**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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**Supplementary Information**

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**Supplementary Methods**

*Synthesis of 3′-(O)-stearoyl gemcitabine*

Gemcitabine HCl were Boc protected by di-*tert*-butyl dicarbonate in the presence of 4-dimethylaminopyridine (DMAP) and triethylamine (TEA) in 1,4-dioxane. After 18 h of reaction at 24°C, the reaction mixture was chromatographed and concentrated in a non-polar product mixture. It was added to methanol and 1 M sodium carbonate. After 4 h, about 1:1 mixture of the two products were obtained, 4-(*N*)-5′-(*O*)-bis(tert-butoxycarbonyl) gemcitabine and 4-(*N*)-tert-butoxycarbonyl gemcitabine. The mixture was column purified, and the 4-(*N*)-5′-(*O*)-bis(tert-butoxycarbonyl) gemcitabine was conjugated with stearic acid in the presence of DMAP and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide (EDCI) in dichloromethane (DCM). The (tert-Butyl carbamates) Boc groups were removed using trifluoroacetic acid (TFA) in DCM to obtain 3′-(*O*)-GemC18 as a white powder. $^{1}$H NMR (300 MHz, THF-d$^{4}$) δ 7.95 (1H, d, *J* = 6.3 Hz, 6-C**H**), 6.32 (1H, t, *J* = 9.3 Hz, 1′-C**H**), 6.10 (1H, brd, 5-C**H**), 5.54–5.45 (1H, m, 3′-C**H**), 4.17–3.69 (3H, overlapping m, 4′-C**H** and 5′-C**H$_2$**), 2.43 (2H, t, *J* = 7.4 Hz, CO–C**H$_2$**), 1.64 (2H, brt, *J* = 7.2 Hz, CO−CH$_2$−C**H$_2$**), 1.40–1.16 (28H, m, C**H$_2$**), 0.89 (3H, brt, *J* = 6.5 Hz, terminal C**H$_3$**). ESI-HRMS $[M+H]^+$ *m/z* calculated for $C_{27}H_{46}F_2N_3O_5$: 530.3400, found: 530.33980.

*Synthesis of 4-(N)-octyl gemcitabine*

Gemcitabine HCl were Boc protected by di-*tert*-butyl dicarbonate in anhydrous dioxane in the presence of 1N KOH. The Boc protected product of 3′-5′-(*O*)-bis(*tert*-butoxycarbonyl) gemcitabine, octanoic acid, and 1-Hydroxy-7-azabenzotriazole (HOAt) in anhydrous DCM were pre-cooled to 4°C, followed by the addition of EDCI. The mixture was de-gassed by vacuum sonication and then stirred at room temperature under argon for about 40 h. Water was added to the reaction mixture and extracted with the mixture of ethyl acetate (EtOAc) and hexane (2:1). The combined organic phase was washed with saturated $NH_4Cl$ and brine and then dried over anhydrous $Na_2SO_4$. The solvent was evaporated, and the residue was purified by column chromatography (EtOAc : hexane, 3:7). The conjugated amide was

isolated as a white powder, and deprotection was performed with TFA in DCM. This solution was stirred at room temperature for 2 h, and excess TFA was removed under reduced pressure. The concentrated sample was co-distilled with DCM for 3 times. The crude sample was chromatographed on silica gel (DCM : ethanol, 94:6).[53] The desired fractions were pooled, and the solvent was evaporated to yield 4-(*N*)-GemC8 as a white powder. $^1$H NMR (300 MHz, THF-d$^4$) δ 10.15 (1H, s, N**H**CO), 8.19 (1H, d, *J* = 7.8 Hz, 6-C**H**), 7.38 (1H, d, *J* = 7.5 Hz, 5-C**H**), 6.26 (1H, t, *J* = 7.7 Hz, 1′-C**H**), 4.38–4.27 (1H, m, 3′-C**H**), 3.93-3.73 (3H, overlapping m, 4′-C**H** and 5′-C**H$_2$**), 2.41 (2H, t, *J* = 7.4 Hz, CO–C**H$_2$**), 1.67–1.62 (2H, m, CO−CH$_2$−C**H$_2$**), 1.40–1.20 (8H, m, C**H$_2$**), 0.89 (3H, t, *J* = 6.9 Hz, terminal C**H$_3$**). ESI-HRMS [M+H]$^+$ *m*/*z* calculated for $C_{27}H_{46}F_2N_3O_5$: 390.18350, found: 390.18295.

