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Mixed liposome approach for ratiometric and sequential delivery of paclitaxel and gemcitabine

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Abstract

Paclitaxel (PTX) and gemcitabine (GEM) are often used in combination due to the synergistic anticancer effects. PTX and GEM combination showed a synergistic effect to SKOV-3 cells at a molar ratio of 1 to 1 and in PTX → GEM sequence. Liposomes were explored as a carrier of PTX and GEM combination. We optimized the drug loading in liposomes varying the preparation method and co-encapsulated PTX and GEM in a single liposome preparation maintaining the maximum loading efficiency of each drug. However, drug release kinetics from the co-loaded liposomes (LpPG) was suboptimal because of the detrimental effect of PTX on GEM release control. Instead, a mixture of LpP and LpG, which were separately optimized according to the desired release kinetics, achieved a greater cytotoxic effect than LpPG, due to the attenuation of GEM release relative to PTX. This study illustrates that co-encapsulation in a single carrier is not always desirable for the delivery of drug combinations, when the activity depend on the dosing sequence. These combinations may benefit from the mixed liposome approach, which offers greater flexibility in controlling the ratio and release kinetics of component drugs.

Keywords

Paclitaxel; Gemcitabine; Ratiometric; Combination delivery; Liposomes

1. Introduction

Cancer is routinely treated with combinations of two or more drugs (1). Combination therapy can achieve high therapeutic efficacy, reduce the dose requirement, and attenuate the development of drug resistance by targeting multiple biological mechanisms (2, 3). For example, paclitaxel (PTX) and gemcitabine (GEM) are used in combination for the treatment of ovarian cancer (4, 5), pancreatic cancer (6, 7), breast cancer (8, 9), and biliary cancer (10). PTX stabilizes microtubules, prevents the formation of normal mitotic

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apparatus, thereby blocking cancer cells at the G₂/M phase (11). GEM, a synthetic analog of cytidine, stops the cancer cell division by inserting its phosphorylated form at the end of the elongating DNA and preventing DNA polymerases from proceeding (12). The two drugs show synergistic anti-cancer effects, especially when they are delivered in an optimal ratio and sequence (13, 14), providing a sound rationale for the PTX/GEM combination therapy. On the other hand, one of the challenges in PTX/GEM therapy is the large difference between the two drugs in aqueous solubility. PTX is virtually insoluble in water (water solubility: ~0.3 µg/mL (15)) and GEM has a water solubility greater than 20 mg/mL (16). The two drugs have very different pharmacokinetics profiles in human (PTX as ABRAXANE (17): terminal half-life of 27 h, mean total clearance of 15 L/h/m², and mean volume of distribution of 632 L/m²; GEM (18): half-life of 42–94 min, clearance of 30.7–92.2 L/h/m², and volume of distribution of 50 L/m²). Therefore, it is difficult to co-localize the two drugs in cancerous tissues in a timely and ratiometric manner, which are critical conditions for achieving synergistic effects.

This challenge creates a demand for delivery systems to co-deliver the two drugs to the same target tissues. One of the strategies is to co-encapsulate the two drugs in a single carrier so that they can circulate together and distribute in the target tissues simultaneously. Several carriers have been developed to co-encapsulate drugs with different solubility values in water (19–24). In particular, liposomes, consisting of a lipid bilayer and an aqueous core (25), are uniquely suitable for coencapsulating hydrophobic drugs and hydrophilic drugs (24, 26). Liposomes are also biocompatible and biodegradable and have a good track record in drug delivery applications. Moreover, liposomes with a stealth surface and an average diameter of less than 300 nm can circulate for a prolonged period and accumulate in selected tumors via the enhanced permeability and retention effect, enabling preferential accumulation of the payload in solid tumors (25).

