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Long circulation and cytotoxicity of PEGylated gemcitabine and its potential for the treatment of pancreatic cancer

Mallaredy Vandana, Sanjeeb K. Sahoo*

Laboratory of Nanomedicine, Institute of Life Sciences, Nalco Square, Chandrasekarpur, Bhubaneswar, Orissa, India

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ABSTRACT

Gemcitabine [2′, 2′-difluoro-2′-deoxycytidine (dFdC)] is a low molecular weight, deoxycytidine analog inhibiting cellular DNA synthesis. Currently, it is the frontline drug approved by Food and Drug Administration (FDA) for the treatment of pancreatic cancer. However, efforts to use gemcitabine as an anti-cancer agent have been limited by its short circulation time and rapid metabolism that reflects in low tumor uptake and intracellular action. Polymer–drug conjugates, in this regard have spawned an approach to improve the cytotoxicity efficiency and bioavailability of gemcitabine by chemical modification. The present study describes the synthesis of a water soluble formulation of PEGylated gemcitabine characterized by FT IR, ^{1}H NMR and RP-HPLC chromatography. The PEGylated gemcitabine has a prolonged circulation time in plasma as studied in an animal model. This eventually caused a marked improvement in the cytotoxicity and apoptosis-inducing activity in pancreatic cancer cell lines (MIA PaCa 2 and PANC 1). Hence, these findings demonstrate the PEGylated gemcitabine is a desirable approach for therapy by intravenous administration. Successful clinical application of this approach can significantly contribute to the treatment of pancreatic cancer.

1. Introduction

Pancreatic adenocarcinoma is a cancer notorious for its late presentation, early and aggressive local invasion with metastatic potential and poor outcome [1]. It is complicated to treat with an overall 5-year survival rate of <5% and a median overall survival of <6 months for the patients [2]. Surgical resection is the only potentially curative modality for treatment; however, with extremely poor prognosis, the need for adjuvant therapies is paramount. Gemcitabine is currently the first line drug available in the market, as one of the best effective chemotherapeutic agent for locally advanced and metastatic pancreatic cancer [3]. It accomplishes its anticancerous activity by incorporation into both DNA and RNA, resulting in masked DNA termination and inhibition of DNA synthase activity [4]. Gemcitabine is known to be a cell cycle-dependent (S-phase-specific) deoxycytidine analogue of the antimetabolite class. It is a prodrug which requires cellular uptake and intracellular phosphorylation [5]. Gemcitabine is transported into the cells by human equilibrative nucleoside transporter-1 (hENT1) via sodium-independent (equilibrative) mechanism and phosphorylated to monophosphate derivative (dFdCMP) by deoxycytidine kinase (dCK), which is then converted to di- and tri-phosphate derivatives (dFdCDP and dFdCTP, respectively) inside the cell. The emerging evidence demonstrates that incorporation of gemcitabine derivatives into DNA is critical for gemcitabine to inhibit cell replication and induce apoptosis in cancer cells [5,6]. dFdCDP inhibits ribonucleotide reductase (RR) liable for catalyzing the reaction that generates the deoxyribonucleotides required for synthesis and repair of DNA. dFdCTP incorporates into DNA as a false nucleoside, inhibiting DNA polymerase and thereby preventing the detection and repair of DNA repairing enzymes (masked chain termination) [7–9].

Although the above described molecular events eventually contribute to the efficiency of gemcitabine for pancreatic cancer treatment, the drug possesses certain drawbacks that are related to its unfavorable pharmacokinetic properties. Like most of the low molecular weight drugs, gemcitabine has very short plasma circulation time [3]. It gets rapidly cleared from the body through renal excretion upon enzymatic conversion to the inactive and more soluble metabolite 2′, 2′-difluorodeoxyuridine (dFdU) by deoxycytidine deaminase (dCDA) expressed in blood, liver, kidney and various tumor tissues [10–12]. Thus, a frequent administration scheduled at high doses is required, in turn leading to myelosuppression, high levels of hepatoxicity & renal toxicity along with

* Corresponding author. Tel.: +91 674 2302094; fax: +91 674 2300728.
E-mail address: sanjeebsahoo2005@gmail.com (S.K. Sahoo).

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toxicity towards other tissues or organs [12,13]. The austerity of such treatment causes the patient to greatly weaken and the cancer, which may have seemed gone, often comes back with a vengeance [14]. Therefore, new therapeutic strategies are needed aiming towards improved pharmacokinetics.

To circumvent the concerns of the high systemic toxicity, and improved body distribution together with prolonged blood circulation; the use of water soluble polymers as macromolecular carrier of low molecular weight conventional drugs is a very promising strategy in anti-cancer therapy [15–18]. Poly (ethylene glycol) (PEG) is a water soluble amphiphillic polymer with high solubility and excellent biocompatibility. And as it is FDA approved for human use, it is frequently used in numerous biomedical applications [18–22]. PEG is commercially available in a variety of molecular weights and has been extensively used as ready-for-use forms by chemical activation for covalent attachment to proteins. These activated PEGs are now being used for conjugation with small organic molecules acting as anti-cancer agents [23–26]. The polymeric therapy referred to as "PEGylated drugs" offer the advantages of long circulation displaying passive tumor targeting due to leakiness of angiogenic tumor blood vessels by the Enhanced Permeability and Retention (EPR) effect; thereby facilitating superior therapeutic approach over the current chemotherapy regime for active or more aggressive cancer [27,28]. Hence, use of a water soluble polymer like PEG act as a platform for drug targeting in non-immunogenic and non-toxic manner by increasing hydrodynamic size after PEGylation and limiting its cellular uptake to the endocytic route leading to slower renal clearance and longer blood circulation time [17,22,27,28]. Consequently, the pharmacokinetics of the drugs gets enhanced after conjugating with PEG.

