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The effect of the acid-sensitivity of 4-(*N*)-stearoyl gemcitabine-loaded micelles on drug resistance caused by RRM1 overexpression

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Abstract

Chemoresistance is a major issue for most gemcitabine-related chemotherapies. The overexpression of ribonucleotide reductase subunit M1 (RRM1) plays a key role in gemcitabine resistance. In this study, we synthesized a new highly acid-sensitive amphiphilic micelle material by conjugating hydrophilic polyethylene glycol with a hydrophobic stearic acid derivative (C18) using a hydrazone bond, which was named as PHC-2. A lipophilic prodrug of gemcitabine, 4-(*N*)-stearoyl gemcitabine (GemC18), was loaded into micelles prepared with PHC-2, a previously synthesized less acid-sensitive PHC-1, and their acid-insensitive counterpart, PAC. GemC18 loaded in acid-sensitive micelles can overcome gemcitabine resistance, and GemC18 in the highly acid-sensitive PHC-2 micelles was more cytotoxic than in the less acid-sensitive PHC-1 micelles. Mechanistic studies revealed that upon cellular uptake and lysosomal delivery, GemC18 in the acid-sensitive micelles was released and hydrolyzed more efficiently. Furthermore, GemC18 loaded in the highly acid-sensitive PHC-2 micelles inhibited the expression of RRM1 and increased the level of gemcitabine triphosphate (dFdCTP) in gemcitabine resistant tumor cells. The strategy of delivering lipophilized nucleoside analogs using highly acid-sensitive micelles may represent a new platform technology to increase the antitumor activity of nucleoside analogs and to overcome tumor cell resistance to them.

Keywords

Hydrazone; nucleoside analogs; cell uptake; intracellular metabolism; lysosomes; dFdCTP/dNTP ratio

1. Introduction

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, dFdC) is a deoxycytidine nucleoside analog, which is approved for the treatment of pancreatic, non-small cell lung, breast, and ovarian

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cancers [1]. Gemcitabine enters cells via a facilitated nucleoside transport mechanism [2] and is phosphorylated into gemcitabine 5'-monophosphate (dFdCMP) by deoxycytidine kinase (dCK) [3]. It is further phosphorylated by other pyrimidine kinases to gemcitabine diphosphate (dFdCDP) and then gemcitabine triphosphate (dFdCTP) [4]. The triphosphate derivative of dFdCTP is intercalated into DNA to inhibit DNA synthesis and induce apoptosis in cells [5]. While many tumor cells show initial sensitivity to gemcitabine therapy, they often acquire resistance over time, which becomes a major issue for gemcitabine-related chemotherapies [6]. The resistance to gemcitabine arises from an altered activation/degradation pathway involving decreased level of nucleoside transporters, reduced level of the activating enzyme dCK, and increased degrading enzymes such as cytidine deaminases (CDA) and 5'-nucleotidase [7]. Moreover, the overexpression of ribonucleotide reductase (RR) expands cellular deoxynucleoside triphosphate (dNTP) pool, which could lead to decreased incorporation of dFdCTP into DNA and reduce the cytotoxicity of gemcitabine, thus playing a key role in gemcitabine resistance [8, 9].

There have been extensive research efforts to overcome gemcitabine resistance, of which the combined utilization of nanoscale drug delivery systems and the lipophilic modification of gemcitabine is an attractive approach. For example, Couvreur's group covalently coupled gemcitabine with 1,1',2-tris-nor-squalenic acid, and the resultant 4-(*N*)-tris-nor-squalenoyl-gemcitabine (SQdFdC) self-assembled into nanoparticles, which were shown to overcome gemcitabine resistance in murine leukemia cells (i.e., L1210 10K) [10], human leukemia cells (i.e., CEM/ARAC8C) [10], and human pancreatic cancer cells (i.e., Panc-1) [11]. It was concluded that SQdFdC nanoparticles enabled the partial circumvention of three well-known resistant mechanisms to gemcitabine, including the down-regulation of nucleoside transporters, insufficient activity of dCK, and inactivation by deaminases [12]. Recently, our laboratory reported that 4-(*N*)-stearoyl gemcitabine (GemC18), a stearic acid amide derivative of gemcitabine, can overcome gemcitabine resistance caused by the overexpression of RR subunit M1 (RRM1), when it is incorporated into solid lipid nanoparticles (SLNs) [13]. We have also found that the internalization of the GemC18-SLNs by endocytosis and the subsequent delivery of SLNs into lysosomes are the key for the GemC18-SLNs to overcome the gemcitabine resistance caused by RRM1 overexpression (Wonganan et al, unpublished data) [14].

Micelle drug delivery systems are characteristic of several advantages in cancer chemotherapy, such as ease of preparation, small and uniform particle size (10–100 nm), high drug loading, and controllable drug release profiles [15, 16]. Acid-sensitive micelles are more attractive because they undergo sharp pH-dependent structural disruption in either the slightly acidic extracellular tumor environment (pH ~6.8) or the more acidic intracellular organelles (e.g., endosomes and lysosomes, pH 5–6), rapidly release the incorporated drug, and consequently enhance the antitumor activity of the drug [17–19]. Previously, we conjugated the hydrophilic polyethylene glycol (PEG, MW 2000) with a hydrophobic stearic acid (C18) using an acid-sensitive hydrazone bond [20], and the resultant PEG-hydrazone-C18 (PHC, hereinafter referred to as PHC-1) molecules self-assemble into nanometer scale micelles when dispersed into an aqueous solution [20]. GemC18 loaded in the PHC-1 micelles showed significantly improved antitumor activity than in the acid-insensitive PEG2000-amide-C18 (PAC) micelles in a B16-F10 murine melanoma tumor model [20]. The accelerated release of GemC18 from the acid-sensitive PHC-1 micelles in the lysosomes and the subsequent hydrolysis of GemC18 to generate the parent drug gemcitabine by lysosome enzymes are thought to be responsible for the improved antitumor activity [20].

These promising results, together with our previous study where GemC18-SLNs were able to overcome gemcitabine resistance caused by RRM1 overexpression [13, 14], inspired us to

further explore whether the acid-sensitive micelles are also able to overcome gemcitabine resistance caused by RRM1 overexpression. To study the effect of acid-sensitivity of the micelles on their ability to overcome gemcitabine resistance, we designed and synthesized a new PEG2000-hydrazone-C18 conjugate (PHC-2) by switching the positions of PEG2000 and C18 around the hydrazone bond. The PHC-2 is more sensitive to acidic condition than PHC-1. Using micelles formed by the newly synthesized PHC-2, the previously synthesized PHC-1 (acid-sensitive), or PAC (acid-insensitive) as carriers for GemC18 (Scheme 1A), we tested whether increasing the acid-sensitivity of the GemC18 nano-carriers will enable the GemC18 to more effectively overcome gemcitabine resistance caused by RRM1 overexpression.

2. Materials and methods

2.1. Materials

Methoxy-polyethylene glycol 2000-aldehyde (PEG2000-aldehyde) was from Creative PEGWorks (Wiston Salem, NC). PEG2000-hydrazone-C18-1 (PHC-1), PEG2000-amide-C18 (PAC), and 4-(*N*-stearoyl gemcitabine (GemC18) were synthesized according to our previously published methods [20]. Gemcitabine hydrochloride (GemHCl) was from U.S. Pharmacopeia (Rockville, MD). Stearic acid, tert-butyl carbazate, sodium dodecyl sulfate (SDS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO), tetrabutylammonium chloride (TBACl) were from Sigma-Aldrich (St. Louis, MO). Pyrene was from Acros Organics (Morris Plains, NJ). The 100-mM 2'-deoxynucleoside 5'-triphosphate (dNTP) set was from Invitrogen (Carlsbad, CA). Gemcitabine triphosphate (dFdCTP) was a gift from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). Lysotracker Red DND-99 was from Molecular Probes (Eugene, OR). Hoechst 33342 was from AnaSpec, Inc. (Fremont, CA). HPLC grade tetrahydrofuran (THF) and methanol were used during HPLC analysis, and all other solvents used in chemical synthesis and cell culture were analytical grade.

