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CR 2945-Conjugated Liposomes for Targeting of Human Pancreatic Cancer Cells

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ABSTRACT–CR 2945, a gastrin/CCK_B receptor antagonist, was conjugated to liposome and tested for the targeting of pancreatic cancer cells *in vitro*. Successful conjugation was confirmed by FTIR and NMR. The size of CR 2945-conjugated liposome was about 500 nm in diameter, with the zeta-potential being −16.5 mV. *In vitro* anti-cancer activity of this formulation with or without gemcitabine encapsulated was tested on human pancreatic cancer cells, PANC-1. The growth inhibitory effect of gemcitabine-encapsulating CR 2945-conjugated liposome was found to be 10-fold more potent than that of gemcitabine-encapsulating non-conjugated liposome, suggesting that CR 2945 could be used as a potential cancer targeting moiety by conjugating into liposome.

Key words–CR 2945, Liposome, Pancreatic cancer, Targeting, Gemcitabine

Pancreatic cancer accounts for approximately 2 – 3% of all malignant neoplasms world wide.[1] It causes around 200,000 deaths yearly and is the fifth most common cause of cancer mortality in the world. At present, only 10 – 20% of the patients with pancreatic cancer have a localized disease that can be considered for resection, but even when resection is performed pancreatic cancer has a poor prognosis.[2] Therefore chemotherapy is important for patients who have the pancreatic cancer.

Gastrin is known to stimulate some colon cancer, gastric cancer and pancreatic cancer *in vitro* (cell) and *in vivo* (xenotransplanted). Gastrin receptor antagonists have been hypothesized to be of clinical relevance in the treatment of gastrin-sensitive colorectal tumors. Some pancreatic cancer cells overexpress gastrin receptor.[3] CR 2945 is a gastrin/CCK_B receptor antagonist.[4] Interruption of binding of gastrin with gastrin receptor of human pancreatic cancer cells by CR 2945 inhibits the growth of human pancreatic cancer cells.[5] As the CR 2945 is a non-peptide material,[6-8] it is easily transformed into the other forms.

Gemcitabine is a pyrimidine antimetabolite that exhibits a broad range of activity against a variety of solid tumor. It is a pro-drug and, once transported into the cell, it must be phosphorylated by deoxycytidine kinase to an active form. Both gemcitabine diphosphate (dFdCTP) and gemcitabine triphosphate (dFdCTP) inhibit the repairing processes for DNA synthesis.[9]

In this study a carboxyl group of CR 2945 is conjugated to an amine group of phosphatidylethanolamine, which will be used for the preparation of liposome and be tested to synergistically inhibit the growth of pancreatic cancer cells by CR 2945-conjugated gemcitabine-encapsulating liposome.

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Experimental

Materials

Dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), L-α-phosphatidylethanolamine (PE), 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and-[2-([2-(8-azaspiro[4.5]dec-8-ylcarbonyl)-4,6-dimethylphenyl]amino-2-oxoethyl)-(R)-1-naphthalenepropanoic acid (CR 2945),1-cyclo-hexyl-3-(2-morpholunethyl)-carbodiimide metho-p-toluene sulfonate (carbodiimide) and 4-morpholinepropanesulfonic acid (Mops) were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY). PANC-1, a human pancreatic cancer cell line, was purchased from Korean Cell Line Bank (Seoul, Korea). Other reagents were analytical grade or cell culture grade.

Synthesis of CR 2945-PE

Phosphatidylethanolamine (PE) in chloroform was deposited as a thin film in a test tube and dried in vacuo. The lipid was suspended in 0.1 M Mops, pH 7.2, at a concentration of 5 μmol/mL. To achieve coupling, 0.2 mL of this suspension was incubated with 0.6 mL of the CR 2945 (15 to 20 mg/mL) in the presence of 8.46 mg carbodiimide, first dissolved in 0.1 mL of 0.1 M Mops buffer pH 7.2. After 4 hr at 20°C, the completion of the reaction was monitored by thin layer chromatography

(TLC). This solution was adjusted to pH 4.0 by acetic acid. The contents of the reaction mixture were precipitated by adjusting the pH to 4.4 with acetic acid, washed several times at the same pH. The precipitate was filtered by glass filter.[10] After filtration of the eluent, the precipitate was dissolved using chloroform. The structural analysis of CR 2945-PE was carried out using FTIR and ^{1}H-NMR (Bruker-Avance DRX-600, Germany).