Recently, efforts have been made in the field of polymer conjugation to increase the therapeutic index of gemcitabine which include, conjugation of gemcitabine to hydrophillic synthetic polymers such as α, β-poly (N-2-hydroxyethyl)-DL-aspartamide (PHEA) and PEG using folic acid as a targeting agent [29,30] Moreover, research in polymer conjugation potentiating the therapeutic index of gemcitabine have also ventured into carrier based radiochemotherapy to image guided delivery of radiochemotherapy using *N*-(2-hydroxypropyl)methacrylamide (HPMA) polymer [31,32]. Apparently, there has been very few published data available for the action of conjugated gemcitabine against pancreatic cancer. The goal of the present study was to utilize the efficiency of the polymer conjugation in the treatment of pancreatic cancer. We therefore designed PEGylated gemcitabine using chemically activated PEG, PEG-*N*-hydroxysuccinimide i.e., PEG–NHS for the conjugation with amine group of gemcitabine hydrochloride, since the protection of the amine function of gemcitabine would prevent its degradation by dCDA; and studied its effect on pancreatic cancer cells. NHS is chosen for amine coupling reactions in polymer due to its higher reactivity at physiological pH in bioconjugation synthesis [17]. Thus, in this paper we are reporting a method for the synthesis of PEGylated gemcitabine by conjugating the amino group at N4 position of gemcitabine to *N*-hydroxysuccinimide derivative of PEG (PEG–NHS) using triethylamine (TEA). The characterization of the PEGylated gemcitabine is done by different analytical techniques like ^{1}H NMR, FT IR spectroscopy and RP-HPLC. Subsequently, *in vitro* release kinetic study is done in buffer mimicking the pH of physiological fluids followed by pharmacokinetic behavior in animal model. The cytotoxicity study of PEGylated gemcitabine in comparison to native gemcitabine is done in two different pancreatic cell lines, MIA PaCa 2 and PANC 1. Additionally, studies of PEGylated gemcitabine for the intracellular distribution, cell cycle effect and mitochondrial membrane potential along with the apoptosis induction is done in PANC 1 cells. Thus, this research aims to increase cytotoxic activity of gemcitabine by improving the bioavailability through polymer conjugation. The intravenous administration of such polymeric conjugates can offers an excellent potential for the therapeutic approach in the treatment of pancreatic cancer.

2. Experimental

2.1. Materials

Gemcitabine hydrochloride (Gemzar) was purchased from Eli Lilly Co. (Indianapolis, IN). mono methoxy PEGs; NHS active esters (PEG–NHS) ($M_w \approx 5$ kDa) was obtained from NOF CORPORATION (Japan). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), 5-bromo-2′-deoxyuridine (BrdU), 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1), Annexin V-FITC, RNase A and propidium iodide were purchased from Sigma–Aldrich Chemicals (St Louis, MO, USA). Dimethylsulfoxide (DMSO), triethylamine (TEA) and acetonitrile of HPLC grade were also obtained from Sigma Aldrich Chemicals, Germany. Micro BCA Protein Assay Reagent was purchased from Thermo Fisher Scientific Inc. (IL, USA). All inorganic salts used were obtained from Qualigens Fine Chemicals, Mumbai. Dialysis membrane (MWCO: molecular weight cut-off = 3.5 kDa) was purchased from Spectra/Por® 6 (Spectrum Laboratories, Inc., CA, USA).

2.2. Cell lines

MIA PaCa 2 and PANC 1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (D-MEM, Invitrogen, CA, USA) supplemented with 1% L-Glutamine, 10% fetal bovine serum (GIBCO, CA, USA), 1% penicillin and streptomycin in a humidified atmosphere of 5% carbon dioxide (CO_2) at 37 °C in an incubator (Hera Cell, Thermo Scientific, Waltham, MA, USA). When the cell confluence of 90% was reached, they were routinely trypsinized and subcultured. All cell culture reagents were purchased from Invitrogen Corporation (CA, USA).

2.3. Synthesis of PEGylated gemcitabine

The PEGylated gemcitabine was synthesized by conjugating gemcitabine to PEG–NHS in dimethylsulfoxide (DMSO), in presence of triethylamine (TEA) [33]. Briefly, the activated PEG–NHS (50 mg) dissolved in 1 ml of DMSO was added slowly to 6 mg of gemcitabine hydrochloride dissolved in 5 ml of DMSO in a dropwise manner under constant magnetic stirring, in presence of 2 mM of TEA (PEG–NHS/Gemcitabine/TEA molar ratio = 1:2:20). The reaction was performed under nitrogen atmosphere at room temperature for 4 h. Then the reaction mixture was dialyzed using a dialysis membrane (MWCO: molecular weight cut-off = 3.5 k Da) against distilled water to remove free and unreacted gemcitabine. Later, the dialyzed solution was freeze-dried using a lyophilizer (LABCONCO Corporation, USA) at temperature of −48 °C and 0.05 mbar to obtain the powdered form of the conjugate.

2.4. Characterization of PEGylated gemcitabine

The characterization of the PEGylated gemcitabine was done by FT IR, ^{1}H NMR and reverse-phase chromatography in HPLC.

2.4.1. Fourier transform infrared spectroscopy

The FTIR spectra for native Gemcitabine, PEG–NHS and PEGylated gemcitabine were obtained from SPECTRUM RX I (Perkin Elmer, FTIR Spectrometer, CA, USA) for characterizing the chemical integrity of the PEGylated gemcitabine as described previously [34,35]. Briefly, the samples were pressed into a potassium bromide