Mouse lung cancer cell line (TC-1, American Type Culture Collection, ATCC, Manassas, VA) was 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. The previously established TC-1-GR cells were cultured in similar RPMI 1640 medium further supplemented with 1 µM GemHCl [13].

2.2. Synthesis and characterization of PEG2000-hydrazone-C18-2 (PHC-2)

2.2.1. Synthesis of stearic hydrazide—Stearic hydrazide was synthesized according to previously published methods with modifications [21, 22]. Briefly, stearic acid (110 mg, 0.773 mmol) was dissolved in 2 mL of anhydrous dimethylformamide (DMF), followed by the addition of tert-butyl carbazate (62 mg, 0.938 mmol) and EDCI (224 mg, 2.337 mmol). The reaction mixture was stirred for 20 h at room temperature, transferred to water (10 mL), and then extracted with ethyl acetate (10 mL × 3). The combined organic layer was washed with brine (20 mL × 3), dried with anhydrous sodium sulfate (Na₂SO₄), and concentrated under reduced pressure. The obtained white solid was purified using a silica gel column (ethyl acetate/hexane, 3:1). The eluent with the *tert*-butoxycarbonyl (Boc)-protected stearic hydrazide was collected and dried to give a white solid (86.1 mg). The white solid was then dissolved in 2.27 mL of dichloromethane (DCM) and 1.36 mL of trifluoroacetic acid (TFA). After one hour of stirring at room temperature, the solvent was evaporated at a reduced pressure, and a white solid was obtained (58 mg, 50.3% yield). *R*_f = 0.42 (chloroform

(CHCl₃)/methanol, 10:1); ¹H NMR (300 MHz, CD₃OD): δ = 7.56 (s, 1H, NH₂NHCO), 2.16 (t, 2H, COCH₂), 1.62 (m, 2H, COCH₂CH₂), 1.27 (m, 28H, (CH₂)₁₄), 0.89 ppm (t, 3H, CH₃); MS [M+H]⁺ *m/z* calculated for C₁₈H₃₈N₂O: 298.5071, found: 298.2984.

2.2.2. Synthesis of PHC-2—PHC-2 was synthesized following previously reported methods with modifications [23, 24]. PEG2000-aldehyde (252 mg, 0.126 mmol) and stearic hydrazide (228 mg, 0.762 mmol) were dissolved in 9 mL of anhydrous ethanol, and the reaction was kept at 75°C for 18 h in the presence of acetic acid (60 μL). The solvent was then evaporated, and the white solid was purified using a silica gel column (CHCl₃/methanol, 10:1 and 20:1) to give PHC-2 as a white solid (127 mg, 43.8% yield). *R*_f = 0.33 (CHCl₃/methanol, 10:1); ¹H NMR (300 MHz, CDCl₃): δ = 8.56 (s, 1H, CONHN=CH), 7.17 (t, 1H, N=CH), 3.5–3.7 (m, 180H, (CH₂CH₂O)₄₅), 3.38 (s, 3H, OCH₃), 2.20 (t, 2H, COCH₂), 1.65 (m, 2H, COCH₂CH₂), 1.25 (m, 28H, (CH₂)₁₄), 0.88 ppm (t, 3H, CH₃).

2.2.3. Acid-sensitive degradation of PHC-2—PHC-2 was dissolved in phosphate buffered saline (PBS, 5 mM, pH 5.5, 6.8, or 7.4) at 2 mg/mL and incubated at 37°C in a water bath. At predetermined time points, sodium hydroxide (NaOH) solution (0.25 N or 0.5 N) was added to the solution to adjust the pH value to 8.0, and the samples were then lyophilized followed by ¹H NMR analysis using a Varian DirectDrive 600 (Palo Alto, CA). The percentage of degradation was calculated using the following equation:

$$\text{Percentage of degradation (\%)} = \frac{S_{\delta 9.79}}{S_{\delta 8.56} + S_{\delta 9.79}} \times 100 \quad (1)$$

Where S is the area of the peak in ¹H NMR spectrum; δ_{8.56} is the chemical shift of the peak corresponding to the proton attached on the nitrogen atom in the hydrazone bond of PHC-2, and δ_{9.79} is the chemical shift of the peak corresponding to the aldehyde proton of PEG2000-aldehyde.

2.2.4. Determination of the critical micellar concentration (CMC) of PHC-2—The CMC value of PHC-2 in water was determined using a pyrene 1:3 ratio method [25]. Briefly, 2 mL of a PHC-2 aqueous solution (0.00046–1 mg/mL) was added in a glass vial containing 1.17 μg of pre-dried pyrene, and the resultant solution was incubated overnight at room temperature while shaking at 200 rpm. Fluorolog3 Fluorimeter (HORIBA Scientific, Edison, NJ) was used to record the emission spectra of pyrene between 350 nm to 450 nm (Ex = 335 nm, slit = 1 nm; Em slit = 2 nm). The CMC value of PHC-2 was calculated by measuring the polarity index of pyrene, *I*₁/*I*₃ (ratio of the intensities of the first and third peaks in the fluorescence emission spectrum of pyrene), as a function of PHC-2 concentration.

2.3. Preparation and characterization of GemC18-loaded PEG-C18 micelles

2.3.1. Preparation of GemC18-loaded PEG-C18 micelles—GemC18 was loaded into PAC, PHC-1 or PHC-2 micelles (i.e., three different forms of PEG-C18) at 5% (W_{GemC18}/W_{PEG-C18}) using a modified thin-film hydration method [20]. Briefly, 0.5 mg of pre-dried GemC18 was hydrated with 1 mL of PEG-C18 aqueous solution (10 mg/mL) under vigorous stirring in a 75°C water bath. Clear micelle solutions were formed within 5 min, which was then cooled down to room temperature with constant water bath sonication. The resultant micelle preparations were lyophilized after filtrating through a 0.2 μm PTFE syringe filter (Nalge Nunc International, Rochester, NY).

2.3.2. Preparation of fluorescence-labeled PEG-C18 micelles—In order to compare the intracellular fate of the PEG-C18 micelles with different acid-sensitivity, a lipophilic fluorescence resonance energy transfer (FRET) pair (DiO and DiI) was loaded into the micelles using the same thin-film hydration method [20]. DiI (0.25 mg) and DiO (0.25 mg) were added into each formulation (10 mg PEG-C18) to achieve a theoretical loading of 5% ($W_{\text{FRET pair}}/W_{\text{PEG-C18}}$). Finally, PEG-C18 micelles loaded with DiO alone (1%, $W_{\text{DiO}}/W_{\text{PEG-C18}}$) were also prepared to determine the intracellular location of the micelles.

