CHAPTER IV

RESULTS AND DISCUSSION

In this study, percentage of total cells with respect to the control represented the antiproliferative effect of PTU or liposomes on U-937 cells. The percentage of viable cells compared to that of the control, on the other hand, indicated toxicity to the cells. A low percentage of viable cells (lower than 90%) was selected as the cutoff point for cytotoxicity. Cell preparations for seeding with less than 90% viable cells were routinely discarded.

1. Entrapment of PTU in liposomes

All liposomal formulations in this study were prepared with PTU at its maximum concentrations possible, in both the organic phase and the aqueous phase. The negatively charged lipids in this study included three different structures, namely dicetylphosphate (DCP), phosphatidylserine (PS), and phosphatidylglycerol (PG). The entrapment of PTU was studied in these liposomes, both with and without cholesterol (CH). Therefore, eight formulations (PC, PC/CH, PC/DCP, PC/DCP/CH, PC/PS, PC/PS/CH, PC/PG, and PC/PG/CH) were used in this study. The results in Figure 6 indicate that addition of cholesterol in liposomes significantly decreased the entrapment of PTU for all formulations ($p < 0.05$ by Student's T-test). At the pH of the aqueous phase used, PTU existed mostly in its unionized form, and association of PTU to the lipid bilayer was expected. Presence of CH in the bilayer would replace PTU molecules in the bilayer, resulting in lower encapsulation of the drug. These results agree well with other work, where both lipophilic and hydrophilic drugs were studied (Chang and Flanagan, 1994). This would not have been the case if PTU had not been associated with the bilayer. In fact, CH is known to increase encapsulation efficiency of hydrophilic drugs and reduce their leakage from liposomes (Allen and Cleland, 1980; Miyajima et al., 1993; Simons and Kramer, 1977).

The charge effects on the entrapment of PTU are shown in Figure 6. The mean entrapment of PTU in neutral liposomes composed of PC was significantly different from that of the negatively charged liposomes ($p < 0.05$). Negatively charged lipids produce a negatively charged interface on the liposomal bilayers. These charged interfaces, in turn, promote the electrostatic repulsion and result in expansion of the aqueous compartments between bilayers within liposome vesicles (Alpar, Bamford, and Walters, 1981; Bangham, de Gier, and Greville, 1967). A significant portion of PTU in these formulations was in the aqueous compartment according to its solubility in HEPES buffer pH 7.4 (1.1 mg/mL). Increase in aqueous compartments would thus increase the entrapment of PTU in liposomes.

Among the negatively charged liposomes, PTU entrapment in DCP liposomes was much lower than in PS and PG liposomes. This difference is statistically significant ($p < 0.05$). DCP is a non-phospholipid component. The difference in the headgroups of these structures may cause the difference in entrapment seen here. Appendix D shows the structures of the three lipids. In addition to the charge effect, the headgroups of PS and PG might also impose the steric effect to the liposomal bilayers. These two effects then added up, resulting in even greater distance between the bilayers in a liposome vesicle.