In this study, we explored liposomes as a carrier of the PTX/GEM combination with an emphasis on ratiometric and sequential delivery. We first demonstrate the challenges in co-encapsulation of PTX and GEM in a single liposome and the limitations in achieving ratiometric and sequential delivery. Alternatively, we encapsulate each drug in separate liposomes according to the desired drug loading and release kinetics and mix them in different ratios. The mixture of liposomes is superior to the dual drug-loaded liposomes in cytotoxic effects. This study shows that the mixed liposome approach helps to increase the flexibility in formulation development and to control the dosing schedule.

2. Materials and Methods

2.1 Materials

N-(carbonyl-methoxypolyethylene-glycol-2000)-1, 2-distearoyl-sn-glycero-3-phospho-ethanolamine (DSPE-PEG₂₀₀₀), dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). PTX was a gift of Samyang Biopharm (Seoul, Korea). Gemcitabine HCl was purchased from Biotang Inc. (Lexington, MA). All other materials, including solvents, were purchased from Sigma Aldrich (St. Louis, MO).

2.2 Preparation of liposomes

DPPC, cholesterol, and DSPE-PEG2000 (6:3:1 in a molar ratio) were dissolved in a mixture of 3 mL chloroform and 1 mL methanol. To prepare PTX-loaded liposomes (LpP, Fig. 1), PTX was added to this solution at 5 wt% of the lipid, and the PTX/lipid solution was transferred to a round-bottom flask and rotated with a rotary evaporator under vacuum at 45 °C to remove the solvent and form a thin lipid film. The dry film was hydrated with 3 mL of phosphate buffered saline (PBS, 10 mM, pH 7.4) for 60 min with slow rotation and a 1 min vortex every 15 min. The liposome suspension was briefly sonicated with an ultrasonic probe (Sonics Vibracell, Newtown, CT), pulsing at a power level of 40% and a 1:1 duty cycle. GEM-loaded liposomes (LpG, Fig. 1) were prepared in one of the following four methods. (i) Regular Loading (Regular): A thin lipid film was formed in the same way as above and hydrated with 3 mL of 0.5 mg/mL GEM solution in PBS. (ii) Remote Loading (RL) (27): The lipid thin film was hydrated with 250 mM ammonium sulfate solution. Blank liposomes were collected by centrifugation at 173,400×g for 20 min. Subsequently, 1.5 mg GEM powder was added to the liposomes suspended in 3 mL PBS (pH 7.4). (iii) Small Volume Loading (SVL) (28): To the pellet of blank liposomes, 1.5 mg GEM powder was added along with 20 µL PBS. (iv) Remote and Small Volume Loading (RL+SVL): To the pellet of blank liposomes containing ammonium sulfate, 1.5 mg GEM powder was added along with 20 µL PBS. In (iii) and (iv) methods, the liposomes and GEM were briefly mixed with 1 min vortex mixing, and the suspension was incubated at 60 °C for 3 h as described in the literature first reporting SVL (28) to facilitate diffusion of GEM into the liposomes. Liposomes containing PTX and GEM (LpPG, Fig. 1) were prepared by the Remote and Small Volume Loading method.

All liposomes were washed with deionized (DI) water 3 times by centrifugation at 173,400 ×g at 4 °C and lyophilized to determine the drug loading efficiency. All other experiments were performed with fresh liposomes.

2.3 Characterization of the liposomes

The particle size and zeta potential of the liposomes were determined using a Malvern Zetasizer Nano ZS90 (Worcestershire, UK). For these measurements, purified liposomes were suspended in phosphate buffer (1 mM, pH 7.4) and sufficiently diluted with the buffer to meet the quality criteria of the equipment. Liposomes were imaged with transmission electron microscopy (TEM). Particles were mounted on a Cu grid with a Formvar and Carbon supporting film and stained with a 2% uranyl acetate solution. Excess stain was removed with a filter paper, and the grid was dried prior to imaging. The samples were imaged with a Philips CM-100 TEM (FEI Company, Hillsboro, OR) operated at 100 kV.