2.3.3. Determination of entrapment efficiency and percentage of drug loading

—In order to determine the entrapment efficiency and the loading percentage of GemC18 in PEG-C18 micelles, the lyophilized GemC18-loaded micelles were dissolved in THF and then subjected to an Agilent 1260 Infinity Quaternary Liquid Chromatographic System (Santa Clara CA) for HPLC analysis. An Agilent ZORBAX Eclipse PlusC18 column (5 μm , 4.6 mm $\times$ 150 mm) was used, and the UV detector was operated at 248 nm. The mobile phase was methanol. The flow rate was 1 mL/min. The percentage of drug loading and the entrapment efficiency were calculated using the following equations:

$$\text{Percentage of drug loading (\%)} = \frac{\text{Weight of GemC18 in micelles}}{\text{Weight of GemC18-containing micelles}} \times 100 \quad (2)$$

$$\text{Entrapment efficiency (\%)} = \frac{\text{Weight of GemC18 in micelles}}{\text{Weight of total GemC18 added}} \times 100 \quad (3)$$

2.3.4. Determination of particle size and zeta potential—The hydrodynamic diameters of GemC18-loaded PEG-C18 micelles were determined using a Malvern Zeta Sizer Nano ZS (Malvern Instruments Ltd., MA). Briefly, lyophilized sample (1 mg) was dissolved in 1 mL of water and filtered through a 0.2 μm PTFE filter prior to measurement. The Zeta potential was determined using the same equipment, but PBS (pH 7.4, 10 mM) was used to dissolve the lyophilized samples to give a final concentration of 1 mg/mL.

2.4. *In vitro* acid-sensitive release of GemC18 from micelles

The *in vitro* release profiles of GemC18 from PHC-2 micelles were determined according to a previously reported method [20]. Briefly, GemC18-loaded PHC-2 micelles were dissolved in PBS (5 mM) with pH values of 5.5, 6.8, or 7.4 (50 $\mu\text{g/mL}$ GemC18) and incubated at 37°C and 150 rpm in a shaking incubator. At predetermined time points, samples were withdrawn and filtered through a 0.2 μm filter. The filtrate (0.2 mL) was lyophilized, re-dissolved in 0.2 mL of methanol, and centrifuged at $15,500 \times g$ for 10 min. GemC18 concentration in the supernatant was analyzed using HPLC.

2.5. *In vitro* cytotoxicity

TC-1 or TC-1-GR cells were seeded into 96-well plates (2500 cells/well). After overnight incubation, the culture medium was replaced with 200 μL fresh medium containing GemHCl, GemC18 (with less than 0.66% of DMSO (v/v) as a solubilizer) or GemC18-loaded PEG-C18 micelles. The molar concentrations of gemcitabine were from 0.0001 to 200 μM . After 48 h of incubation, the cell viability was evaluated using an MTT assay [20]. DMSO alone at 0.66% was not significantly cytotoxic to TC-1 and TC-1-GR cells after 48 h of incubation (e.g., cell viability in TC-1-GR cells, $96.03 \pm 7.51\%$, vs. $100.00 \pm 5.10\%$ in DMSO free medium, $n = 6$, $P > 0.05$). The values of half inhibitory concentration (IC_{50}) were expressed as the molar equivalent GemHCl concentration required to reduce the

absorbance to 50% of that in untreated control wells. The resistance index was calculated by dividing the IC₅₀ value of each formulation in TC-1-GR cells by that in TC-1 cells.

2.6. Cellular uptake and intracellular metabolism of GemC18

TC-1-GR cells (2×10^5 cells/well) were seeded in a 12-well plate and incubated overnight. The cells were then treated with GemC18 or GemC18-loaded PEG-C18 micelles (10 µg/mL GemC18) for another 2 or 6 h, lysed with 1% SDS, lyophilized, and analyzed using HPLC. To inhibit endocytosis, the cell uptake was carried out at 4°C for 2 h. To inhibit specific endocytosis mechanism, TC-1-GR cells were pre-treated with chlorpromazine (5 µg/mL), filipin (2.5 µg/mL), wortmannin (3 µg/mL) or cytochalasin B (20 ng/mL) for 30 min followed by another 2 h of incubation with the GemC18-loaded micelles. Chlorpromazine, filipin, wortmannin, and cytochalasin B are inhibitors of clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis and phagocytosis, respectively [26–29]. To evaluate the intracellular metabolism of GemC18, the TC-1-GR cells were cultured in GemC18-containing medium for 2 h. The medium was then changed to fresh medium containing 0 or 50 mM NH₄Cl. After another 16 h of incubation, the amount of GemC18 in the cells was determined, which was divided by the amount of GemC18 initially taken up by the cells (i.e., immediately after the 2 h incubation) to determine the percentage of GemC18 that remained in the cells. The effect of alkalinizing lysosomal pH on the intracellular metabolism of the GemC18 was evaluated by comparing the percentage of GemC18 in the cells after 16 h of incubation in the presence or absence of NH₄Cl.

2.7. Cell apoptosis assay

TC-1-GR cells (2×10^4 cells/well) were seeded in a 24-well plate and incubated overnight at 37°C, 5% CO₂. The culture medium was then replaced with fresh medium containing different GemHCl or GemC18 formulations (50 µM GemHCl-equivalent), which were removed 2 h later and replaced with fresh culture medium. The cells were then cultured for 24 additional hours, harvested, resuspended in 0.1 mL of PBS (1% FBS), and stained with 0.1 mL of Guava Nexin[®] reagent (Millipore Corporation, Billerica, MA) for 20 min at room temperature in dark. The stained cells were filtrated through a cell strainer (70µm, BD Biosciences, Durham, NC) and analyzed using a Guava easyCyte 8HT Flow Cytometry System (Millipore Corporation). Four populations of cells can be distinguished, including viable cells (annexin V negative, 7-aminoactinomycin D (7-AAD) negative), early apoptotic cells (annexin V positive, 7-AAD negative), late apoptotic or dead cells (annexin V positive, 7-AAD positive), and cell debris (annexin V negative, 7-AAD positive), which are located in the lower left, lower right, upper right, and upper left quadrants of the cytograms, respectively. In order to test the effect of alkalinizing the lysosomal pH on the pro-apoptotic activity of various formulations, the 24 h incubation was also carried out in the presence of 50 mM NH₄Cl.

2.8. Intracellular fate of PEG-C18 micelles

TC-1-GR cells (5×10^4 cells/well) were seeded in a 35-mm poly-D-lysine coated glass bottom dish (Mattek Corporation, Ashland, MA) and incubated overnight. To study the intracellular localization of PEG-C18 micelles, the cells were incubated with DiO-loaded PEG-C18 micelles (100 µg/mL in culture medium) (Ex/Em, 488/501 nm) for 2 h, followed by 20 min of incubation with 500 nM LysoTracker Red DND-99 (Ex/Em, 577/590 nm) and 5 additional minutes of incubation with 5 µg/mL Hoechst33342 (a nuclear dye, Ex/Em, 345/478 nm). The cells were then rinsed with PBS and examined with a Leica TCS-SP5 confocal microscope with an oil immersion objective (63 × 1.4 NA) (Leica Microsystems GmbH, Mannheim, Germany).

In order to compare the intracellular fate of PEG-C18 micelles with different acid-sensitivity, the cells were incubated with 100 $\mu\text{g/mL}$ DiI/DiO-loaded PEG-C18 micelles for 2 h, followed by six additional hours of incubation after the micelle-containing medium was replaced with fresh medium. The cells were then examined using a Leica TCS-SP5 confocal microscope. Fluorescence images were acquired with the excitation wavelength of DiO (488 nm), and the spectral filter of 555–655 nm (for DiI detection) was used to record the FRET effect. All images were obtained with the same gain and offset. Seven to nine cells were randomly selected in the confocal images and analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD) to quantify the change of the FRET effect. The area of each cell was outlined based on its bright field image, and the intensity of the FRET spots was measured after thresholding. Only spots bigger than 1 pixel were included.