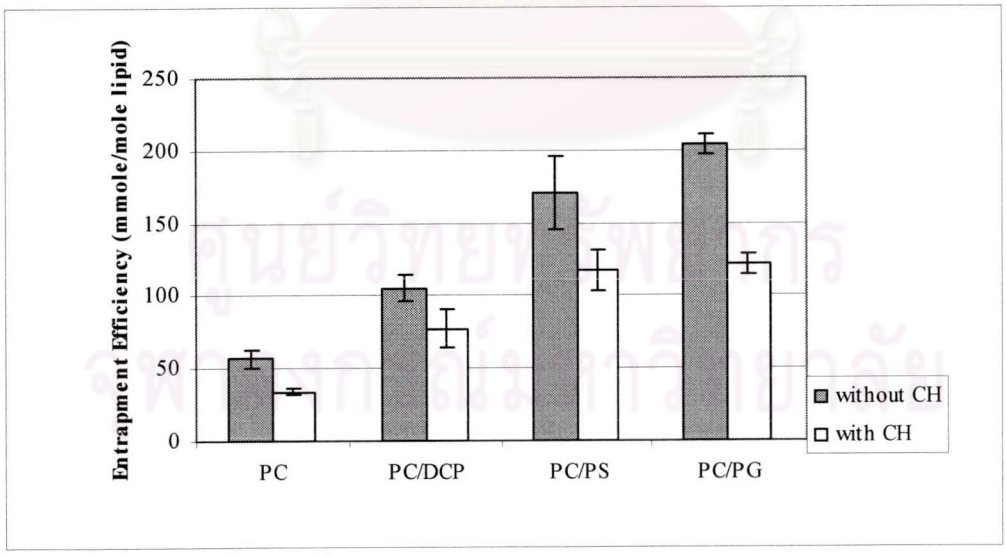


Figure 6: Entrapment efficiency of PTU liposomes with different lipid compositions. The charged lipid and cholesterol were included at 10 and 30 mol%, respectively. Each bar represents mean ± S.D. (n = 4).

2. Effect of PTU on cell growth

The aim of this experiment was to select appropriate concentrations of PTU. The required concentrations were those without cytotoxicity. In addition, to clearly distinguish the effects of the carrier systems, antiproliferation should be minimal as a solution. The antiproliferative effect of PTU solution at various concentrations is illustrated in Figure 7. The results in percentage of viable cells are presented in Figure 8.

Both the antiproliferative effect and cytotoxicity of PTU solution increased as PTU concentration increased. Cytotoxicity of PTU solution was clearly seen at PTU doses of 220 and 330 μg/mL. Thus, PTU concentration of 5.5 μg/mL was selected for further experiments where formulation effects on cell growth were studied.

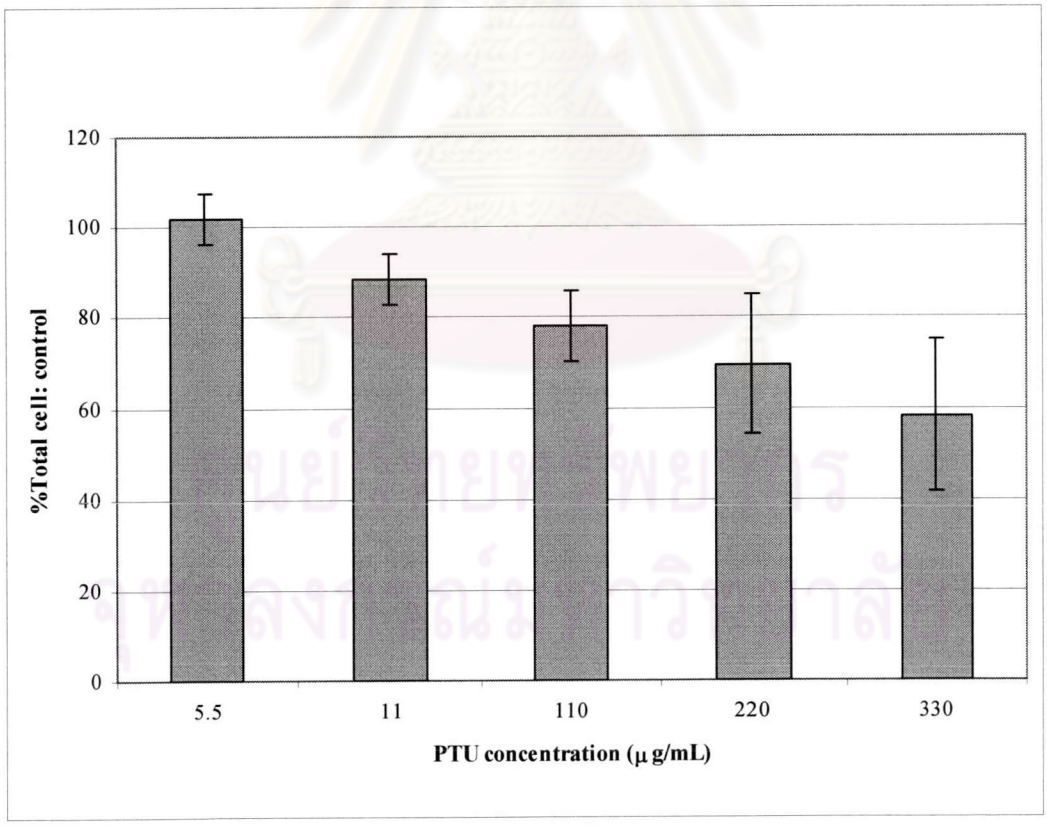


Figure 7: Effect of PTU on cell growth. Each bar represents mean ± S.D. (n = 4).