To determine the loading efficiency of PTX and/or GEM in liposomes, the lyophilized liposomes were accurately weighed and dispersed in acetonitrile. The liposome suspension was immersed in a bath of cold water and sonicated for 2 h. The suspension was then diluted with an equal volume of DI water and centrifuged to obtain a clear supernatant. The supernatant was filtered through a 0.45 µm syringe filter and analyzed with high pressure liquid chromatography (HPLC). HPLC analysis was performed with an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) equipped with a C18 Column (25 cm × 4.6 mm,

particle size 5 μm) (Supelco, St. Louis, MO). The mobile phase was a 50:50 mixture of water and acetonitrile (for PTX) or a 90:10 mixture of water and acetonitrile (for GEM), and run at a flow rate of 1 mL/min. PTX and GEM were detected at 227 nm and 269 nm, respectively. **Under these conditions, PTX was detected at 14.4 min out of total 20 min elution time, and GEM at 5.2 min out of 10 min elution time.** Drug loading efficiency was calculated as the weight ratio of drug to liposomes.

2.4 In vitro drug release kinetics

Freshly prepared liposomes equivalent to 170 μg PTX and/or 60–90 μg GEM were dispersed in a 1 mL PBS solution and placed in a dialysis cassette (1 mL capacity, MWCO 30–50 KDa, Spectra/Por®). The cassette was then placed in 30 mL of PBS containing 0.2% Tween 80 (PBST) and incubated at 37 °C with constant agitation using a Labquake shaker (Thermo Fisher Scientific, Waltham, MA). At predetermined time points (0.5, 3, 7, 24, 48, 72, 96, and 120 h), the release medium was completely replaced with fresh medium. The sampled medium was mixed with an equal volume of acetonitrile, filtered through a syringe filter with a 0.45 μm pore size, and analyzed with HPLC. **The samples obtained from LpPG were analyzed twice for PTX and GEM using respective HPLC conditions.**

2.5 Cytotoxicity test

SKOV-3 human ovarian cancer cells (ATCC, Manassas, VA) were grown in RPMI-1640 medium containing 10% FBS, 100 U/mL of penicillin, and 100 $\mu\text{g/mL}$ of streptomycin. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 and harvested with 0.05% trypsin-EDTA. The cells were maintained in culture for up to 30 passages.

SKOV-3 cells were incubated with free drugs and liposomes, and their viability was measured with the MTT assay. Cells were seeded in a 96-well plate at a density of 10,000 cells per well and grown overnight. Subsequently, the cells were treated with free PTX, GEM, PTX/GEM mixture in 1:1 molar ratio, single or dual drug liposomes, or liposome combinations over a range of concentrations and incubated for 72 h. Briefly, liposomes were diluted to a series of concentrations with cell culture medium. Each concentration was set up in quadruplicate. To determine the optimal treatment sequence, SKOV-3 cells were exposed to PTX and GEM sequentially (PTX $\rightarrow$ GEM) or vice versa (GEM $\rightarrow$ PTX) for 24 h per drug, followed by 24 h incubation in drug-free medium.

After the incubation with free drug or liposomes, cells were treated with 20 μL of a 5 mg/mL MTT solution and incubated for 4 h. One hundred μL of stop/solubilization solution was added, and the plate was left in dark overnight. The absorbance of solubilized formazan in each well was measured with a microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 562 nm. The absorbance of the medium was subtracted from the absorbance of all wells. The measured sample absorbance was then normalized to that of the untreated control cells.

The half maximal inhibitory concentration (IC_{50}) was determined using GraphPad Prism 6 (GraphPad, San Diego, CA). The combination index (CI) of the PTX/GEM combination was calculated as: $CI = D_1 / (D_x)_1 + D_2 / (D_x)_2$, where D_1 and D_2 were the doses of GEM and

PTX in the combination at the IC_{50} level and $(Dx)_1$ and $(Dx)_2$ were the IC_{50} values of GEM and PTX as a single treatment, respectively (3). CI values of 0.9–1.1, >1.1, and <0.9 were interpreted as additivity, antagonism, and synergism, respectively.