2.9. Western blot

TC-1-GR cells (3×10^5 cells/well) were seeded in a 6-well plate followed by overnight incubation at 37°C, 5% CO_2 . Fresh culture medium containing 1 μM of GemHCl-equivalent GemHCl, GemC18 or GemC18-loaded PEG-C18 micelles was added to the cells. After 24 additional hours of incubation, the cell lysates were prepared using Pierce® RIPA lysis buffer (Thermo Scientific, Pittsburgh, PA) containing the Halt™ protease inhibitor cocktail (Thermo Scientific). The TC-1-GR protein lysate (40 μg) was separated on a 7.5% Mini-Protea® TGX™ precast gel (Bio-Rad, Hercules, CA). Immunoblotting for RRM1 was performed using a rabbit polyclonal antibody against RRM1 (Aviva System Biology, San Diego, CA) and a polyclonal anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Aviva). β -Actin (mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a control. Bands of RRM1 and β -actin were detected using an enhanced chemiluminescence method (Pierce ECL Western Blotting Substrate, Thermo Scientific).

2.10. Determination of cellular dNTP and dFdCTP concentrations

TC-1-GR cells (1.5×10^6 cells/well) were seeded in a 75 cm^2 flask and incubated overnight at 37°C, 5% CO_2 . The cells were then incubated with GemHCl, GemC18 or GemC18-loaded PEG-C18 micelles (10 μM GemHCl-equivalent) for 24 h and harvested by trypsinization and centrifugation. The cell pellet were resuspended in 0.1 mL of PBS and treated with 6% (v/v) trichloroacetic acid for 10 min in an ice bath with occasional vortexing. After centrifugation at $15,500 \times g$ for 10 min at 4°C, the supernatant was collected and stored at -80°C . In each 100 μL of the sample, 4.3 μL of saturated sodium carbonate was added to neutralize the pH before analyzing the dNTP and dFdCTP concentrations using HPLC.

The HPLC conditions for dNTPs and dFdCTP analyses were adapted from previously published methods [30, 31]. An Agilent 1260 Infinity Quaternary Liquid Chromatographic System equipped with an Agilent ZORBAX Eclipse Plus C18 column (3.5 μm , 4.6 $\text{mm} \times 150 \text{ mm}$) was used for the analysis of both compounds. For the analysis of dNTPs, 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 ; and the detection wavelength was 254 nm. For the analysis of dFdCTP, 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 ; and the detection wavelength was 271 nm.

2.11. Data analysis

All data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were completed by performing analysis of variance followed by Fisher's protected least significant difference procedure. A *P* value of ≤ 0.05 (two-tail) was considered significant.

3. Results and discussion

3.1. Synthesis and characterization of PHC-2

Previously, we synthesized an acid-sensitive PEG-hydrazone-C18 (PHC-1) by conjugating PEG2000-hydrazide with octadecanal [20]. To develop a more acid-sensitive micelle material, we designed and synthesized a new PEG-hydrazone-C18, PHC-2, by conjugating PEG2000-aldehyde with stearic hydrazide (Scheme 1B). Stearic acid and Boc-protected hydrazine were coupled in the presence of EDCI to synthesize Boc-protected stearic hydrazide, which was further treated with TFA to give stearic hydrazide. The disappearance of the methyl proton peak of the Boc group at 1.47 ppm (Fig. S1 in supporting information) and the appearance of the hydrazide proton (CONHNH_2) peak at 7.56 ppm demonstrated the formation of stearic hydrazide (Fig. S2). The stearic hydrazide was then conjugated with PEG2000-aldehyde to obtain PHC-2, which was evidenced by: (1) the chemical shift of the aldehyde proton of PEG from 9.79 ppm (Fig. S2) to 7.17 ppm (Fig. 1A), likely due to the replacement of the aldehyde oxygen by the less electronegative nitrogen; (2) the chemical shift of the hydrazide proton (CONHNH_2) from 7.56 ppm (Fig. S2) to 8.56 ppm (CONHN=CH) (Fig. 1A), likely due to the effect of π -bond from the newly formed carbon-nitrogen double bond.

The characteristic changes in the chemical shift during the formation of the hydrazone bond were also used to evaluate the acid-sensitive degradation of the newly synthesized PHC-2. As shown in Fig. 1B, after the PHC-2 was incubated at different pH values for 2 h, the area of peak **b**, which corresponds to the NH proton (CONHN=CH , 8.56 ppm) of PHC-2, decreased as a function of time, while the area of peak **a**, which represents the aldehyde proton (CHO , 9.79 ppm) of PEG2000-aldehyde, increased gradually, indicating the degradation of PHC-2, and the generation of PEG2000-aldehyde. The degradation of the PHC-2 and the generation of the PEG2000-aldehyde were faster at lower pH (Fig. 1B). Further quantification of the degradation of PHC-2 by equation (1) revealed that PHC-2 was very sensitive at pH 5.5, with 49.1% degradation after only 2 h of incubation, and a complete degradation after 6 h (Fig. 1C). Even at pH 7.4, 54.2% of PHC-2 was degraded after 24 h of incubation (Fig. 1C). Therefore, PHC-2 is more acid-sensitive than the previously reported PHC-1, which at pH 5.5 degraded 31.7% after 2 h, and 46.2% after 6 h [20]. It was reported that the acid-sensitivity of the hydrazone bond is affected by the substituents on the carbonyl reaction partner, with electron donating substituents facilitating the protonation of the -C=N nitrogen and the subsequent acid-sensitive hydrolysis [32], which may explain why replacing the alpha oxy group of PEG in the PHC-1 with a more electron donating alkyl chain of stearic hydrazide in the PHC-2 further increased the acid-sensitivity of PHC-2, relative to PHC-1 (Scheme 1A).

The CMC value is an important parameter indicating the micelle forming ability of amphiphilic molecules. The CMC of PHC-2 was determined by fluorescence spectroscopy using pyrene as a probe, which is sensitive to the polarity change of its microenvironment [33]. Fig. 1D showed the polarity index (I_1/I_3) of pyrene as a function of the PHC-2 concentration. After fitting the data into a decreasing sigmoid of the Boltzmann type, the CMC value of PHC-2 was calculated to be $36.4 \pm 13.8 \mu\text{g/mL}$, which is in the same order of magnitude as the CMC values of PHC-1 ($72.5 \pm 0.3 \mu\text{g/mL}$) and PAC ($63.5 \pm 14.2 \mu\text{g/mL}$) [20], likely because all three PEG-C18 compounds have the same hydrophobic and

hydrophilic chains, just being conjugated together with different chemical bonds and/or in different orientations.

3.2. Preparation and characterization of GemC18-loaded PHC-2 micelles

The conjugation of stearic acid to gemcitabine enabled its incorporation into solid lipid nanoparticles and liposomes [34, 35]. Previously, we loaded 4-(*N*-stearoyl) gemcitabine, the lipophilic amide derivative of gemcitabine (GemC18), into the acid-sensitive PHC-1 micelles and acid-insensitive PAC micelles using a modified thin-film hydration method [20]. In the present study, we used the same method to incorporate GemC18 into the PHC-2 micelles. The GemC18-loaded PHC-1 micelles and PAC micelles were also prepared for comparisons.

