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Stearoyl gemcitabine nanoparticles overcome resistance related to the over-expression of ribonucleotide reductase subunit M1

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ABSTRACT

Gemcitabine is a deoxycytidine analog used in the treatment of various solid tumors. However, tumors often develop resistances over time, which becomes a major issue for most gemcitabine-related chemotherapies. In the present study, a previously reported stearoyl gemcitabine nanoparticle formulation (GemC18-NPs) was evaluated for its ability to overcome gemcitabine resistance. In the wild type CCRF-CEM human leukemia cells, the IC_{50} value of GemC18-NPs was 9.5-fold greater than that of gemcitabine hydrochloride (HCl). However, in the CCRF-CEM-AraC-8C cells that are deficient in the human equilibrative nucleoside transporter-1, the IC_{50} of GemC18-NPs was only 3.4-fold greater than that in the parent CCRF-CEM cells, whereas the IC_{50} of gemcitabine HCl was 471-fold greater than that in the parent CCRF-CEM cells. The GemC18-NPs were also more cytotoxic than gemcitabine HCl in the deoxycytidine kinase deficient (CCRF-CEM/dCK$^{-/-}$) tumor cells. Similar to gemcitabine HCl, GemC18-NPs induced apoptosis through caspase activation. Another gemcitabine-resistant tumor cell line, TC-1-GR, was developed in our laboratory. In the TC-1-GR cells, the IC_{50} of GemC18-NPs was only 5% of that of gemcitabine HCl. Importantly, GemC18-NPs effectively controlled the growth of gemcitabine resistant TC-1-GR tumors in mice, whereas the molar equivalent dose of gemcitabine HCl did not show any activity against the growth of the TC-1-GR tumors. Proteomics analysis revealed that the TC-1-GR cells over-expressed ribonucleotide reductase M1, which was likely the cause of the acquired gemcitabine resistance in the TC-1-GR cells. To our best knowledge, this represents the first report demonstrating that a nanoparticle formulation of gemcitabine overcomes gemcitabine resistance related to ribonucleotide reductase M1 over-expression.

1. Introduction

Gemcitabine (2′-2′-difluorodeoxycytidine, dFdC) is a deoxycytidine analog, which is used to treat various solid tumors such as ovarian cancer, non-small cell lung cancer, pancreatic cancer, and breast cancer [1,2]. It is also an attractive candidate for combination therapy because of its favorable toxicity profile [3]. Combination therapies with cisplatin, etoposide and mitomycin are active against many other solid tumors such as bladder cancer, gastric cancer, and esophageal cancer [4,5].

However, tumors acquire resistance over time, which becomes a major issue for most gemcitabine-related chemotherapies [6]. The resistance is related to the mechanism of action of gemcitabine. Gemcitabine is transported into cells by nucleoside transporters such as the human equilibrative nucleoside transporter-1 (hENT1) [7]. Decreased expression of hENT1 confers lower gemcitabine toxicity in cells by blocking the cellular uptake of gemcitabine [8]. After cellular uptake, gemcitabine is transformed by a deoxycytidine kinase (dCK) into gemcitabine monophospate, which is further phosphorylated to gemcitabine diphosphate (dFdCDP), and then gemcitabine triphosphate (dFdCTP) [9]. The metabolite, dFdCTP, is intercalated into DNA by DNA polymerase alpha to inhibit DNA synthesis and induce cells to undergo apoptosis [10]. The dFdCDP acts as a ribonucleotide reductase (RR) inhibitor [2,11], which leads to increased incorporation of gemcitabine into DNA. On the other hand, gemcitabine is deaminated to its inactive form by adenosine or cytidine deaminases (CDA) [12,13]. Thus, nucleoside transporters, dCK, deaminases, RR, and the accumulation of dFdCDP seem to be important for the development of resistance to gemcitabine. Of particular importance, the RR is believed to play a key role in resistance to gemcitabine in many tumor cells in culture [14,15] and *in vivo*[16,17], and there is evidence that the effectiveness of gemcitabine treatment is correlated to the level of ribonucleotide reductase M1 (RRM1) expression in tumor cells. For example, clinically, non-small cell lung cancer patients with a low level of RRM1 mRNA expression had a significantly longer median survival when treated with gemcitabine/cisplatin [18,19], but patients with biliary tract cancers and a higher expression of RRM1 were resistant to gemcitabine treatment [20].