2.6 Statistical analysis

All data were expressed as the means $\pm$ standard deviations. Statistics were performed using ANOVA with recommended post-hoc multiple comparisons tests. A p value of <0.05 was considered statistically significant.

3. Results

3.1 Optimal ratio and treatment sequence of PTX/GEM combination

The IC_{50} values of PTX and GEM in SKOV-3 cells were 22.1 ± 2.8 nM and 22.0 ± 7.4 nM, respectively. The PTX/GEM combination, mixed in a 1:1 molar ratio, showed an IC_{50} value of 11.8 ± 2.1 nM. These values yielded a CI of 0.535, indicating that the PTX/GEM combination in 1:1 ratio was synergistic. Maintaining this ratio, we then investigated the effect of the dosing sequence of free drugs, varying the order of treatments (PTX→GEM and GEM→PTX). The cell responses to the combination were dependent on the treatment sequence, with PTX→GEM showing the lower IC_{50} (Fig. 2), consistent with other studies using different cell lines (9, 14). This result indicates that PTX/GEM combination in 1:1 molar ratio, delivered sequentially (PTX→GEM), will offer an optimal synergistic effect against SKOV-3 cells.

3.2 Characterization of GEM-, PTX-, and PTX/GEM liposomes

Liposomes containing GEM and/or PTX were prepared using different loading methods (Table 1). The z-averages of the liposomes were around 200 nm with polydispersity index (PDI) values of ~0.2 (mid-range polydispersity), irrespective of the encapsulated drug(s) or preparation methods. In all methods, liposomes were formed by hydration of the thin dry phospholipid film. **The drug-loaded liposomes were slightly larger than blank liposome.** The zeta potentials of liposomes were negative, **irrespective of drug loading**, reflecting the net negative charge of DSPE-PEG₂₀₀₀. The TEM image of LpPG shows vesicle structures with an average diameter of <200 nm (Fig. 3), consistent with dynamic light scattering measurement. The particle size and zeta potential of LpPG were stable during storage at 4 °C.

PTX loading efficiency ranged from 4.5 to 6 wt%, irrespective of the production methods (Table 1). The loading efficiency was slightly higher than the theoretical loading (5 wt%), suggesting that the lipid components might have been preferentially lost during washing due to the relative polarity. In contrast, GEM loading varied dramatically according to the preparation method. With the regular loading method, where GEM was added during the hydration of the lipid film, GEM loading efficiency was only 0.5 wt%. GEM loading improved slightly to 1.3 wt% with the use of remote loading (RL) but substantially improved to >4 wt% with the small volume loading (SVL). Combination of RL and SVL did not improve the loading beyond the level achieved with SVL. PTX and GEM could be loaded in a single liposome (LpPG) maintaining the respective loading efficiency.

3.3 *In vitro* drug release kinetics

The PTX encapsulated in the lipid bilayer of liposomes had similar release profiles, irrespective of GEM co-encapsulation. Less than 25% of the total PTX was released from LpP (Fig. 4a) and LpPG (Fig. 4b) in 48 h. In contrast, GEM release was affected by the presence of both PTX and ammonium sulfate. GEM release from LpPG was quick: 66.1% of total GEM was released in 48 h (Fig. 4b). On the other hand, LpG (GEM liposomes without PTX co-encapsulation) prepared by the same method (RL+SVL) released 37.9% of the total GEM in 48 h, less than LpPG in the same time period (Fig. 4c). LpG prepared by SVL (without RL) showed faster initial release (34.8% vs. 23.4% of LpG (RL+SVL) in 3h, $p < 0.05$ by Sidak's multiple comparisons test) (Fig. 4c). These results indicate that PTX embedded in the liposome membrane expedited GEM release by compromising the diffusion barrier function of the membrane, whereas RL helped attenuate initial drug release by forming an ionic complex of GEM and sulfate.