*Synthesis of 4-(N)-steroyl cytarabine (cytarabine-C18, Ara-C-C18)*

The 2′-3′-5′-(*O*)-tris(*tert*-butoxycarbonyl) Ara-C was prepared by di-*tert*-butyl dicarbonate in anhydrous dioxane in the presence of 1N KOH. The conjugation of 2′-3′-5′-(*O*)-tris(*tert*-butoxycarbonyl) Ara-C with stearic acid was performed in HOAt and EDCI in anhydrous DCM under argon for about 40 h at room temperature. After conjugation, Boc groups were removed by TFA in DCM at room temperature for 2 h. The crude sample was chromatographed on silica gel (DCM : ethanol, 94:6).[53] The desired fractions were pooled, and the solvent was evaporated to yield 4-(*N*)-Ara-C-C18 as a white powder.$^1$H NMR (300 MHz, THF-d$^4$) δ 7.9 (1H, brd, 6-C**H**), 7.4 (1H, brd, 5-C**H**), 6.2 (1H, 1′-C**H**), 4.4–4.2 (6H, overlapping m,2′-C**H$_2$**, 3′-C**H**, 4′-C**H** and 5′-C**H$_2$**), 2.4 (2H, brt, CO–C**H$_2$**), 1.6–1.7 (2H, m, CO−CH$_2$−C**H$_2$**), 1.40–1.20 (28H, m, C**H$_2$**), 0.9 (3H, brt, terminal C**H$_3$**). ESI-HRMS [M+Na]$^+$ *m*/*z* calculated for $C_{27}H_{47}N_3O_6Na$: 532.3357, found: 532.3354.

*Determination of the content of GemC18 in (endo)lysosomes*

TC-1-GR (3 x 10$^6$ cells) were seeded into 100-mm cell culture dishes and incubated overnight at 37°C, 5% $CO_2$. The medium was then replaced with fresh medium containing 20 μM of 4-(*N*)-GemC18 in dimethyl sulfoxide (DMSO) or in 4-(*N*)-GemC18-SLNs and incubated for 3 h at 37°C, 5% $CO_2$. The

culture medium was removed. Cells were washed three times with cold PBS and incubated with 1 ml of protease solution (1 mg/ml) in protective buffer at 4°C for 15 min. To stop protease activity, 100 µl of FBS was added. The suspension was then centrifuged at 300 x g for 5 min. The cell pellet was resuspended in 500 µl of protective buffer supplemented with 500 ppm digitonin to permeabilize the plasma membrane. After 15 min of incubation at 4°C, the cells were then centrifuged at 500 x g for 5 min. The supernatant was collected and mixed with calcium chloride to a final concentration of 8 mM to precipitate the rough endoplasmic reticulum and any mitochondria that were in the fraction. The mixture was centrifuged at 5,000 x g for 15 min. The supernatant was then collected, lyophilized, dissolved in methanol and centrifuged. The concentration of GemC18 in the supernatant was determined using HPLC.

*Quantification of intracellular deoxynucleotide and nucleoside triphosphate*

Cells were washed with ice-cold PBS and harvested. The cell pellets were resuspended in PBS and de-proteinized with 6% trichloroacetic acid (TCA), vortexed for 20 s, ice-bathed for 10 min, and then vortexed again for 20 s. Acidic cell extracts were centrifuged at 13,000 x g for 10 min at 4°C. The supernatant was neutralized with 5 M $K_2CO_3$. Separation and quantification of deoxynucleotides and nucleoside triphosphate were carried out using HPLC.

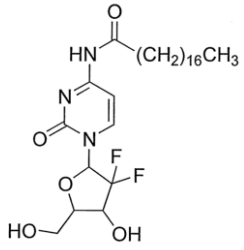
*Quantification of intracellular gemcitabine triphosphate*

Cells were washed with ice-cold PBS and harvested. The cell pellets were resuspended in PBS and treated with 40% TCA. The mixture was vortexed and placed in an ice bath for 20 min. Acidic cell extracts were centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was collected and mixed with a freshly prepared trioctylamine:freon (1,1,2-trichlorotrifluoroethane) mixture. After centrifugation at 16,000 x g for 2 min at 4°C, the organic layer was carefully removed. The extraction procedure was repeated three times to clean and neutralize the sample. The final aqueous layer was analyzed for gemcitabine triphosphate using HPLC.

