CHAPTER III

MATERIALS AND METHODS

Materials

1. Ammonium molybdate (Fluka, Switzerland, Lot no. 232685)
2. Antibiotic-antimycotic (penicillin G, streptomycin sulfate, amphotericin B) (Hyclone, USA, Lot no. AMB15564)
3. Calcein (Sigma, USA, Lot no. 20K0575)
4. Chloroform, AR grade (Merck, Germany, Lot no. K20164645348)
5. Cholesterol (Sigma, USA, Lot no. 11H8488)
6. Dibasic sodium phosphate anhydrous (Merck, Germany, Lot no. F997086532)
7. Dicetylphosphate (Sigma, USA, Lot no. 10K1593)
8. Dulbecco's Modified Eagle's Medium (GibcoBRL, USA, Lot no. 1105635)
9. Fetal bovine serum (Biochrom KG, Germany, Lot no. 4374, 633A, 634A, and 044EE)
10. Fiske-Subbarrow reducer (Sigma, USA, Lot no. 107H6249)
11. HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Sigma, USA, Lot no. 88H5446)
12. Hydrogen peroxide (Merck, Germany, Lot no. K28735787)
13. L-Glutamine (Hyclone, USA, Lot no. AMB15523)
14. Methanol, AR grade (Merck, Germany, Lot no. K29532209131)
15. Multiwell plates (Nunc, Denmark)
16. Phosphatidylcholine (Phospholipon®90 Nattermann Phospholipid GmbH, Cologne, Germany, Lot no. 770991)
17. Phosphatidylglycerol (Sigma, USA, Lot no. 21K5202)
18. Phosphatidylserine (Sigma, USA, Lot no. 69H7015)
19. Polycarbonate membranes 100 and 200 nm (Avestin, Canada)
20. Potassium chloride (Merck, Germany, Lot no. 036TA915536)
21. Potassium dihydrogen phosphate (Merck, Germany, Lot no. 3M633293M)
22. Propylthiouracil (supplied by Sriprasit Pharma Co., Ltd., Lot no. 9220747)

23. RPMI 1640 medium (GibcoBRL, USA, Lot no. 1106055)
24. Sephadex G-50 (Sigma, USA, Lot no. 115H0876)
25. Sodium bicarbonate, tissue culture grade (Sigma, USA, Lot no. 90K0818)
26. Sodium chloride (Fluka, Switzerland, Lot no. 71379)
27. Sodium hydroxide (Merck, Germany, Lot no. B870498625)
28. Sterilization filtration membranes 0.22 μm (cellulose acetate membrane) (Iwaki, Japan)
29. Sulfuric acid (Analar, England, Lot no. K2361283165112)
30. Tissue culture flasks (Nunc, Denmark)
31. Trypan blue (4% solution)
32. Trypsin-EDTA (GibcoBRL, USA, Lot no. 1025956)
33. α-Tocopherol (Approx. 95%, Sigma, USA, Lot no. 53H0444)

Equipment

1. Analytical balance (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland)
2. Autoclave (HA-3D, Harayama Manufacturing Corporation, Japan)
3. Counting chamber (BOECO, Germany)
4. Flex-column (Kontes, USA)
5. Fluorescence microscope (BX-FLA, Olympus, Japan)
6. Hand-held extruder (LiposoFast^{TM}, AVESTIN, Canada)
7. Hot air oven (UL50, Memmert, Germany)
8. Humidified carbon dioxide incubator (Model 3164, Forma Scientific, USA)
9. Inverted microscope (CK2, Olympus, Japan)
10. Laminar air flow hood (HBB 24485, Holten, Denmark)
11. Light microscope (Leica ATC2000, Olympus, Japan)
12. pH meter (Beckman, USA)
13. Rotary evaporator (RE120, Buchi, Switzerland)
14. Shaking incubator (Innova 4230, New Brunswick Scientific, USA)
15. Ultracentrifuge (L80, Beckman, USA)
16. UV spectrophotometer (Model 7800, Jasco Corporation, Japan)
17. Vortex mixer (G-560E, Scientific industries, USA)

Methods

1. Preparation of liposomes

1.1 Blank liposomes

Liposomes were prepared by film-hydration method at total lipid concentration of 5 mg/mL. The composition of liposomes comprised phosphatidylcholine (PC), cholesterol, and a charged lipid (dicetylphosphate (DCP), phosphatidylserine (PS), or phosphatidylglycerol (PG)) at a molar ratio of 6:3:1. Alpha-tocopherol (0.1 mol%) was used as an antioxidant in all preparations. Briefly, the lipids were dissolved in chloroform in a round-shaped flask. The solution was evaporated to a thin film under vacuum using rotary evaporator. The lipid film was kept under vacuum for 6 hours to eradicate traces of the organic solvent. To prepare blank liposomes, the aqueous phase (47 mM HEPES buffer pH 7.4, I = 0.2) was added to hydrate the lipid film. The liposomal vesicles formed spontaneously after lipid hydration. The resultant liposomal suspension was shaken at 4 ^{o}C for 12 hours in a refrigerated shaker (New Brunswick Scientific, USA) to allow complete formation of liposomal vesicles.