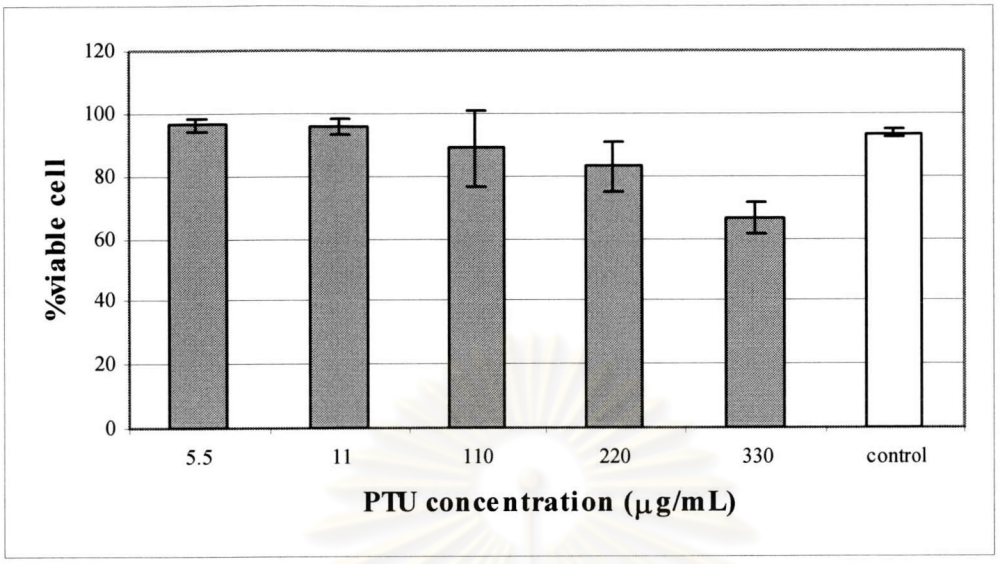


Figure 8: Percentage of viable cells at various PTU concentrations. Each bar represents mean ± S.D. (n = 4).

3. Effects of phosphatidylcholine (PC) on cell growth

There are reports regarding potential growth inhibitory effects associated with blank liposomes in some cell lines (Allen et al., 1981; Yoshioka et al., 1990). These effects seem to depend on the composition of the liposomes and the cell line studied. Thus, it was necessary to determine the lipid concentration at which cytotoxicity to the cells was devoid. Figures 9 and 10 display the antiproliferative effect and cytotoxicity of empty PC liposomes used in this study. Significant antiproliferation was seen at and above lipid concentration of 0.3 mg/ml. There was no statistical significance between the two lower doses ($p > 0.05$ by Tukey's test). No cytotoxicity was seen in all the doses studied ($p > 0.05$) though there was some such trend at higher concentrations (3.5 and 7 mg/mL). In a previous study, Allen et al. (1981) demonstrated that it might be necessary to keep empty liposome concentrations to levels below 60 μM in order to prevent certain cultured cell lines from liposome cytotoxicity. In most other studies on liposome-cell interaction, however, lipid was generally used at 100 μM (Lee, Hong, and Papahadjopoulos, 1992; Miller et al., 1998). Thus, the lipid concentration of 0.075 mg/mL, which was equivalent to 100 μM, was selected for further experiments. It is

worth noting that if incubation time was longer than 24 hours, significant cytotoxicity was seen at high concentrations (0.3 mg/mL and up, data not shown).