As shown in Table 1, the entrapment efficiency of all the three micelles was around 100% when GemC18 was loaded at 5% (i.e., $W_{\text{GemC18}}/W_{\text{PEG-C18}} = 0.5 \text{ mg/10mg}$). The high entrapment efficiency may be attributed to the high affinity between the C18 chains of the GemC18 and the PEG-C18s. It was reported that fatty acid chains stabilize and hold together the phospholipid bilayers of the cell membrane by a variety of forces such as van der Waals interaction [36]. All the GemC18-loaded PEG-C18 micelles were around 35 to 40 nm, with a slightly negative zeta potential (Table 1). The similar physicochemical properties of the three micelles permit the use of them to study the effect of their acid-sensitivity on their ability to overcome gemcitabine resistance.

Acid-sensitive release of GemC18 from PHC-2 micelles was investigated at different pH. As seen in Fig. 2, the release rate of GemC18 from PHC-2 micelles was pH- and time-dependent. The GemC18-loaded PHC-2 micelles were relatively stable at pH 7.4. The percentage of GemC18 remaining in the micelles was $90.0 \pm 2.3\%$ and $71.3 \pm 9.8\%$ after 2 and 8 h of incubation, respectively, while it released all the GemC18 at 24 h. The release rate was higher at pH 6.8, with only $84.4 \pm 3.5\%$ of the GemC18 remaining in the micelles after 2 h, and a complete release within 5 h. The PHC-2 micelles released GemC18 even faster at pH 5.5; only $57.2 \pm 5.2\%$ of the GemC18 was in the micelles after 2 h of incubation and all GemC18 was released from the micelles within 3 h. The release of GemC18 from PHC-2 micelles is in fact faster than from our previously reported PHC-1 micelles [20]. The faster acid-sensitive degradation of the PHC-2 likely led to a faster destruction of the micelle structure, and consequently the faster release of the GemC18 from the PHC-2 micelles.

3.3. The cytotoxicity of GemC18-loaded acid-sensitive micelles in TC-1-GR cells

In order to investigate if the GemC18-loaded PEG-C18 micelles can overcome gemcitabine resistance caused by RRM1 overexpression, the TC-1-GR cells previously developed in our laboratory were used [13]. The IC_{50} values of each formulation in sensitive TC-1 cells or resistant TC-1-GR cells were summarized in Table 2. GemHCl showed the strongest cytotoxicity in TC-1 cells, with an IC_{50} value of $17.8 \pm 6.0 \text{ nM}$, while the GemC18 in solution and in micelles were less effective, showing increased IC_{50} values, which was in agreement with our previous results that GemHCl was more potent in killing sensitive tumor cells than GemC18, either in a solution or in nanoparticles [13, 20]. However, the IC_{50} value of GemHCl increased to $6.48 \pm 0.21 \text{ }\mu\text{M}$ in resistant TC-1-GR cells, showing a resistance index of 364.1 (Table 2). GemC18 in solution and GemC18 in acid-insensitive PAC micelles overcame gemcitabine resistance to some extent, showing a decreased resistance index. Acid-sensitive micelles were found to overcome resistance to a higher extent. The acid-sensitive PHC-1 micelles were more cytotoxic than GemHCl in TC-1-GR cells, with a 6.4-fold decreased resistance index. Most importantly, the PHC-2 micelles that are more acid-sensitive showed a 7.5-fold decrease in IC_{50} value and 35.1-fold decrease in resistance index, as compared with GemHCl (Table 2).

Hydrophobic modification of the amine group of gemcitabine with stearic acid has been reported to protect it from deamination [35]. The hydrophobized prodrug of gemcitabine, GemC18, might be able to enter cells either by simple passive diffusion when given as a solution or by endocytosis when incorporated into nanoparticles, thus potentially bypassing the requirement for active transport by nucleoside transporters. However, in the present study, it is unlikely that the GemC18-loaded acid-sensitive micelles overcome gemcitabine resistance by the above two well-known mechanisms, because the cytidine deaminase and mouse equilibrative nucleoside transporter 1 (ENT1) were expressed in similar levels in both cell lines [13]. On the other hand, TC-1-GR cells overexpress RRM1 as compared to TC-1 cells, which explains why the TC-1-GR cells are resistant to gemcitabine both *in vitro* [8, 37] and *in vivo* [38, 39]. Therefore, the GemC18-loaded acid-sensitive micelles can overcome gemcitabine resistance caused by RRM1 overexpression, and GemC18 loaded in the more acid-sensitive PHC-2 micelles was more effective than loaded in the less acid-sensitive PHC-1 micelles.

3.4. Cellular uptake of various GemC18 formulations and the uptake mechanisms

Cellular uptake is an important determinant of the cytotoxicity of a chemotherapeutic drug. We evaluated the uptake of GemC18 by incubating TC-1-GR cells with GemC18 in a solution or in three different PEG-C18 micelles for 2 or 6 h. In general, the percentage of GemC18 that was internalized by the cells was significantly less when GemC18 was in micelles than in solution (Fig. 3A), likely because GemC18 in solution simply diffused into the cells, while the GemC18-loaded micelles entered the cells by endocytosis. As a matter of fact, it has been reported that the cellular uptake of small molecular drugs is generally higher when it was in the free form than in a particulate delivery system [20, 40]. Among the three different micelles, the percentage of GemC18 entered the cells was not different after 2 h of incubation (Fig. 3A). However, after 6 h of incubation, the percentage of GemC18 in PHC-2 micelles that was taken up by the cells was 1.16- and 1.19-fold higher than that in PAC and PHC-1 micelles, respectively ($P < 0.05$). The higher percentage of GemC18 uptake in PHC-2 micelles by TC-1-GR cells after 6 h of incubation may be due to the extracellular release of GemC18 from the micelles during incubation. As seen in Fig. 2, around 20% of the GemC18 that was loaded in the PHC-2 micelles was released after 6 h of incubation at pH 7.4, while only less than 8% was released from the PHC-1 and PAC micelles even after 8 h of incubation at the same pH [20].

In order to exclude the possibility that the GemC18 released extracellularly from PHC-2 micelles may have led to its increased cytotoxicity, we pre-incubated TC-1-GR cells with the three different micelles for 2 h and then replaced the medium with drug-free fresh medium, which should allow the same percentage of GemC18 to enter the cells for all three micelles as shown in Fig. 3A. Cell apoptosis was analyzed 24 h later. Pre-treatment of the cells with GemC18 alone or GemHCl in solution did not significantly increase the percentage of apoptotic cells, as compared to pre-treatment with drug-free medium alone. However, pre-treatment with the GemC18-loaded micelles induced cell apoptosis, and the extent of apoptosis was larger for cells pretreated with the acid-sensitive PHC-1 and PHC-2 micelles, more extensive for the PHC-2 micelles (Fig. 3B). Quantification of the cell population after different treatments is summarized in Fig. 3C. Less than 1% of early apoptotic cells and around 3% of late apoptotic and dead cells were found in the untreated control, GemHCl, and GemC18 groups. However, the percentage of the early apoptotic cell increased to $2.0 \pm 0.1\%$, $2.5 \pm 0.4\%$ and $9.4 \pm 0.8\%$ in TC-1-GR cells treated with GemC18-loaded PAC, PHC-1, and PHC-2 micelles, respectively. Similarly, the percentage of the late apoptotic and dead cells also increased in TC-1-GR cells pre-treated with acid-sensitive micelles, especially with the more acid-sensitive PHC-2 micelles. Finally, similar to other previously reported acid-sensitive micelles, when administered *in vivo*, our PHC-2 micelles

may respond to the slightly acidic tumor microenvironment (pH 6.8) and release the GemC18 incorporated in them extracellularly before the micelles are internalized by endocytosis, which may decrease the ability of the acid-sensitive micelles to overcome gemcitabine resistance. Strategies that make the micelles less sensitive to the slightly acidic tumor microenvironment (pH 6.8), but more sensitive in the more acidic lysosomal environment (pH 5.5) will likely address this concern.

