarriers (Ganta et al., 2008; Jana et al., 2014).

In this study, chlorambucil and gemcitabine, two commonly used anticancer chemotherapy drugs with completely different mechanisms of action, were chosen to form amphiphilic conjugate (Fig. 1). Chlorambucil gemcitabine conjugate can self-assemble into nanoparticles which probably aggregate in tumor site via enhanced permeability and retention effect (EPR effect). We investigated in vitro and in vivo anticancer efficacy of chlorambucil gemcitabine conjugate nanoparticles. The aim of our study is to establish the validity of using chlorambucil gemcitabine conjugate nanoparticles as therapeutics for cancer.

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http://dx.doi.org/10.1016/j.ejps.2015.08.013
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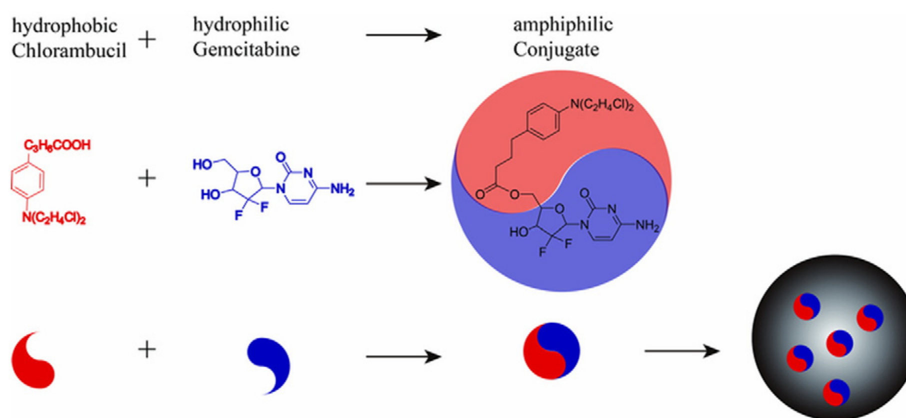

Fig. 1. Design of chlorambucil gemcitabine conjugate nanoparticles.

2. Materials and methods

2.1. Materials and animals

Chlorambucil was obtained from J&K Chemical Ltd. (Shanghai, China). Gemcitabine was purchased from Demo Medical Tech Co., Ltd. (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dicyclohexylcarbodiimide (DCC) were purchased from Shanghai Jingchun Reagent Co., Ltd. (Shanghai, China). Trifluoroacetic acid (TFA), di-*tert*-butyl dicarbonate (DBDC) and 4-dimethylaminopyridine (DMAP) were supplied by Energy Chemical Ltd. (Shanghai, China). 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine iodide (DiR) was obtained from Biotium, Inc. (Hayward, CA). *N*,*N*-dimethyl formamide (DMF), dichloromethane (DCM), petroleum ether (PE), ethyl acetate (EA) and other chemicals in analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Human hepatoma cell line SMMC-7721, gastric cancer cell line SGC-7901, glioblastoma cell line U87 and cervical cancer cell line Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) culture medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 mg/mL streptomycin and 100 U/mL penicillin), Human breast adenocarcinoma cell line MCF-7 cells were maintained in DMEM with 20% FBS and 1% antibiotic solution. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

Athymic female mice (BALB/c strain) (4–6 weeks, 15–17 g weight) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and maintained in an SPF environment under controlled temperature (24 °C). Animal experiments were performed in accordance with the CAPN (China Animal Protection Law) and protocols were approved by the Shanghai JiaoTong University Animal Care and Use Committee.

2.2. Synthesis and characterization of chlorambucil gemcitabine conjugate

Gemcitabine was protected as previously described. To a solution of gemcitabine (80 mg, 0.3 mmol) in 0.8 mL of DMF was added DBDC (660 mg, 3.0 mmol). The reaction mixture was maintained at 37 °C for 4 h, and was then subjected to flash chromatography (PE EA 1:1 to EA) to give 4-*N*-3′-*O*-bis(*tert*-Butoxycarbonyl)gemcitabine (106 mg, 75%).