3.4 Cytotoxic effects of liposome combinations

We compared the cytotoxic effects of LpPG and a mixture of LpP and LpG (prepared with RL + SVL) at 1:1 PTX to GEM molar ratio in SKOV-3 cells. The mixture of LpP and LpG showed a lower IC_{50} value than LpPG (7.04 nM vs. 13.49 nM) in SKOV-3 cells, with a greater extent of suppression at higher concentrations (Fig. 5).

4. Discussion

Appropriate drug ratios and dosing sequences are critical to efficient combination therapy of cancer. It has been demonstrated that the synergistic effect of PTX/GEM combination is ratio- and schedule-dependent, as confirmed by the present study. PTX/GEM was synergistic in killing SKOV-3 cells at a 1:1 molar ratio, and the effect was more pronounced when PTX was administered before GEM. The significance of sequential dosing may be explained by the fact that PTX induces the inactivation of enzymes that can destroy GEM (13). In addition, with the simultaneous or reverse sequence (GEM→PTX) administration, GEM plays a dominant role in cell cycle arrest (29), thus undermining the PTX-induced hyperploidy (14). Additional mechanisms account for the merit of PTX→GEM sequential delivery *in vivo*: PTX reduces the stromal density and improves intratumoral transport of subsequent therapy by directly affecting stromal cells (6) or by inducing partial apoptosis of tumor cells (30).

Having identified a synergistic ratio and dosing sequence of GEM and PTX against SKOV-3 cells, we first attempted to load the two drugs in a single liposome (LpPG), as co-encapsulation will maximize the co-localization of the two drugs in the same tissues. However, the release kinetics was the opposite of the optimal sequence, with GEM releasing faster than PTX. Given the comparison with GEM release from LpG (GEM liposomes without PTX), it is likely that PTX in the membrane has compromised the diffusion barrier function of the liposome membrane. As an alternative, we mixed LpP and LpG in a ratio equivalent to 1:1 PTX to GEM molar ratio, where LpG retained GEM relatively better than LpPG (GEM release at 48 h of 37.9% vs. 66.1%). The cytotoxicity result showed that LpP + LpG mixture was more efficient than LpPG due to the attenuation of GEM release.

It is worth noting the strategies employed to improve GEM loading in liposomes and to sustain the drug release. As a hydrophilic drug, GEM is difficult to encapsulate in the aqueous compartment of liposomes by simple hydration. We combined two known strategies, RL and SVL, which increase the drug loading efficiency in different mechanisms. The former relies on a pH difference across the liposome membrane, as routinely used in doxorubicin loading (25). Here, the pH difference (pH 3 vs. pH 7) creates a concentration gradient of an unionized drug, providing a driving force for the drug to diffuse into the liposomes and precipitate in the liposomes as an ion complex with sulfate (doxorubicin- $\text{NH}_3)_2\text{SO}_4$ that does not easily pass the membrane (25). However, GEM does not ionize in the liposomes as extensively as doxorubicin, due to its low pKa value (pH 3.6 vs. pH 8.68 of doxorubicin); thus, the RL method is less effective for GEM loading (28). This limitation was overcome with the use of a small external volume, which generates the maximum concentration gradient across the liposomal membrane by keeping the external GEM at the saturation solubility and, thus, provides a driving force for GEM to enter liposomes. The RL and SVL methods, combined together, increased GEM loading substantially, from 0.5% to 4.2 %.