Given that all three micelles delivered the same amount of GemC18 into the TC-1-GR cells within 2 h, their different pro-apoptotic activities are likely due to different rates of intracellular metabolism of GemC18 after cellular uptake. In fact, data in Fig. 3A showed significantly more GemC18 in solution was internalized by the TC-1-GR cells after 2 h of incubation with the GemC18 in micelles, but the GemC18 in solution failed to induce any significant apoptosis, indicating that how the GemC18 entered the cells is critical for it to induce apoptosis in the gemcitabine resistant TC-1-GR cells.

The lipophilic GemC18 alone likely enters the cells by simple passive diffusion, whereas the GemC18-loaded micelles likely enter the cells by endocytosis. To confirm whether GemC18 in solution and in micelles entered the cells by different routes, we first determined the cell uptake of GemC18, in a solution or in micelles, by TC-1-GR cells at 4°C. Endocytosis is energy-dependent, and thus is inhibited when the cells are incubated at 4°C. As shown in Fig. 4A, the uptake of GemC18 alone in solution by TC-1-GR cells did not significantly change when the cells were incubated at 4°C, as compared to at 37°C. However, the uptake of GemC18 in micelles was almost completely inhibited when incubated at 4°C, suggesting that the uptake of GemC18 in solution occurred independent of energy, while the uptake of GemC18 loaded in micelles was via an energy-dependent endocytosis. In fact, the uptake of the GemC18-loaded micelles by TC-1-GR cells was likely by clathrin-mediated endocytosis, because, as shown in Fig. 4B, only chlorpromazine, the inhibitor of clathrin-mediated endocytosis, significantly inhibited the uptake of the GemC18-loaded micelles ($P < 0.01$), whereas filipin, wortmannin and cytochalasin B did not.

3.5. Cellular level acid-sensitivity of various PEG-C18 micelles

Clathrin-mediated endocytosis is the main mechanism for the internalization of macromolecules and plasma membrane constituents for most cell types [41], which delivered the internalized drug delivery systems to the endolysosomal systems, where the acidic condition has been utilized in designing drug delivery systems for acid-sensitive release of drugs carried by the delivery systems [42–44]. In order to confirm the lysosomal delivery of these PEG-C18 micelles, DiO-labeled PEG-C18 micelles were used. As seen in Fig. 5A, the green fluorescence of DiO-labeled micelles almost completely overlapped with the LysoTracker Red, a marker specific to late endosomes and lysosomes, indicating all three PEG-C18 micelles were delivered to the lysosomes after endocytosis. Fluorescence resonance energy transfer (FRET) technique was further used to study the cellular level acid-sensitivity of the PEG-C18 micelles. When the micelles that are incorporated with both DiO (donor, Ex/Em 488/501 nm) and DiI (acceptor, Ex/Em 501/565 nm) are excited at 488 nm, it is expected that they will give a red fluorescence from DiI because of the energy transfer from the donor to the acceptor within the Förster distance. The intensity of the red fluorescence, FRET effect, gradually decreases with the increased distance between the FRET pair when the micelles dissociate and release the FRET pair. As shown in Fig. 5B, a bright red color was observed when TC-1-GR cells were pre-incubated with all three DiO/DiI-loaded PEG-C18 micelles. After 6 h of incubation, no significant change in the fluorescent intensity was observed in cells pre-incubated with the acid-insensitive PAC micelles. However, a significant decrease in the fluorescent intensity was observed in cells pre-incubated with the acid-sensitive PHC-1 or PHC-2 micelles, and the fluorescence almost completely disappeared in cells that were pre-incubated with the more acid-sensitive PHC-2

micelles. A quantification of the fluorescent intensity of the confocal images showed that $95.8 \pm 44.3\%$, $53.5 \pm 18.7\%$ and $13.0 \pm 5.6\%$ of the original fluorescent intensity remained in cells that were pre-incubated with the PAC, PHC-1, and PHC-2 micelles, respectively (Fig. 5C). The fluorescent intensity in cells treated with the PHC-2 micelles was significantly lower than that in cells treated with PHC-1 and PAC micelles (Fig. 5C) ($P < 0.01$). The intracellular acid-sensitivity of those three micelles is in agreement with the acid-sensitivity of the micelle materials and the acid-sensitive release of GemC18 from these micelles. Indeed PHC-2 molecules were completely degraded after 6 h of incubation at pH 5.5 (Fig. 1C), and a complete release of GemC18 from the PHC-2 micelles was observed as early as after 3 h of incubation at the same pH (Fig. 2).

3.6. The effect of the acidic condition of lysosomes on cell apoptosis and the metabolism of GemC18

Lysosomal delivery of the GemC18-loaded PEG-C18 micelles and the subsequent different acid-sensitive release of the GemC18 from the micelles in the lysosomes appeared be responsible for their different ability to overcome gemcitabine resistance. To further confirm this hypothesis, ammonium chloride (NH_4Cl), a weak base lysosomotropic alkalization agent, was used to neutralize lysosomes [45]. TC-1-GR cells that had been pre-treated with either GemC18 or GemC18-loaded micelles for 2 h were further incubated for 24 h with fresh medium with NH_4Cl (50 mM). Cell apoptosis was analyzed after Annexin V and 7-AAD staining. As shown in Fig. 6A, $13.7 \pm 1.0\%$ of the cells that were pre-treated with GemC18 were apoptotic (Annexin V positive), which was not different from the untreated control. The percentage of apoptotic cells increased to $16.6 \pm 0.7\%$, $18.8 \pm 1.5\%$ and $21.1 \pm 2.0\%$ for cells that were pre-treated with GemC18-loaded PAC, PHC-1 and PHC-2 micelles (Fig. 6A). For comparison, the percentages of the apoptotic cells in TC-1-GR cells that received the same pre-treatments were incubated for additional 24 h in fresh medium in the absence of NH_4Cl are shown in Fig. 6B. In the absence of NH_4Cl , the percentage of the apoptotic cells were $3.8 \pm 1.1\%$, $3.8 \pm 0.8\%$, $7.2 \pm 0.8\%$, $10.1 \pm 1.2\%$ and $28.1 \pm 3.2\%$ for cells that were untreated or pre-treated with GemC18, PAC, PHC-1, or PHC-2 micelles respectively.

It should be noted that NH_4Cl (50 mM) also induced a certain degree of apoptosis in the control group, even though it did not show cytotoxic effect in the same incubation period when determined using MTT assay (data not shown). Therefore, we normalized the pro-apoptotic activity of various GemC18 formulations in TC-1-GR cells in the presence or absence of NH_4Cl , by dividing the percentage of apoptotic cells induced by GemC18 formulations by the percentage of apoptotic cells induced when TC-1-GR cells were cultured in GemC18-free medium, in the presence or absence of NH_4Cl . As shown in Fig. 6C, NH_4Cl did not change the relative pro-apoptotic activity of the GemC18 in solution. However, when GemC18 was delivered into the cells by the micelles, its pro-apoptotic activity was inhibited by NH_4Cl , especially for the acid-sensitive micelles, more significantly for the PHC-2 micelles. In other words, NH_4Cl decreased the pro-apoptotic activity of the GemC18-loaded PEG-C18 micelles, but not GemC18 in solution, likely because the NH_4Cl inhibited the acid-sensitive release of GemC18 from the micelles when the lysosomal pH was increased by NH_4Cl . It is also noted that the pro-apoptotic activity of the GemC18 in micelles, especially in the acid-sensitive PHC-1 and PHC-2 micelles, was not completely inhibited by NH_4Cl , likely because the acid-sensitive micelles, especially the PHC-2 micelles, can be destabilized even at pH 7.4 (Fig. 2) [20].