1.2 PTU-loaded liposomes

PTU liposomes were prepared by incorporating PTU in both aqueous (1.1 mg/mL PTU in HEPES buffer) and lipid (0.53 mg PTU/100 mg lipid) phases. The method used was the same as described in 1.1. The resultant liposomal suspension was routinely examined under a light microscope to ascertain that the product was devoid of PTU crystals.

1.3 Calcein-loaded liposomes

Calcein was used as a water-soluble fluorescent marker at its self-quenching concentration (approximately 80 mM). Calcein (0.05 g) was dissolved in 0.3 N sodium hydroxide (1 mL). The pH of the solution was adjusted to 7 and the solution was used as the aqueous phase for liposome preparation. Liposomes were prepared by the method described above. Liposomal suspensions was extruded through two-stacked 0.2 μm polycarbonate membranes with a hand-held extruder. The extrusion process was repeated through 0.1μm membranes to further reduce liposome size. Non-encapsulated calcein was separated from calcein-loaded liposomes by gel filtration using Sephadex G-50 (0.1 mL suspension loaded on a 1 x 21 cm gel bed). After gel filtration, lipid contents were assayed as described below in Section 2.2. The calcein-loaded liposomal suspension was used within the same day of preparation to avoid liposome leakage and bleaching of the fluorescent marker.

2. Determination of PTU encapsulation efficiency

The liposome suspension was separated into the supernatant containing the free drug and the pellet containing the entrapped drug by ultracentrifugation (60,000 rpm, 4 ^{o}C for 6 hours). The PTU content in the liposomal pellet was assayed and used to calculate the encapsulation efficiency. The PTU in the supernatant was also assayed for routine monitoring of percent analytical recovery.

2.1 Quantitative analysis of PTU in liposome preparations

An aliquot of liposomal suspension was centrifuged at 60,000 rpm at 4^{o}C for 6 hours in an ultracentrifuge. A known amount of saturated solution of PTU in HEPES buffer was added to the aliquot to aid the process of centrifugation. The PTU content in the supernatant and in the pellet was assayed according to the following assay protocol.

2.1.1 Determination of PTU in the liposomal pellet

The pellet separated from 1 mL of liposomal suspension was dissolved in methanol:chloroform (8:2) in a 10-mL volumetric flask, and the solution was adjusted to volume. The solution was further diluted until the concentration was within the desired range. This final solution was assayed by UV spectrophotometry at 275 nm. The presence of lipid components did not interfere with the assay (Rattanatraiphop, 2000).

2.1.2 Determination of PTU in the supernatant

The supernatant was appropriately diluted with methanol and analyzed by UV spectrophotometry at 275 nm.

2.2 Assay of phospholipids (New, 1997)

The phosphorus content of phospholipids was determined by Bartlett assay (Bartlett, 1959). In this method, phospholipid phosphorus was acid hydrolyzed to inorganic phosphate and converted to phospho-molybdic acid by the addition of ammonium molybdate. The phospho-molybdic acid was reduced to a blue-colored compound by amino-naphthyl-sulfonic acid (Fiske-Subbarrow reducer). The intensity of the blue color was measured spectrophotometrically at 800 nm, and the concentration was determined from a calibration curve of phosphate standard solutions prepared from potassium phosphate. The phospholipids used for the preparation of liposomes contain one mole of phosphorus per mole of phospholipids.

2.2.1 Preparation of phosphate standard solutions

The anhydrous potassium dihydrogen phosphate was dried at 105 ^{o}C for 4 hours in a hot air oven. A stock solution of phosphate standard was prepared by

accurately weighing 43.55 mg of dried anhydrous potassium phosphate into a 100-mL volumetric flask. The content in the flask was dissolved in double-distilled water and adjusted to volume. The final concentration of phosphorus was 3.2 μmol/mL. Aliquots of phosphate stock solution (2, 3, 4, 5, 6, 7, and 8 mL, respectively) was transferred to seven 100-mL volumetric flasks. The solutions were adjusted to volume with double-distilled water. The final concentrations of phosphorus were 0.064, 0.096, 0.128, 0.160, 0.192, and 0.224 μmol/mL, respectively.

2.2.2 Preparation of sample solutions

The liposomal suspension was diluted with double-distilled water to give a concentration of approximately 1 mg/mL of phospholipid(s) before being subjected to further assay procedure.