Co-encapsulation of synergistic drug combination in a single liposome has an important advantage in equalizing pharmacokinetics and biodistribution of multiple drugs (31, 32). However, such systems may not be necessary or desirable for drug combinations that show variable effects according to the dosing sequence. In this regard, the advantage of the mixed liposome approach is multifold: (i) the drug ratio can be readily modified; (ii) each liposome can be prepared in a manner optimal for controlling the drug release; and (iii) flexible control of drug release kinetics is possible based on combinations of multiple liposomes (e.g., LpP + LpG, LpP + LpG + LpPG, LpG + LpPG, etc). A prerequisite of mixed liposome approach is that those liposomes have equal size, shape, charge, and surface chemistry so that they have the same pharmacokinetics and biodistribution profiles. This may not be a difficult expectation as liposome size and shape are readily controlled by lipid membrane extrusion and the charge and surface chemistry depend on the lipid compositions. A new surface modification method developed in our laboratory (33) can further help equalize the surface chemistry of multiple liposomes.

5. Conclusion

We used liposomes for the delivery of PTX and GEM combination. PTX and GEM could be co-loaded in the lipid membrane and aqueous core of a single liposome, respectively. However, drug release kinetics from the co-loaded liposomes (LpPG) was opposite to the desired PTX→GEM sequence due to the detrimental effect of PTX on GEM release control. A mixture of LpP and LpG, which were separately prepared to achieve desired drug release kinetics, showed a greater cytotoxic effect than LpPG. This study shows that the mixed liposome approach is more appropriate than co-encapsulation for the delivery of PTX/GEM combination, whose effect is dependent on the dosing sequence.

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Fig. 1.
Schematic description of liposomes containing PTX and/or GEM.

Fig. 2. Cytotoxicity of GEM/PTX combinations on SKOV-3 cells given in different sequences. $n = 3$ tests. Average $\pm$ standard deviation. GEM $\rightarrow$ PTX vs. PTX $\rightarrow$ GEM: $p < 0.01$ by Extra sum-of-squares F test.

LpPG

Fig. 3.
Transmission electron microscope image of LpPG. Scale bar: 100 nm.

Fig. 4.

In vitro release kinetics of (a) LpP, (b) LpPG, and (c) LpG. n=2 (a and b) or 3 (c) independent and identical batches. Average $\pm$ standard deviation.

Fig. 5.

Cytotoxicity of LpP + LpG vs. LpPG. GEM:PTX = 1:1 molar ratio for both LpP + LpG and LpPG. $n = 4$ tests. Average $\pm$ standard deviation. LpP+LpG vs. LpPG: $p < 0.01$ by Extra sum-of-squares F test.

Table 1

Size and zeta potential of liposomes^a

Liposomes	Description	Preparation method	Z-average (nm)	PDI ^b	Zeta Potential (mV)	Drug loading efficiency (%) ^c
LpP	PTX liposomes	Regular	206.7 ± 0.7	0.26 ± 0.03	-18.8 ± 3.9	5.3 ± 1.0
LpG	GEM liposomes	Regular	160.2 ± 1.5	0.36 ± 0.05	-23.1 ± 1.8	0.5 ± 0.2
LpG	GEM liposomes	RL	168.8 ± 1.0	0.24 ± 0.04	-25.6 ± 2.2	1.3 ± 0.2
LpG	GEM liposomes	SVL	175.9 ± 0.8	0.21 ± 0.03	-24.9 ± 2.1	4.3 ± 0.8
LpG	GEM liposomes	RL+SVL	164.2 ± 0.3	0.14 ± 0.03	-24.4 ± 2.3	4.2 ± 0.3
LpPG	Liposomes containing both PTX and GEM	RL+SVL	205.8 ± 0.9	0.23 ± 0.09	-21.8 ± 3.4	PTX: 6.5 ± 0.4 GEM: 4.2 ± 0.4
LpBlank	Blank liposomes	RL	142.2 ± 9.4	0.66 ± 0.13	-26.1 ± 1.1	Not applicable

^aThe data are presented as means ± standard deviations of three experiments.

^bThe polydispersity index (PDI) was obtained by cumulant analysis, as described in the International Standard on DLS ISO13321 Part 8 (Malvern DLS technical note MRK656-01).

^cDrug loading efficiency = mass of loaded drug / mass of liposomes