Enzymes in the lysosomes such as cathepsin B (a cysteine protease) and cathepsin D (an aspartic protease) are likely involved in the hydrolysis of GemC18 to produce the active gemcitabine [35]. In fact, increasing the pH in the lysosomes with NH_4Cl not only inhibited the release of GemC18 from acid-sensitive micelles, but also inhibited the hydrolysis of

GemC18 (Fig. 6D). The intracellular stability of GemC18 when delivered into the cells as a solution or various micelles was investigated in the presence or absence of NH_4Cl . As shown in Fig. 6D, $22.0 \pm 0.7\%$ of GemC18 that was internalized by TC-1-GR cells as a solution was recovered after 16 h, and NH_4Cl did not affect the percentage of recovery. The recovered percentage of GemC18 was decreased when it was delivered into the cells using the micelles, to a larger extent for the PHC-1 and PHC-2 micelles. In fact, only $9.1 \pm 1.0\%$ of GemC18 that was delivered into the cells by the PHC-2 micelles was recovered after 16 h of incubation in the absence of NH_4Cl (Fig. 6D). It is possible that the faster release of GemC18 from the acid-sensitive micelles in the lysosomes resulted in the faster hydrolysis of GemC18 and the faster generation of gemcitabine. NH_4Cl did not affect the intracellular degradation of GemC18 when it was given in a solution (Fig. 6D). It only slightly slowed down the degradation of GemC18 when given by the PAC micelles ($P > 0.05$), but significantly slowed down the intracellular degradation of GemC18 when given by the PHC-1 and PHC-2 micelles ($P < 0.01$), to a larger extent by the PHC-2 micelles, likely because it inhibited the acid-sensitive release of the GemC18 from the micelles and the enzymatic hydrolysis of the GemC18 to generate the gemcitabine. Therefore, normal acidic lysosomes are required for the GemC18 to induce apoptosis when it is delivered into cells by acid-sensitive micelles.

3.7. The effect of various GemC18 formulations on RRM1 expression and cellular level of dFdCTP

Finally, to gain insight on how the GemC18 loaded in the highly acid-sensitive PHC-2 micelles more effectively overcome gemcitabine resistance caused by RRM1 overexpression, we investigated the effect of the GemC18-loaded micelles on RRM1 expression and on the levels of dNTPs and dFdCTP in TC-1-GR cells. As shown in Fig. 7A, after 24 h of incubation with GemHCl, GemC18 in solution, or GemC18-loaded PAC, PHC-1 or PHC-2 micelles, only GemC18-loaded PHC-2 micelles significantly decreased the expression of RRM1 in TC-1-GR cells; the GemC18-loaded PHC-1 micelles also slightly decreased RRM1 level. The effect of different GemHCl or GemC18 formulations on the total dNTP pool size in TC-1-GR cells was also studied. As shown in Fig. 7B, GemHCl significantly increased the dNTP pool size from 410.1 ± 42.9 to 535.6 ± 43.4 pmol/ 10^6 cells ($P < 0.05$). It is known that gemcitabine up-regulates RRM1 level [46]. GemC18 in solution, GemC18-loaded PAC and PHC-1 micelles all did not show any significant effect on the dNTP pool size, as compared with untreated control ($P > 0.05$). However, GemC18 loaded in the highly acid-sensitive PHC-2 micelles significantly decreased the dNTP pool size of TC-1-GR cells to 73.0% ($P < 0.05$) of the GemHCl group (55.9% of the untreated control ($P < 0.01$)). This is in agreement with the significant down-regulation of the RRM1 level in TC-1-GR cells by GemC18 in the highly acid-sensitive PHC-2 micelles (Fig. 7A), because ribonucleotide reductase, comprised of RRM1 and RRM2 subunits, is responsible for the conversion of NDPs to dNDPs, which are then converted to dNTPs.

Gemcitabine is a prodrug; it is its active metabolite dFdCTP that inhibits tumor cell growth by competing with cellular dNTPs for incorporation into DNA. Thus, we further measured the cellular dFdCTP level after TC-1-GR cells were treated with different GemHCl or GemC18 formulations. Incubation of TC-1-GR cells with GemC18 loaded in the highly acid-sensitive PHC-2 micelles more than doubled the intracellular dFdCTP level, as compared to incubation with GemHCl (Fig. 7C), whereas GemC18 in the PAC and PHC-1 micelles only slightly increased the intracellular dFdCTP level, and GemC18 alone in a solution did not show any effect, as compared to GemHCl (Fig. 7C). Given that the dFdCTP inhibits DNA synthesis by competing with cellular dNTPs for incorporation into DNA [47, 48], we calculated the intracellular dFdCTP/dNTP ratio. As seen in Fig. 7D, when TC-1-GR cells were treated with the GemC18-loaded PHC-2 micelles, the intracellular dFdCTP/dNTP

ratio was close to 4-fold higher than that in cells treated with GemHCl. Therefore, the ability of GemC18-loaded PHC-2 micelles to decrease RRM1 expression and to increase the intracellular dFdCTP, and thus dFdCTP/dNTP ratio, likely explains why the GemC18 loaded in the highly acid-sensitive PHC-2 micelles can more effectively overcome gemcitabine resistance caused by RRM1 overexpression than GemC18 loaded in the less acid-sensitive PHC-1 micelles.

Previously, we have shown that the TC-1-GR cells are resistant to gemcitabine because they overexpress RRM1 [13]. The TC-1-GR cells also have a significantly higher level of dNTPs than the parent TC-1 cells (Wonganan et al., unpublished data) [14]. After incubation with gemcitabine (same concentration, same time period), the intracellular level of dFdCTP in TC-1-GR cells was significantly less than in TC-1 cells (Wonganan et al., unpublished data) [14], which explains why the TC-1-GR cells are resistant to gemcitabine. However, when the TC-1-GR cells were treated with GemC18 in our solid-lipid nanoparticles (GemC18-SLNs), RRM1 expression was decreased and the intracellular level of dFdCTP was increased (Wonganan et al., unpublished data) [14], which explains why our GemC18-SLNs can overcome gemcitabine resistance caused by RRM1 overexpression. It becomes clear recently that the GemC18-SLNs are able to overcome gemcitabine resistance in TC-1-GR cells because they deliver the GemC18 into lysosomes, and it seems that the gemcitabine hydrolyzed from GemC18 in lysosomes can be more efficiently convert to dFdCTP (Wonganan et al., unpublished data). In the present study, we showed that our GemC18-loaded PHC-2 micelles are more cytotoxic than PHC-1 micelles in the gemcitabine resistant TC-1-GR cells that overexpress RRM1, likely because the GemC18 in the highly acid-sensitive PHC-2 micelles is more quickly released in the lysosomes after endocytosis, hydrolyzed to release gemcitabine, and the gemcitabine is then more efficiently phosphorylated by dCK to dFdCMP, which is then sequentially further phosphorylated to dFdCDP and dFdCTP.