2.2.3 Treatment of the samples

The procedure for treating the samples was as follows:

Fifty microliters of diluted liposome suspension was added to empty test tubes. The sample was dried down and resuspended in 0.5 mL of double-distilled water. A calibration curve was set up by pipetting of 0.5 mL of the standard solutions into separate tubes, together with a blank (0.5 mL of distilled water). Each of the resuspended samples and the standard solutions was added with 0.4 ml of 5 M sulfuric acid and then incubated at 180-200 ^{o}C for an hour in a hot air oven preheated at 200 ^{o}C for 30 minutes. After the tubes were cooled down by standing them at room temperature, 0.1 mL of the freshly diluted hydrogen peroxide (10% v/v) was added. The tubes were then incubated at 180-200 ^{o}C for another 30 minutes in the hot air oven until colorless solutions were obtained. The solutions were cooled down to room temperature. Acid-molybdate solution (4.6 mL) was added to each tube, and the content of the tube was mixed well by vortexing. The solutions were reduced with 0.2 mL of Fiske-Subbarrow reducer. The tubes were then covered and placed in a boiling water bath for 7 minutes. After the tubes were cooled down, the absorbances of the

blue-colored solutions were measured at 800 nm against distilled water. The phosphorus content of liposomal suspension was calculated as follows:

$$\text{Phosphorus content } (\mu g/mL) = A \times B \times C$$

A = concentration (μmol of phosphorus/ml) calculated from the absorbance

B = dilution factor

C = molecular weight of lecithin

2.3 Calculation of encapsulation efficiency

Encapsulation efficiency was defined as the fraction of PTU found in the liposomal pellet and expressed as millimole of drug per mole of lipid.

$$\text{Encapsulation efficiency} = \frac{\text{Amount of PTU in pellet (mg/mL) / MW of PTU}}{\text{Amount of phospholipids (mg/mL) / MW of phospholipids}}$$

When other lipids were present, the calculation was modified so that the encapsulation efficiency represents the amount of PTU per mole of total lipid.

3. Effects of PTU /liposomes on macrophage U-937 cell growth

3.1 Preparation of PTU solutions

PTU was dissolved in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and sterilized by filtration through 0.22 μm cellulose acetate membrane filtration units (Iwaki, Japan).

3.2 Preparation of liposomal formulations

3.2.1 Preparation of blank and PTU-loaded liposomes

Liposomes with various lipid compositions (PC, PC/CH, PC/DCP, PC/DCP/CH, PC/PS, PC/PS/CH, PC/PG, and PC/PG/CH) were prepared as described in Sections 1.1 and 1.2. The liposomal pellets harvested by centrifugation were redispersed with sterile RPMI 1640 with 10% fetal bovine serum (FBS). The resultant liposomal suspensions were extruded through sequential two-stacked 0.2 μm and 0.1 μm polycarbonate membranes by a hand-held extruder. After extrusion, liposomal preparations were then sterilized by filtration through 0.22 μm sterile membrane.

3.2.2 Preparation of calcein-loaded liposomes

The calcein-loaded liposomes prepared as described in Section 1.3 were sterilized by filtration through 0.22 μm sterile membrane. The liposomal preparations were used as such without further quantification for calcien or lipid contents.

3.3 Cell culture

3.3.1 Maintenance of the human histiocyte/macrophage U-937 cell line

The human histiocyte/macrophage U-937 cells were grown in RPMI 1640 enriched with 10% FBS as a suspension. Cells were routinely seeded in 25 cm^2 tissue culture flasks and passed every 3 days with a passage ratio of 1:5 (v/v). Cultures were maintained in a CO_2 incubator at 95% humidity, 5% CO_2, at 37 ^{o}C. Doubling time of the U-937 was approximately 48 hours under these conditions.

3.3.2 Maintenance of the fibroblast BALB/c 3T3 cell line

BALB/c3T3 fibroblast cells were routinely seeded in 25 cm^2 tissue culture flasks at a concentration of 2 x 10^5 cells in 5 mL. BALB/c 3T3 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 8% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 100 U/ml amphotericin B, and 2 mM glutamine (DMEM+). The cells were routinely passed at no more than 70% confluency. Briefly, the cell monolayers were washed with 2 mL phosphate-buffered saline (PBS) and incubated for 2 minutes with 0.05% (w/v) trypsin-EDTA. Cells were redispersed with fresh medium after trypsin-EDTA was discarded. The dispersed fibroblasts were reseeded in the 25-cm^2 flask for further passages (2 x 10^5 cells/ 5 mL DMEM+). Cultures were maintained in a CO_2 incubator at 95% humidity, 5% CO_2, at 37 ^oC.