Preparation of Liposomes

All reagents and glassware used for liposome preparation were sterile and all open tube manipulations, except rotary evaporation (Heidolph VV2000, IWAKI glass Co., Ltd.), were carried out in a clean bench. Liposomes were prepared by the reverse-phase evaporation method.[11] Briefly, gemcitabine solution was prepared for encapsulation at 8 mM (pH 5.5~6.0, 290 mOsm/kg) in PBS. The lipid mixtures of DPPC : Chol : CR 2945-PE (6:3:1 molar ratio) and DPPC : Chol : PE (6:3:1 molar ratio) were dissolved in chloroform, and then the organic solvent was evaporated from the mixtures. Formed lipid films were suspended in 1 ml of freshly hydrated diethyl ether, to which was added 1 ml of aqueous drug solution. The mixture was sonicated for 5 min, and ether was then eliminated by rotary evaporation. The liposome was downsized using LIPEX™ extruder (Northern Lipid INC, Vancouver, Canada) through 0.2 μm pore size polycarbonate membranes for 10 times. CR 2945-conjugated liposomes were separated from unencapsulated free drug by gel chromatography using a sterile 1 × 12 cm Sephadex® G-75 column eluted with PBS. The encapsulation efficiency of gemcitabine hydrochloride was determined by Bligh and Dyer extraction method.[12] The concentration of gemcitabine hydrochloride was measured spectrophotometrically at 232 nm.

Characterization of liposomes

The size distribution of liposome was determined using dynamic laser-light scattering (Malvern Zetasizer®, Malvern Instruments Ltd., England) at 24°C using He-Ne laser light source (at 670 nm).

Cell culture

PANC-1, human pancreatic carcinoma cells, were maintained in DMEM medium supplemented with 10% FBS at 37°C in a 5% CO_2 incubator (Sanyo Electric Co. Ltd., Japan).[13] Cells were normally grown in 25 cm^2 or 75 cm^2 polystyrene tissue culture flasks (Nunc, Denmark) until they became approximately 80% confluent. After the cells were trypsinized, 100 μl of single cell suspension (5×10^4 cells/well) were seeded in 96-well flat-bottomed microassay plates (Nunc, Denmark), and incubated for 16 hr before addition of the CR 2945 conjugated liposome

In Vitro Growth Inhibition Assay

Growth inhibition was assessed using the MTT assay.[14] Briefly, MTT was dissolved in 1× PBS at 5 mg/ml, filtered through 0.2 μm polycarbonate membrane filter (NALGENE® Filterware, Naperville, IL) to sterilize. At the end of the incubation, 15 μl of MTT solution was added. Plates were incubated for additional 3 hr at 37°C in an incubator, then MTT containing medium was aspirated off and 150 μl of DMSO was added to dissolve the formed formazan. Absorbance was measured at 570 nm using an ELISA reader (Dynatech MR5000, Dynatech Laboratories, U.S.A.). Percent growth was calculated according to the following equation;

$$\% \text{ Growth} = \frac{[OD_{570}(\text{sample}) - OD_{570}(\text{original})]}{[OD_{570}(\text{control}) - OD_{570}(\text{original})]} \times 100$$

Results and Discussion

Synthesis of CR 2945-PE

The conjugation of carboxyl group of PE and amine group

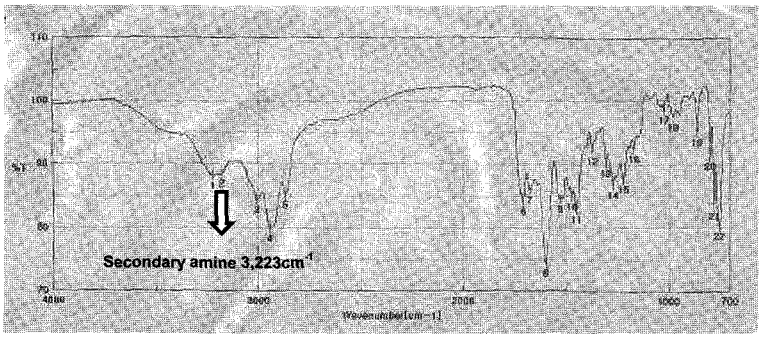

Figure 1–FT-IR spectra of CR 2945-PE.

of CR 2945 was performed by carbodiimide. IR spectra of CR 2945-PE with peptide bond exhibited the characteristic band of primary amine at 3,223 cm^{-1} (Figure 1). ^{1}H-NMR spectra of CR 2945-PE exhibits a peak at δ = 8.830 ppm which is assigned to protons of the peptide bond (Figure 2). Figure 2 also shows that the naphthalene group of CR 2945-PE appears at δ = 7.437 ~7.297 as in the spectra of free CR 2945. Thus, the ^{1}H-NMR spectra of CR 2945-PE shows the successful conjugation of alkyl chain of PE to CR 2945.