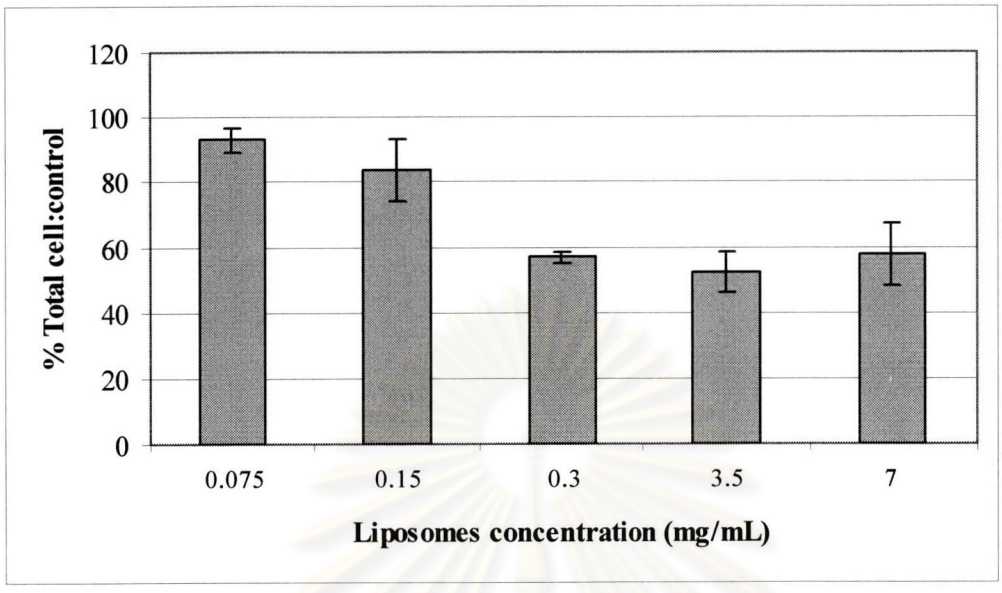


Figure 9: Antiproliferative effect of phosphatidylcholine (PC) liposomes on U-937 macrophages. Each bar represents mean ± S.D. (n = 4).

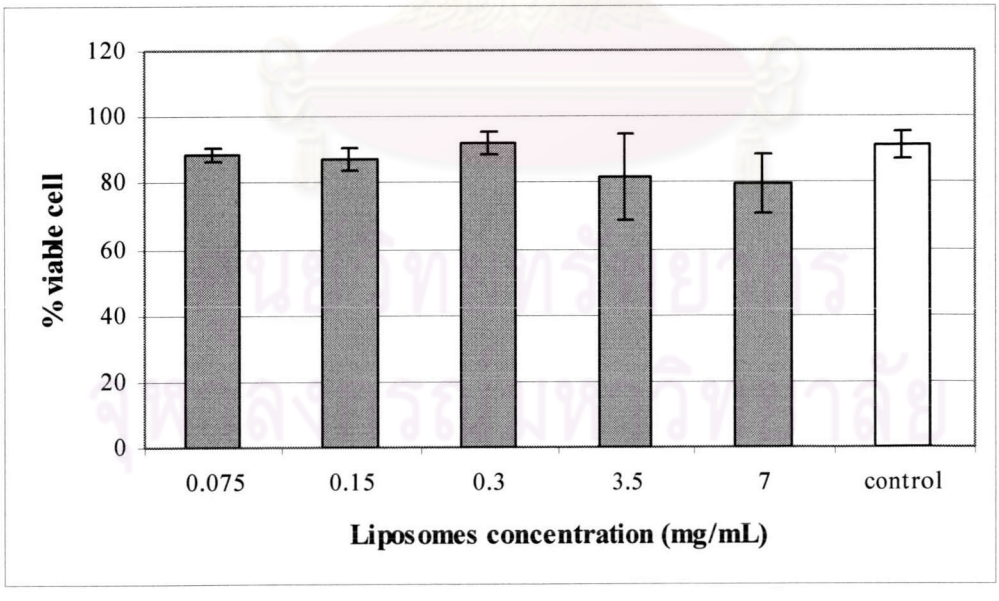


Figure 10: Percentage of viable cells of U-937 macrophages treated with various PC liposome concentrations. Each bar represents mean ± S.D. (n = 4).

