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NANOMEDICINE

Just getting into cells is not enough: Mechanisms underlying 4-(*N*)-stearoyl gemcitabine solid lipid nanoparticle's ability to overcome gemcitabine resistance caused by RRM1 overexpression

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ABSTRACT

Gemcitabine is a deoxycytidine analog that is widely used in the chemotherapy of many solid tumors. However, acquired tumor cell resistance often limits its use. Previously, we discovered that 4-(*N*)-stearoyl gemcitabine solid lipid nanoparticles (4-(*N*)-GemC18-SLNs) can overcome multiple acquired gemcitabine resistance mechanisms, including RRM1 overexpression. The present study was designed to elucidate the mechanisms underlying the 4-(*N*)-GemC18-SLNs' ability to overcome gemcitabine resistance. The 4-(*N*)-GemC18 in the 4-(*N*)-GemC18-SLNs entered tumor cells due to clathrin-mediated endocytosis of the 4-(*N*)-GemC18-SLNs into the lysosomes of the cells, whereas the 4-(*N*)-GemC18 alone in solution entered cells by diffusion. We substantiated that it is the way the 4-(*N*)-GemC18-SLNs deliver the 4-(*N*)-GemC18 into tumor cells that allows the gemcitabine hydrolyzed from the 4-(*N*)-GemC18 to be more efficiently converted into its active metabolite, gemcitabine triphosphate (dFdCTP), and thus more potent against gemcitabine-resistant tumor cells than 4-(*N*)-GemC18 or gemcitabine alone. Moreover, we also showed that the RRM1-overexpressing tumor cells were also cross-resistant to cytarabine, another nucleoside analog commonly used in cancer therapy, and 4-(*N*)-stearoyl cytarabine carried by solid lipid nanoparticles can also overcome the resistance. Therefore, formulating the long-chain fatty acid amide derivatives of nucleoside analogs into solid lipid nanoparticles may represent a platform technology to increase the antitumor activity of the nucleoside analogs and to overcome tumor cell resistance to them.

1. Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC) is a deoxycytidine analog with antitumor activity against a wide variety of solid tumors. Unfortunately, tumor cells often develop resistance to gemcitabine, which limits its clinical efficacy [1]. The mechanism of gemcitabine resistance is closely related to its mechanism of action and intracellular metabolism [2]. Gemcitabine is transported into cells by nucleoside transporters such as the human equilibrative nucleoside transporter 1 (hENT1) [3]. Upon entering cells, gemcitabine is phosphorylated by deoxycytidine kinase (dCK) to gemcitabine monophosphate (dFdCMP) and subsequently by nucleotide kinase to its active metabolites, gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP) [4]. The dFdCTP is incorporated into DNA to inhibit DNA synthesis [5], and dFdCDP inhibits ribonucleotide reductase (RR), an enzyme that is required for the synthesis of deoxynucleotides (dNTPs) [6]. Because dFdCTP competes with deoxycytidine triphosphate (dCTP) for incorporation into DNA, a decrease in cellular concentration of dNTPs (i.e., dNTP pool) further increases the incorporation of dFdCTP into DNA [4,5]. Therefore, tumor cells that have decreased expression of nucleoside transporters and/or dCK or increased expression of the large and/or small subunits of the RR (RRM1 and/or RRM2) are resistant to gemcitabine [7,8].

Recently, there is increasing evidence that certain gemcitabine nanoparticles can overcome tumor cells resistance to gemcitabine [9–14]. Previously we discovered that the 4-(*N*)-stearoyl gemcitabine solid lipid nanoparticles (4-(*N*)-GemC18-SLNs) developed in our laboratory are more cytotoxic than gemcitabine in gemcitabine resistant tumor cells that are deficient in hENT1 or dCK [11]. More importantly, the 4-(*N*)-GemC18-SLNs can overcome gemcitabine resistance caused by the overexpression of RRM1, both *in vitro* and *in vivo* [11]. This finding is clinically relevant considering that data from numerous clinical studies have documented the inverse correlation between RRM1 expression in tumor cells and their sensitivity to gemcitabine. Clinical studies in non-small cell lung and pancreatic cancer patients who received gemcitabine-based therapy revealed that patients with low levels of RRM1 expression showed a better response and a longer

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survival than those with high RRM1 levels [15–18]. Moreover, the treatment benefit from gemcitabine in pancreatic cancer patients with disease recurrence was observed only in patients with low RRM1 [19]. A strong expression of RRM1 was also detected in biliary tract cancer patients and intrahepatic cholangiocarcinoma patients who were resistant to gemcitabine [20,21]. Finally, recent data in patients with advanced nasopharyngeal carcinoma also revealed that the progression-free survival of patients with RRM1-positive expression is shorter than patients with RRM1-negative expression [22].

In the present study, we identified the mechanisms by which our 4-(*N*)-GemC18-SLNs overcome gemcitabine resistance caused by RRM1 overexpression.

2. Materials and methods

2.1. Cell line

Mouse lung cancer cell line (TC-1, ATCC # CRL-2785) were cultured in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin, all from Invitrogen (Carlsbad, CA). The previously established TC-1-GR cells were cultured in similar RPMI 1640 medium further supplemented with 1 μM of gemcitabine HCl [11].