Characterizations of liposomes

Figure 3 shows the sizes distribution of CR 2945-conjugated liposomes, where there are two peaks around 230 nm and 700 nm with the average size of 500 nm in diameter. The first peak at 230 nm nearly corresponds to the pore size of the membrane used for the extrusion, however, the second peak at 700 nm seems to be much bigger than that of the pore size of membrane. Increase in the size of liposome, as shown in the second peak, is probably due to the fusion of the liposomes during the

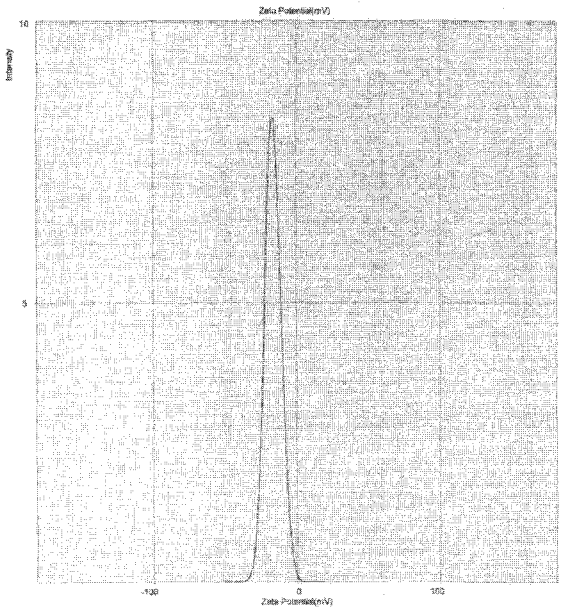

Figure 4–Zeta potential of CR 2945-conjugated liposomes.

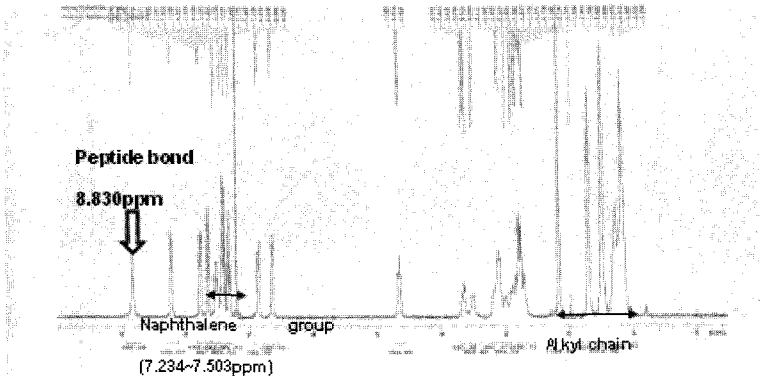

Figure 2–^{1}H-NMR spectra of CR 2945-PE.

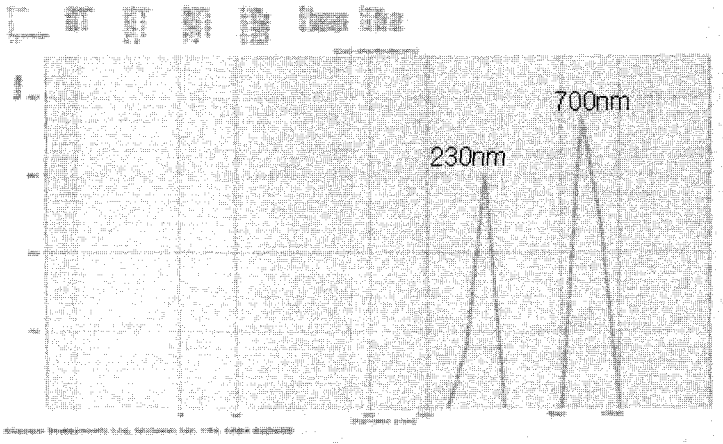

Figure 3–Dynamic laser light scattering of CR 2945-conjugated liposomes.

storage. Increased size of the liposome usually exerted decreased drug delivery efficiency, which resulted in a decreased anti-cancer activity. Encapsulation efficiency of gemcitabine into the liposome is found to be about 84% as determined by the method of Bligh and Dyer. Figure 4 shows the zeta potential of CR 2945-conjugated liposomes, where the CR 2945-conjugated liposomes represent weak negative charge of −16.5 mV.