4. Formulation effects on proliferation of U-937

4.1 Effects seen at lipid concentration of 0.075 mg/mL

4.1.1 Antiproliferative effect of neutral liposomes without cholesterol

Figure 11 depicts antiproliferation of neutral liposomes without cholesterol (PC liposomes) in the U-937 cells. It was clearly seen that PC had some antiproliferative action on U-937 cells at 0.075 mg/mL. At comparable lipid concentrations, PTU liposomes gave similar results to blank liposomes. This might reflect the low entrapment efficiency of the formulation. Thus, most of the effect seen was from PC. This argument is supported by the observation that additional PTU solution significantly increased the antiproliferation of PTU liposomes ($p < 0.05$). These results suggest that PTU entrapped in liposomes might not be promptly available to the cells. This would be in agreement with the scenario of liposomes entering the cells via endocytosis or phagocytosis. Drugs entrapped in liposomes need to be released from endosomes or phagosomes into the cytoplasm before they can exert any biological action. The tendency to reach the cytoplasm depends on the physicochemical properties of the molecule as well as the carrier system. It is possible that some drug molecules will be carried through the cell without being released into the cytoplasm (Straubinger et al., 1983). However, the highest antiproliferative effect was seen with blank liposomes with additional PTU solution. The effect seen was statistically different from the effect of blank liposomes ($p < 0.05$). PTU solution only did not show any antiproliferation. Thus, the effect of blank liposomes seemed to be synergistic to the antiproliferative effect of PTU. The mechanism underlying this observation is not clearly understood. However, similar observations with blank liposomes have been previously reported with other drugs (Katragadda et al., 2000). Theoretically, liposome-cell interaction could lead to either increased permeability of the cell membrane to the drug or increased susceptibility of the cell to drug effects. This is based on the fact that PC, which is invariably the main structural lipid used in most studies, is a surfactant.

4.1.2 Antiproliferative effect of neutral liposomes with cholesterol

The results of neutral liposomes with cholesterol composed of PC and CH (PC/CH liposomes) are shown in Figure 12. The effects on U-937 proliferation showed a comparable profile to that seen with PC liposomes.

Allen et al. (1991) found that inclusion of cholesterol in PC liposomes resulted in a decrease in the uptake by cultured mouse bone marrow macrophages. Such trend was not well evident here since there was no difference in the antiproliferative effect between the two formulations, especially in blank liposomes ($p > 0.05$). The only exception was in the blank liposomes with additional PTU solution. In this case, the antiproliferative effect was not a direct indicator of liposome uptake into the cells due to the confounding effect from PTU solution.

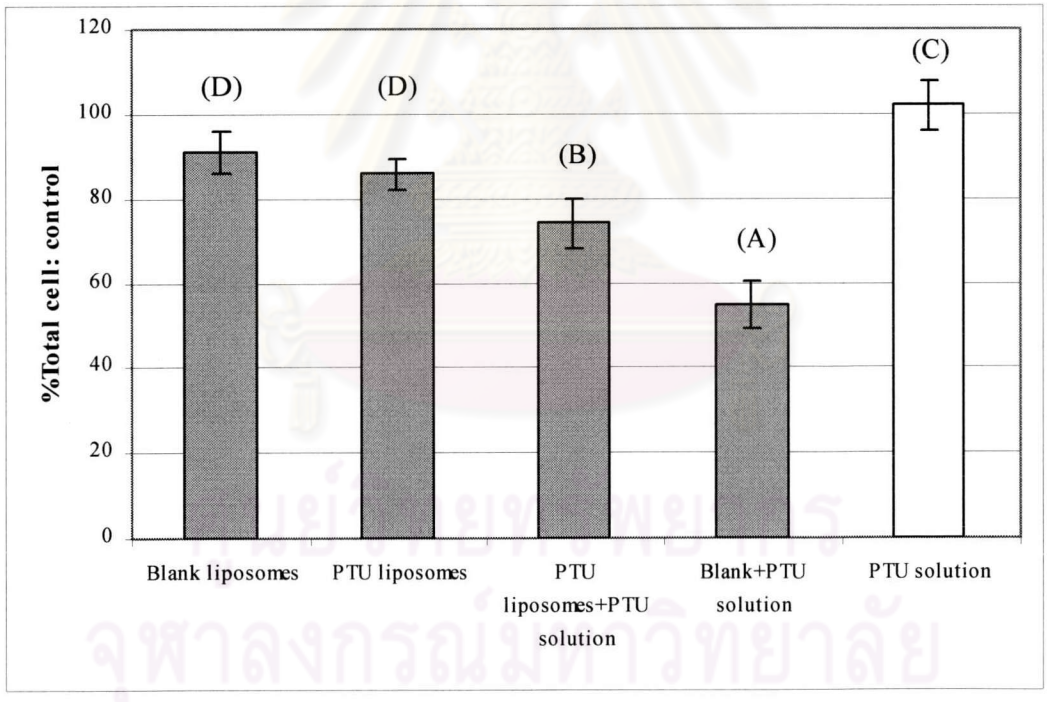


Figure 11: Antiproliferative effect of PC liposomes at lipid concentration of 0.075 mg/mL. Each bar represents mean ± S.D. (n = 4). Bars with the same alphabet belong to the same group by ANOVA and Tukey's test.