There have been extensive research efforts to overcome gemcitabine resistance. For example, amino acid ester prodrugs of gemcitabine were synthesized, and they were not as sensitive as gemcitabine to

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deamination by CDA [21]. In order to facilitate the uptake of gemcitabine by cells with decreased expression of nucleotide transporters, a lipophilic gemcitabine pro-drug was synthesized by esterifying gemcitabine at the 5′ position with an elaidic fatty acid [22]. Gemcitabine was also conjugated with cardiolipin to increase its uptake [23]. A phospholipid gemcitabine conjugate was shown to overcome both nucleoside transporter-deficiency and dCK-deficiency in cancer cells in culture [24], but it is unknown whether the phospholipid gemcitabine conjugate was effective *in vivo*.

An increasing amount of evidence had pointed to the promise of using nanoparticles to combat cancer cell resistance to chemotherapy [25], but the utilization of a gemcitabine nanoparticle formulation to overcome gemcitabine resistance is limited. Most relevantly, Reddy et al. reported that a nanoparticle formulation of gemcitabine prepared using gemcitabine covalently coupled to 1,1′,2-tris-nor-squalenic acid (4-(*N*)-tris-nor-squalenoyl-gemcitabine) was more cytotoxic than gemcitabine in two gemcitabine resistance cell lines, a human leukemia cell line (CCRF-CEM-AraC-8C) and a murine leukemia cell line (L1210 10K) in culture [26]. The L1210 10K cells were dCK deficient, and the CCRF-CEM-AraC-8C cells were hENT1 deficient [26].

Previously, a novel stearoyl gemcitabine nanoparticle formulation was developed in our laboratory by incorporating a stearic acid amide derivative of gemcitabine, stearoyl gemcitabine (GemC18), into solid lipid nanoparticles engineered from lecithin/glycerol monostearate-in-water emulsions [27]. In mice with pre-established model mouse or human tumors, the stearoyl gemcitabine nanoparticles (GemC18-NPs) were significantly more effective than gemcitabine HCl in controlling tumor growth [27]. The improved anti-tumor activity of the GemC18-NPs was not simply due to the GemC18 *per se* because the same GemC18 dissolved in Tween 20 micelles failed to show any significant anti-tumor activity in mice [27]. In an effort to develop a strategy to overcome resistance to gemcitabine, the feasibility of overcoming tumor resistance to gemcitabine using the GemC18-NPs was evaluated. It was discovered that the GemC18-NPs can overcome gemcitabine resistance related to the over-expression of RRM1, not only in culture, but also in mice.

2. Materials and methods

2.1. Materials and cell lines

Tween 20, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), stearic acid (C18), caspase-3 assay kit, iodoacetamide, urea, and thiourea were from Sigma-Aldrich (St. Louis, MO). Gemcitabine HCl was from U.S. Pharmacopeia (Rockville, MD). Soy lecithin was from Alfa Aesar (Ward Hill, MA). Glycerol monostearate was from Gattefosse Corp. (Paramus, NJ). N-3,4-tridhydroxybenzamide (didox) was from Cayman Chemical (Ann Arbor, MI). Biolytes, Bio-Safe Coomassie blue staining solution, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), laemmli sample buffer, β-mercaptoethanol, nitrocellulose membrane, strip IPG (pH 3–10), Tris–HCl gel, and precision plus protein standards were from Bio-Rad (Hercules, CA). The duplex small interfering RNA (siRNA) oligonucleotides for RRM1 (UUAAUAACUGGGCUUCUGGGCUCUC and GAGAGCCCAGAAGCCCAGUUAUUAA), the negative universal control siRNA (Cat. No: 12935–300), and the Lipofectamine™ RNAiMAX were from Invitrogen (Carlsbad, CA).