Chlorambucil (120 mg, 0.39 mmol) and DCC (98 mg, 0.48 mmol) were dissolved in 6 mL of chloroform. The mixture was stirred for 1 h, and then added to a solution of 4-*N*-3′-*O*-bis(*tert*-butoxycarbonyl) gemcitabine (122 mg, 0.26 mmol) and DMAP (32 mg, 0.26 mmol) in 3 mL of chloroform; the resulting mixture was stirred for 48 h at room temperature. The white solids were removed by filtration. The filtrate was concentrated and purified by flash chromatography, and then dissolved in DCM and treated with 0.5 mL TFA; the resulting mixture was stirred overnight and subjected to flash chromatography (PE EA 1:1 to EA) to give compound **1** (61 mg, 42%). Compound **1** ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.81 (m, 2H), 2.47 (m, 4H), 3.50 (brs, 1H), 3.70 (m, 9H), 4.15 (m, 1H), 5.33 (m, 2H), 5.86 (d, J = 7.6 Hz, 1H), 6.24 (t, J = 8.8 Hz, 1H), 6.67 (d, J = 8.4 Hz, 2H), 7.03 (d, J = 8.4 Hz, 2H), 7.56 (s, 1H), 7.67 (s, 1H), 7.71 (d, J = 7.6 Hz, 1H). The abbreviations s, d, t, m, brs stand for singlet, doublet, triplet, multiplet and broad singlet. ^{13}C NMR (400 MHz, DMSO-d_6): δ 171.6, 170.3, 165.2, 153.9, 144.6, 141.5, 129.3, 129.3, 112.0, 94.83, 78.55, 69.95, 69.75, 69.48, 59.75, 59.20, 52.23, 41.16, 33.08, 32.42, 26.47, 20.75, 14.08 (Supporting Information, Fig. S1 and Fig. S2). ESI MS *m*/*z* Calcd: 548.14, found: 549.15 for $[M + H]^+$.

2.3. Preparation and characterization of the chlorambucil gemcitabine conjugate nanoparticles.

Chlorambucil gemcitabine conjugate nanoparticles were prepared using the nanoprecipitation method. A solution of compound **1** (1 mL at 1 mg/mL) in DCM was mixed with 1 mL of distilled water; the mixture was sonicated for 1 min using a probe sonicator. DCM was completely evaporated under a vacuum using a Rotavapor at room temperature to obtain an aqueous suspension of compound **1** nanoparticles (CG-1). By using similar nanoprecipitation technique, RhoB or DiR loaded CG-1 was also prepared. The average size and zeta potential of nanoparticles were measured by dynamic light scattering (DLS) (ZetaSizer Nano ZS90, Malvern Instrument, USA). The diameter mean values were calculated from the measurements performed in triplicate. The morphology of nanoparticles was observed using transmission electron microscopy (TEM) (Tecnai G2 Spirit Biotwin, FEI, USA) without negative staining, and one drop of nanoparticles solution (0.2 mg/mL) was placed on a copper grid and air-dried before measurements.

2.4. Cytotoxicity assay

In vitro cytotoxicity of CG-1 was evaluated by MTT assay with SMMC-7721, Hela, MCF-7 SGC-7901 and U87 cells. Cells were seeded in 96-well plates at a density of 2000 cells per well in 100 μL of complete culture medium and incubated for 18 h attachment; the medium was then replaced by 200 μL of culture medium containing chlorambucil, gemcitabine and CG-1 at various concentrations ranging from 0.1 to 30 μM. After 48 h, the medium was replaced with 100 μL of 0.5 mg/mL MTT in culture medium and 3 h later the MTT solution was replaced with 150 μL of DMSO solution. The plates were shaken at 100 rpm for 15 min at 37 °C before the relative color intensity was measured at the analysis wavelength of 490 nm using a powerwave XS microplate reader (Bio-Tek, USA). Cells without drug treatment were used as the control and all experiments were carried out with four replicates.

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