Anticancer Activity of CR 2945 after PE conjugation

The growth inhibitory effect of free CR 2945 and PE-conjugated CR 2945 is shown in Figure 5. As the CR 2945 *per se* has an anticancer activity, the maintenance of the activity after the conjugation process is very critical. In 48 hr exposure experiment, CR 2945-conjugated DPPC liposome was as potent as free CR 2945 (at 72 M), suggesting that the anticancer activity remained after the conjugation and liposome preparation.

For the lipid toxicity test, parallel studies were carried out with the same DPPC liposome with no CR 2945 conjugated, but there was no inhibition of cell growth at the lipid concentrations up to 2 mM (data not shown). This concentration is more than 10 times higher than the lipid concentrations used in the growth inhibition experiment.

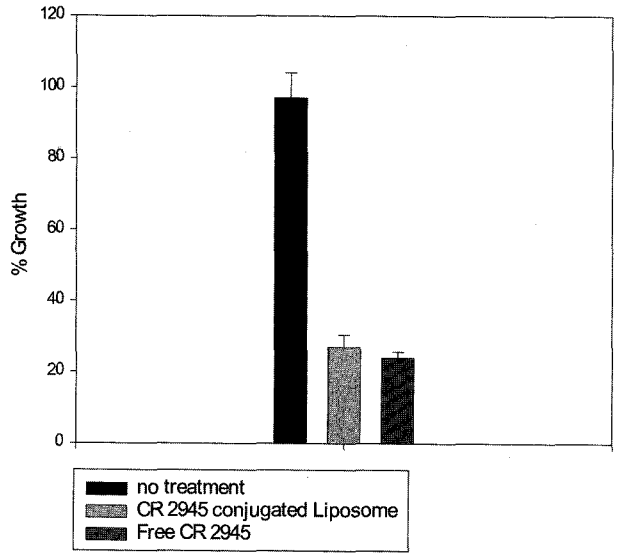

Figure 5–Cytotoxicity of free CR 2945 and CR 2945-conjugated liposome (no gemcitabine encapsulated) in PANC-1 cells. The concentration of CR 2945 was 72 M and the same concentration was kept for the CR 2945-conjugated liposome treatment.

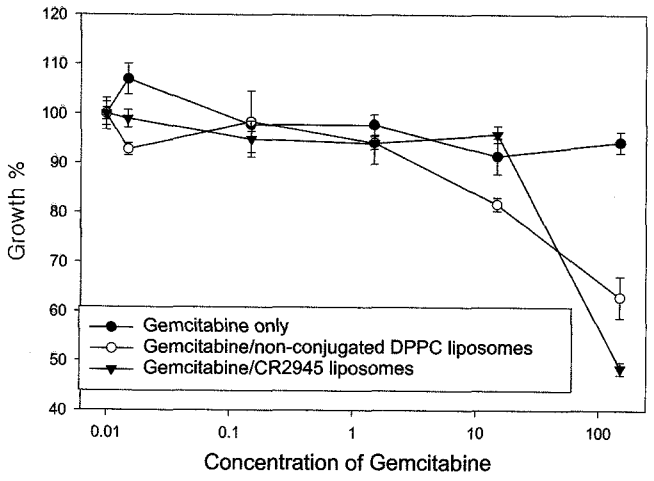

Figure 6–Growth inhibition of PANC-1 cells by free gemcitabine, gemcitabine in non-conjugated DPPC liposomes and gemcitabine in CR 2945-conjugated liposomes.

Synergistic Growth Inhibition of CR 2945 conjugated liposomes with Gemcitabine

Using gemcitabine as a model anticancer drug, not only the targeting ability but the anticancer effect of CR 2945 was tested on the human pancreatic carcinoma cells (PANC-1). Figure 6 shows that gemcitabine-encapsulated CR 2945-conjugated liposome is 10-fold more potent than gemcitabine-encapsulated non-conjugated one. Free gemcitabine did not show any growth inhibitory effect at the concentration ranges up to 100 M. This is probably due to the high solubility of gemcitabine, resulting in poor penetration into the cells, whereas encapsulation of the drug into the liposome can facilitate the penetration into the cells and result in the enhanced anticancer activity of the drug. This result might suggest that gemcitabine is a so-called "liposome-dependent drug" as the cellular penetration and the resulting pharmacological activity are significantly improved by the encapsulation into the liposome.

Based on these observations, CR 2945 might be used as a useful anticancer moiety not only by itself but as a conjugated form with delivery systems such as liposome, especially for the pancreatic carcinoma cells. Therefore, CR 2945-conjugated liposome should be capable of acting in vivo by selective delivery of payload to the tumor cells especially in situations where these cells overexpress gastrin/CCK_B receptors on their surfaces.

Acknowledgment

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