Conclusion

In the present study, we synthesized a new acid-sensitive PHC-2 molecule that self-assembles into micelles. GemC18 loaded in the PHC-2 micelles can overcome gemcitabine resistance caused by RRM1 overexpression. Using the highly acid-sensitive PHC-2 micelles, the previously synthesized less acid-sensitive PHC-1 micelles or acid-insensitive PAC micelles as the carriers for GemC18, we showed that the increased acid-sensitive release of the GemC18 from its delivery systems once internalized into lysosomes via endocytosis and the rapid hydrolysis of gemcitabine from the GemC18 in the lysosomes are critical for the gemcitabine to effectively down-regulate RRM1 expression, increase the intracellular dFdCTP level, and ultimately kill the tumor cells that overexpress RRM1. The strategy of conjugating a nucleoside analog with a (long-chain) fatty acid and delivering the resultant derivative using highly acid-sensitive micelles may represent a new platform technology to increase the antitumor activity of nucleoside analogs and to overcome tumor cell resistance against them.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Characterization of PHC-2. (A) ^1H NMR spectrum of PHC-2 in CDCl_3 . (B) ^1H NMR spectra of PHC-2 in CDCl_3 after incubation up to 24 h at pH 7.4, 6.8 or 5.5, 37°C . a, CHO from PEG-aldehyde, 9.79 ppm; b, CONHN=CH from PHC-2, 8.56 ppm. (C) Time- and pH-dependent degradation profiles of PHC-2 based on ^1H NMR spectra in B. (D) The plot of I_1/I_3 values versus the concentrations of PHC-2 in water ($n = 3$).

Fig. 2.
In vitro release of GemC18 from PHC-2 micelles in PBS of different pH values (5.5, 6.8, or 7.4) at 37°C ($n = 3$).

Fig. 3. Cellular uptake and apoptosis studies. **(A)** The percentage of GemC18 internalized by TC-1-GR cells after incubation with GemC18, in a solution or in different micelles, for 2 h or 6 h ($n = 3$). a, $P < 0.05$, PHC-2 vs. PAC or PHC-1 for 6 h. **(B)** Representative cytograms of cell apoptosis analysis of TC-1-GR cells after treatment with GemHCl, GemC18, GemC18-loaded PAC, PHC-1 or PHC-2 micelles. Untreated cells were used as control. **(C)** The percentage of different TC-1-GR cell populations (nuclear debris, early apoptotic, late apoptotic and dead, and viable cells) after the treatment with GemHCl, GemC18, GemC18-loaded PAC, PHC-1 or PHC-2 micelles ($n = 3$).

Fig. 4. Mechanisms of the cellular uptake of GemC18 in solution or in various micelles. **(A)** A comparison of cellular uptake GemC18 in solution or in micelles at 4°C vs. 37°C ($n = 3$). a, $P < 0.01$ vs. the control. **(B)** The effect of various specific endocytosis inhibitors on the cellular uptake of GemC18 in three different PEG-C18 micelles ($n = 3$). b, $P < 0.01$ vs. the control.

Fig. 5.

A comparison of the intracellular fate of PEG-C18 micelles with different acid-sensitivity. (A) Colocalization of DiO-loaded PEG-C18 micelles (green) and lysosomes (red). Cell nuclei are in blue. Bar, 20 μm . (B–C) The change of FRET effect in TC-1-GR cells. Cells were pre-incubated with DiI/DiO-loaded PEG-C18 micelles for 2 h, followed by six additional hours of incubation in fresh medium. Bar, 20 μm . Values in (C) are derived from the confocal micrographs in B ($n = 7-9$). a, $P < 0.05$ vs. PAC; b, $P < 0.01$ vs. PAC and PHC-1.

Fig. 6.

The effect of NH₄Cl on the pro-apoptotic activity of GemC18 in solution or in micelles against TC-1-GR cells and the intracellular degradation of GemC18. The percentage of apoptotic cells after TC-1-GR cells were incubated in the presence (**A**) or absence (**B**) of NH₄Cl for 24 h. Cells were pre-incubated for 2 h with GemC18, GemC18-loaded PAC, PHC-1 or PHC-2 micelles. Cells pre-incubated with fresh medium were used as a negative control ($n = 3$). (**C**) The effect of NH₄Cl on the pro-apoptotic activity of the GemC18 in TC-1-GR cells, relative to the negative untreated control. (**D**) The percentage of GemC18 remaining in TC-1-GR cells 16 h after they were incubated in the presence or absence of NH₄Cl after internalization. Cells were pre-treated with GemC18, GemC18-loaded PAC, PHC-1 or PHC-2 micelles for 2 h to allow the internalization of GemC18 ($n = 3$). a, $P < 0.01$, 0 mM vs. 50 mM NH₄Cl.

Fig. 7.

The effect of different formulations of GemHCl or GemC18 on RRM1 expression and dNTP and dFdCTP levels in TC-1-GR cells. **(A)** Immunoblotting analysis of RRM1 expression in TC-1-GR cells treated with GemHCl, GemC18, GemC18-loaded PAC, PHC-1, or PHC-2 micelles. Untreated cells were used as the control (Shown are typical data from more than 3 replicates). **(B)** Cellular dNTP level after treatment with GemHCl, GemC18, GemC18-loaded PAC, PHC-1, or PHC-2 micelles ($n = 3$). Untreated cells were used as the control. a, $P < 0.05$ vs. control; b, $P < 0.05$ vs. GemHCl; c, $P < 0.01$ vs. GemHCl; d, $P < 0.05$ vs. control. **(C)** Cellular dFdCTP level after treatment with GemHCl, GemC18, GemC18-loaded PAC, PHC-1, or PHC-2 micelles ($n = 3$). e, $P < 0.01$ vs. GemHCl and GemC18; f, $P < 0.01$ vs. others. **(D)** The ratio of dFdCTP to total dNTPs in TC-1-GR cells after treatment with GemHCl, GemC18, GemC18-loaded PAC, PHC-1, or PHC-2 micelles ($n = 3$). g, $P < 0.01$ vs. others; h, $P < 0.05$ vs. others.

Scheme 1.

Schematic representation of micelle formation and the reaction process. **(A)** Chemical structures of the three PEG-C18 micelle materials, illustration of the preparation of GemC18-loaded micelles, and the expected acid-sensitive release profiles of GemC18 from the micelles. **(B)** The scheme of the synthesis of PHC-2 conjugate.

Table 1Characterization of GemC18-loaded PEG-C18 micelles ($n = 3$)

	Drug loading (%)	Entrapment efficiency (%)	Particle size (nm)	Zeta potential (mV)
GemC18-PAC micelles	4.7 ± 0.03	98.8 ± 0.7	37.9 ± 0.5	-2.1 ± 0.5
GemC18-PHC-1 micelles	4.9 ± 0.2	103.1 ± 4.4	34.9 ± 4.1	-1.9 ± 1.3
GemC18-PHC-2 micelles	4.6 ± 0.2	97.5 ± 5.0	37.7 ± 0.8	-2.5 ± 0.7

Table 2

IC₅₀ values of various GemHCl and GemC18 formulations in TC-1 and TC-1-GR cells ($n = 4-5$) and the calculated resistance indices

	IC ₅₀		Resistance index
	TC-1 (nM)	TC-1-GR (μM)	
GemHCl	17.8 ± 6.0	6.48 ± 0.21	364.1
GemC18 in solution	75.2 ± 4.9	10.50 ± 1.12	139.5
GemC18-PAC micelles	120.7 ± 32.8	12.23 ± 0.75	101.3
GemC18-PHC-1 micelles	85.3 ± 4.9	4.88 ± 2.93	57.2
GemC18-PHC-2 micelles	82.9 ± 4.2	0.86 ± 0.03	10.4