2.2. Syntheses of 4-(N)-stearoyl gemcitabine (4-(N)-GemC18), 3′-(O)-stearoyl gemcitabine (3′-(O)-GemC18), 4-(N)-octyl gemcitabine (4-(N)-GemC8), and 4-(N)-stearoyl cytarabine (4-(N)-Ara-C-C18) and the incorporation of them into nanoparticles

The 4-(*N*)-GemC18 was synthesized as previously described by us [23]; 3′-(*O*)-GemC18, 4-(*N*)-GemC8, and 4-(*N*)-Ara-C-C18 were synthesized according to a literature protocol with slight modifications [24,25] (see Supplement for detailed methods and Fig. S1 for structures). The compounds were incorporated into solid lipid nanoparticles (SLNs) as previously described to prepare 4-(*N*)-GemC18-SLNs, 3′-(*O*)-GemC18-SLNs, 4-(*N*)-GemC8-SLNs, and 4-(*N*)-Ara-C-C18-SLNs, respectively [23]. The 4-(*N*)-GemC18-incorporated poly (lactic-*co*-glycolic) acid nanoparticles (4-(*N*)-GemC18-PLGA-NPs) were prepared using a solvent displacement method as previously described [26]. The concentration of 4-(*N*)-GemC18 in the nanoparticles was determined using a HPLC method as previously described [27]. Table S1 includes diameters, zeta potentials, and polydispersity indices of the nanoparticles.

2.3. Hydrolysis of gemcitabine from 4-(N)-GemC18-SLNs and 3′-(O)-GemC18-SLNs in cell culture medium

The 4-(*N*)-GemC18-SLNs or 3′-(*O*)-GemC18-SLNs (10 μM of GemC18) were incubated in 1 ml of RPMI 1640 medium supplemented with 10% FBS at 37 °C, 5% CO_2. At predetermined time points, medium was collected and analyzed for gemcitabine concentration using an HPLC method as previously described [28].

2.4. Western blot analysis

Immunoblotting for RRM1 protein was performed as previously described [11]. β-Actin (detected using mouse monoclonal antibody) was used as a control.

2.5. Quantification of intracellular dNTPs, NTPs, and dFdCTP

Intracellular dNTP, NTP, and dFdCTP were extracted and analyzed (using HPLC) as previously described with slight modifications [29–31] (see Supplement methods). An Agilent 1260 Infinity Quaternary Liquid Chromatographic System equipped with an Aglient ZORBAX Eclipse Plus C18 column (3.5 μm, 4.6 mm × 150 mm) was used for the analysis for all compounds. For dNTPs and NTPs analyses, the mobile phase consisted of two solutions: 10 mM KH_2PO_4/10 mM tetrabutylammonium chloride (TBACl) (pH 7.0) with 0.25% methanol (A) and 50 mM KH_2PO_4/5.6 mM TBACl (pH 7.0) : methanol (70:30, v/v) (B). Run was started at 60% A followed by a linear gradient to 40% A over 30 min and held at 40% A for 40 min. The flow rate was 1.0 ml/min. The injection volume was 40 μl. The detection wavelength was 254 nm. Figs. S2B–C show the HPLC histograms of dNTPs and NTPs in TC-1 and TC-1-GR cells, respectively. For dFdCTP analysis, two solutions were used: 10 mM KH_2PO_4/10 mM TBACl (pH 7.0) with 0.25% methanol (A) and 250 mM KH_2PO_4/10 mM TBACl (pH 7.0) : methanol (85:15, v/v) (B). They were mixed at 50:50 (v/v). The flow rate was 1.2 ml/min. The injection volume was 20 μL. The detection wavelength was 271 nm.

2.6. In vitro cytotoxicity assay

TC-1 and TC-1-GR cells were seeded into 96-well plates (3×10^3 cells/well). After overnight incubation at 37 °C, 5% CO_2, cells were treated with various concentrations of gemcitabine HCl, cytarabine (Ara-C), gemcitabine derivatives in nanoparticles, or the Ara-C derivative in nanoparticles for 48 h. Cell viability was determined using an MTT assay [11].

2.7. Inhibition of RRM1 expression by siRNA silencing

TC-1-GR cells were transfected with RRM1 siRNA or control siRNA complexed with Lipofectamine™ RNAiMAX (Invitrogen) following the manufacturer's instruction [11]. The siRNA-transfected cells were re-seeded (3×10^4 cells/well) into 96-well plates 48 h after transfection and incubated overnight at 37 °C, 5% CO_2. Cells were then treated with Ara-C for 48 additional hours, and the cytotoxicity was evaluated using an MTT assay.

2.8. In vitro cellular uptake assay

Cellular uptake was performed as previously described [27]. To inhibit endocytosis, cell uptake was carried out as described above but at 4 °C [23]. To inhibit specific endocytosis mechanisms, cells were pretreated with chlorpromazine (5 μg/ml), filipin (2.5 μg/ml), wortmannin (3 μg/ml), or cytochalasin B (20 ng/ml) in RPMI 1640 medium for 30 min at 37 °C before performing the uptake study. Chlorpromazine, filipin, wortmannin, and cytochalasin B are inhibitors of clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and phagocytosis, respectively [32–34]. The concentrations of the inhibitors were the highest concentrations that did not affect the viability of TC-1-GR cells in 2.5 h (Fig. S3).

2.9. Fluorescence microscopy

TC-1-GR (1.5×10^5 cells/well) were seeded in a 35 mm poly-D-lysine-coated glass-bottom dish (Mattek Corporation, Ashland, MA) and incubated overnight at 37 °C, 5% CO_2. Cells were incubated with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)-fluorescein-labeled SLNs (100 μg/ml of DOPE-fluorescein) for 2 h [23]. The nanoparticle-containing medium was then replaced with fresh medium and incubated for 0, 2, or 6 additional hours. Intracellular localization of fluorescein-labeled SLNs was monitored as previously described [27].

2.10. Quantitation of GemC18 in lysosomes

The lysosomal fraction was prepared using a cell fractionation method described previously with slight modifications [35,36] (see Supplement for more details). The activity of cathepsin B in the fraction was confirmed to be significantly higher than in the cytoplasmic fraction. The concentration of GemC18 in the fraction was determined