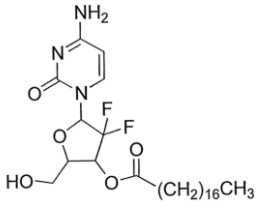
**Supplementary Figures**

**Figure S1**

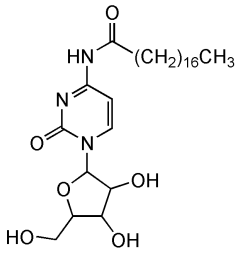
**A** **B** **C**




4-(*N*)-GemC18




3′-(*O*)-GemC18




4-(*N*)-Ara-C-C18

**Figure S1.** Chemical structure of **(A)** 4-(*N*)-GemC18 **(B)** 3′-(*O*)-GemC18 and (**C**) 4-(*N*)-Ara-C-C18

**Figure S2**

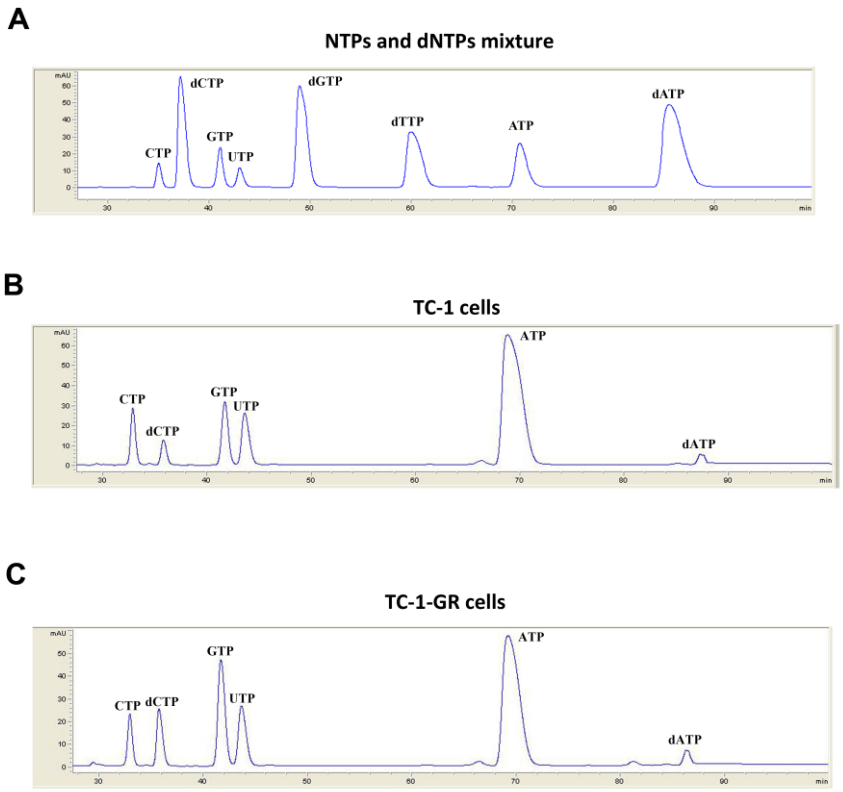



**Figure S2. (A)** Typical HPLC chromatograms of a standard mixture of cytidine triphosphate (CTP), deoxycytidine triphosphate (dCTP), guanosine triphosphate (GTP), uridine triphosphate (UTP), deoxyguanosine triphosphate (dGTP), deoxythymidine triphosphate (dTTP), adenosine triphosphate (ATP) and deoxyadenosine triphosphate (dATP). **(B-C)** HPLC chromatograms representative of intracellular nucleoside triphosphates and deoxynucleotides in TC-1 **(B)** and TC-1-GR cells **(C)**.