3.4 Selection of PTU and lipid doses

3.4.1 Dose response of PTU solution

Dose response of PTU was determined to select the appropriate concentration range for further experiments. Five concentrations of PTU in RPMI 1640 with 10% FBS (5.5, 11, 110, 220, and 330 µg/mL) were incubated with the U-937 (final seeding density = 1x10^5 cells/mL, final volume = 2 mL/well) for 24 hours under the culture conditions described above. At the end of incubation, cells were stained with 0.4% trypan blue solution for 1-2 min and counted. The total cell count was compared with that of the control, and the percentage calculated. The results were assessed in terms of the antiproliferative effect and the toxicity of PTU to the U-937 cells. Percentage of viable cells compared to that of the control was used to determine toxicity to the cells.

3.4.2 Effects of PC on cell growth

Empty liposomes containing PC at various lipid concentrations were prepared by the method described in Section 1.1. The antiproliferative activity and toxicity in the U-937 macrophages were evaluated to select the lipid concentration at which antiproliferative activity of the lipid was sufficiently low and toxicity to the cells was not evident.

Briefly, the extruded PC liposomes were incubated with the U-937 (final seeding density = $1x10^5$ cells/mL) at various lipid concentrations (7, 3.5, 1, 0.3, 0.15, and 0.075 mg/mL) in 24-well culture plates for 24 hours. The final volume was 2 mL/well. The cultures were maintained in a CO_2 incubator at 95% humidity, 5% CO_2, at 37 °C. At the end of the incubation period, cells were stained by 0.4% trypan blue solution for 1-2 min and were counted for both viable cells and dead cells. The criteria for antiproliferative effect and toxicity to cells were the same as in Section 3.4.1. The experiments were performed in quadruplicate (4 wells/batch)with four batches of each liposomal preparation.

3.5 Formulation effects on proliferation of U-937 cells

The experiments were performed under the protocol comparable to that described in Sections 3.4.1-2. The U-937 cells at the starting density of $1x10^5$ cells/mL were treated with 6 different conditions for each lipid composition as follows:

a) Blank liposomes

b) PTU liposomes

c) PTU liposomes with additional PTU solution

d) Blank liposomes with additional PTU solution

e) PTU solution

f) Control (culture medium)

The amount of liposomal suspension used was calculated from the lipid assay to give the final concentration of 0.075 mg of total lipid/mL in the incubation medium in all cases. Except for Treatments a, b, and f, PTU concentration was kept at 5.5 μg/mL in the incubation medium. The amount of PTU in Treatment b was varied according to difference in the encapsulation efficiency between formulations. Thus, in Treatment c, the amount of PTU added was also varied according to the formulation tested to keep PTU concentration at 5.5 μg/mL.

Briefly, the macrophage suspension was centrifuged at 1,000 rpm and washed with PBS buffer. The fresh medium was added, and the cell suspension was counted with a hemocytometer under the microscope. The seeding density was used according to the cell counts to make the final starting density of cells in the incubation medium $1x10^5$ cell/mL. Cultures with more than 90% viability were used in all cases. The treatments and controls were randomly assigned to each individual well. Four wells were assigned for each treatment. Cultures were maintained in a CO_2 incubator at 95% humidity, 5% CO_2, at 37 ^oC for 24 hours. At the end of incubation, an aliquot of the content of each well was collected and stained with 0.4% trypan blue. Cells were counted under an inverted microscope. The experiments were performed in quadruplicate (4 wells/batch) with four batches of each liposomal preparation. Percentage of total treatment/control cells and percentage of viable cells were determined as follows:

$$\text{Percentage of total cell} = (v+d)_{\text{Treatment in well number n}} \times 100/(v+d)_{\text{Control}}$$

$$\text{Percentage of viability} = [v/(v+d)]_{\text{Treatment in well number n}} \times 100$$

where v = no. of viable cells and d = no. of dead cells.

3.6 Uptake of calcein-loaded PC liposomes by the U-937 and the BALB/c 3T3 cell lines

The calcein-loaded liposomes were incubated with the two different cell lines, the U-937 and the BALB/c 3T3. The cell density and the liposome concentration were specified at $1x10^5$ cells/mL and 0.15 mg total lipid/mL, respectively.

The calcein-loaded liposomes were incubated with each cell preparation for 5, 12, and 24 hours in 6 well plates. Cultures were maintained in a CO_2 incubator at 95% humidity, 5% CO_2, at 37 ^{o}C. After incubation the content of each well was washed with PBS to remove the medium. Cells were redispersed in fresh medium and observed under a fluorescence microscope at 470 nm excitation wavelength and 509 nm emission wavelength. A treatment with calcein solution at 80 mM was performed and served as a control for nonspecific binding of the fluorescent dye to the cell surface.

4. Statistical analysis

Data analysis was performed on SPSS 9.0. Kolmogorov-Smirnov normality test on pooled samples was used to justify the valid assumptions for parametric tests. If the normality test showed that the distribution of data did not significantly deviate from normality, the analysis of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) was used. The data were transformed using a sine-log function as necessary in order to obtain the required normality.