Human leukemia cell line, CCRF-CEM (# CCL-119), and mouse lung cancer cell line, TC-1 (# CRL-2785), were from the American Type Culture Collection (Rockville, MD). CCRF-CEM-AraC-8C cells (hENT1 deficient), CCRF-CEM/dCK$^{-/-}$ cells (dCK deficient), and CCRF-CEM-AraC-8D cells (dCK deficient) were kindly provided by Dr. Buddy Ullmann (Oregon Health & Science University, Portland, OR), Dr. Margaret Black (Washington State University, Pullman, WA), and Dr. Beverly S. Mitchell (Stanford University School of Medicine, Stanford, CA), respectively. The cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (all from Invitrogen).

2.2. Preparation of stearoyl gemcitabine nanoparticles

GemC18-NPs were prepared as previously described [27]. Briefly, 3.5 mg of soy lecithin, 0.5 mg of glycerol monostearate, and 5 mg of GemC18 were placed into a 7 mL glass vial. One mL of de-ionized and filtered (0.22 μm) water was added into the mixture, which was then maintained on a 70–75 °C hot plate while stirring, with occasional water-bath sonication (Bransonic Ultrasonic Cleaner, Danbury, CT), until the formation of homogenous slurry. Tween 20 was added in a step wise manner to a final concentration of 1% (v/v). The resultant emulsions were allowed to cool to room temperature while stirring to form nanoparticles. Particle size and zeta potential were measured using a Malvern Zetasizer Nano ZS (Westborough, MA). In a short 20-day preliminary stability study, the size of the nanoparticles did not change significantly when the nanoparticles in aqueous suspension were stored in ambient condition (Supplemental Fig. S1). When stored at 37 °C in PBS (pH 6.01 or 7.4) for 72 h, no significant particle size increase, nor GemC18 degradation, was observed (Supplemental Fig. S2).

2.3. Development of TC-1-GR cell line

The gemcitabine resistant cell line, TC-1-GR, was developed by culturing TC-1 cells with gradually increasing concentration of gemcitabine HCl over a 3-month period as previously described [28]. The maximum concentration used was 1 μM. Cells were grown in 75 cm^2 flasks in RPMI 1640 containing 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in 5% CO_2 at 37 °C.

2.4. In vitro cytotoxicity assay

Cells (10,000/well for leukemia cells, 5000/well for TC-1 and TC-1-GR cells) were seeded in 96-well plates. After overnight incubation, they were further incubated in the presence of various concentrations of gemcitabine HCl, the equivalent amount of GemC18-NPs (no more than 40 μM of GemC18), or didox, an inhibitor of RRM1, for 48 h (TC-1 or TC-1-GR cells) or 72 h (CCRF-CEM, CCRF-CEM-AraC-8C, CCRF-CEM-AraC-8D, and CCRF-CEM/dCK$^{-/-}$) at 37 °C, 5% CO_2. To compare the cytotoxicities of GemC18-NPs and GemC18, TC-1-GR cells (5000/well) were allowed to grow for 24 h and incubated with different concentrations of GemC18 in GemC18-NPs or in trace amount of dimethyl sulfoxide (DMSO) for 48 h. RPMI 1640 medium alone or medium with trace amount of DMSO were used as a control. GemC18-free nanoparticles equivalent to GemC18-NPs that contain 10 μM or less GemC18 were not toxic to TC-1-GR cells (less than 50 μM in the CCRF-CEM cells and derivative lines). The number of cells alive was quantified using an MTT assay. Absorbance was measured at 570 nm and 630 nm using a BioTek Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT). Penicillin and streptomycin in the cell culture medium did not significantly affect the cytotoxicity of the GemC18-NPs (Supplemental Table S1). The fraction of affected (killed) cells (Fa) and the fraction of unaffected (live) cells (Fu) at every dose were calculated, and the Log (Fa/Fu) values were plotted against the Log (concentration of gemcitabine) [29]. IC_{50} was the dose at Log (Fa/Fu) = 0. The experiment was repeated at least three times.

2.5. Assay of caspase-3 activity

Caspase-3 activity was determined using a Sigma caspase-3 assay kit. Briefly, CCRF-CEM cells (10,000 cells/well in 150 μl) were seeded in 96-well plates. After overnight incubation, the cells were treated for 72 h with gemcitabine HCl or GemC18-NPs at concentrations ranging from 0.22 to 314 nM. At the end of incubation, the cells were washed with PBS (10 mM, pH 7.4), centrifuged (3000×*g* for 5 min at 4 °C),