**Figure S3**

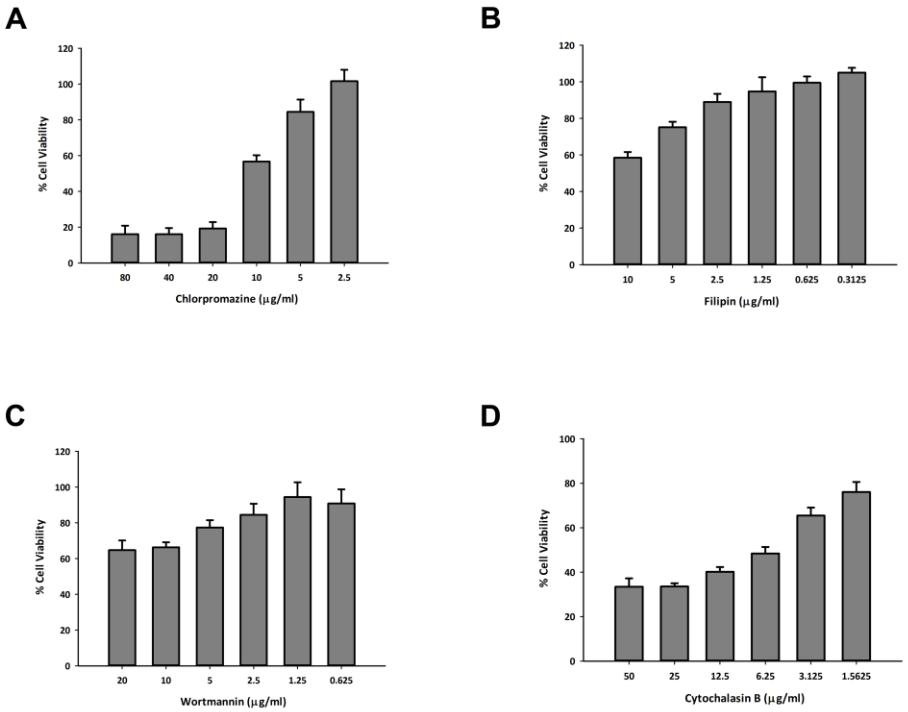



**Figure S3.** The cytotoxicity of chlorpromazine **(A),** filipin **(B)**, wortmannin **(C)**, and cytochalasin B **(D)** in TC-1-GR cells after 2.5 h of incubation (n = 4). For cytochalasin B, we ultimately used 20 ng/ml in the inhibition study because at 20 ng/ml of cytochalsin B, the viability of the TC-1-GR cells was close to 100%.

**Figure S4**

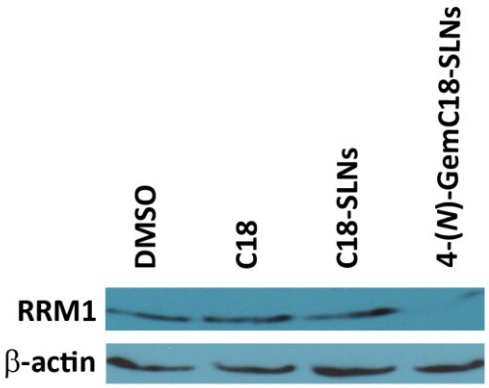



**Figure S4.** Representative Western blot of RRM1 protein levels in TC-1-GR cells after 48 h of treatment with DMSO (vehicle control), stearic acid (C18) dissolved in DMSO, C18 incorporated into solid lipid nanoparticles (C18-SLNs), or 4-(*N*)-GemC18-SLNs. The concentration of 4-(*N*)-GemC18 was 4 μM; the C18 concentration was equivalent to 4 μM.

**Supplemental Table S1.** Particle size, zeta potential, and polydispersity index (PDI) of gemcitabine derivative and Ara-C derivative nanoparticles used in this study. Data shown are mean ± S.D. from at least 3 determinations.

| | **Diameter (nm)** | **Zeta potential (mV)** | **PDI** |
|---|---|---|---|
| **4-(*N*)-GemC18-SLNs** | 146 ± 12 | -40.3 ± 2.1 | 0.29 ± 0.05 |
| **3′-(*O*)-GemC18-SLNs** | 143± 12 | -41.1 ± 3.6 | 0.27 ± 0.04 |
| **4-(*N*)-GemC8-SLNs** | 168 ± 19 | -45.3 ± 0.5 | 0.29 ± 0.03 |
| **4-(*N*)-GemC18-PLGA-NPs** | 212 ± 20 | -42.4 ± 1.2 | 0.16 ± 0.03 |
| **4-(*N*)-Ara-C-C18-SLNs** | 186 ± 13 | -33..4 ± 0.9 | 0.23 ± 0.02 |