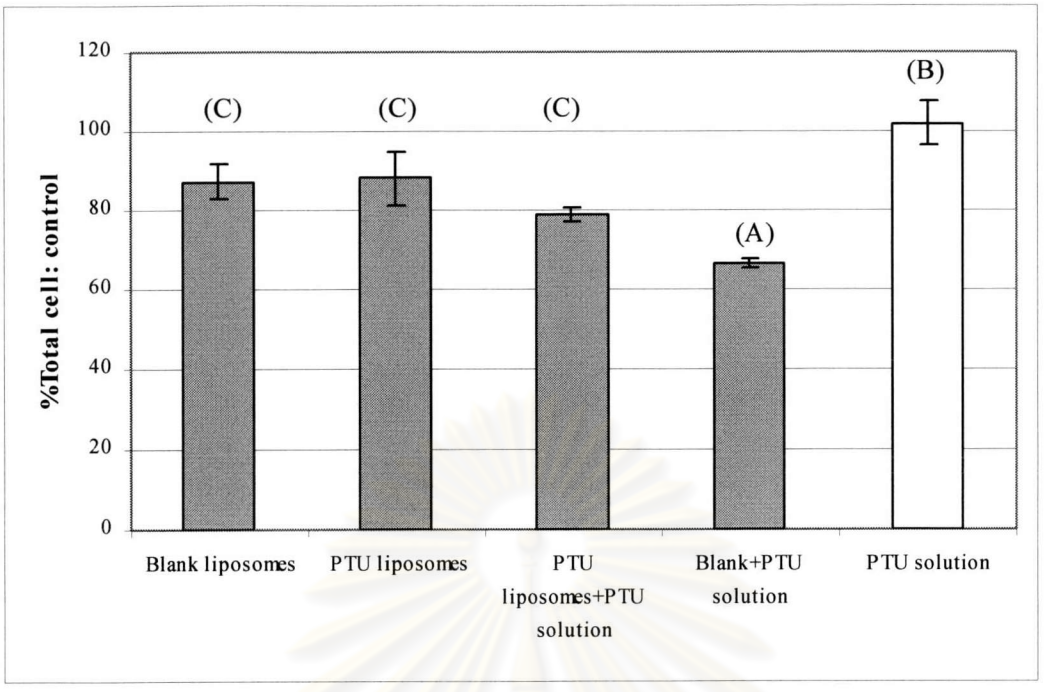


Figure 12: Antiproliferative effect of PC/CH at lipid concentration of 0.075 mg/mL. Each bar represent mean ± S.D. (n = 4). Bars with the same alphabet belong to the same group by ANOVA and Tukey's test.

4.1.3 Antiproliferative effect of negatively charged liposomes containing PC and DCP without and with cholesterol

The results of PC/DCP liposomes (Figures 13 and 14) are compared with those of PC liposomes in Figure 11. The results indicate no significant differences between PC and PC/DCP liposomes regardless of cholesterol. This was in contrast with previous works done in mouse liver and U-937 cell lines (Katragadda et al., 2000; Lui and Lui, 1996). Katragadda et al. (2000) reported that inclusion of DCP in DPPC liposomes increased the uptake of stavudine in U-937 cells. However, the effect reported was modest. The small discrepancy may be the results from the difference in the structural lipid (PC versus DPPC) as well as the different measurement tools used (antiproliferation versus uptake of the radiolabeled drug) in this present study. Thus, inclusion of DCP seemed to have minimal impact on liposome uptake by the U-937 cell line.

Although the entrapment efficiency was much higher with PC/DCP liposomes, there seemed to have no significant effect on antiproliferation. Again, most

of the effect seen was probably from the lipid (Figures 13 and 14). It was also clear that there was no effect seen from inclusion of CH.

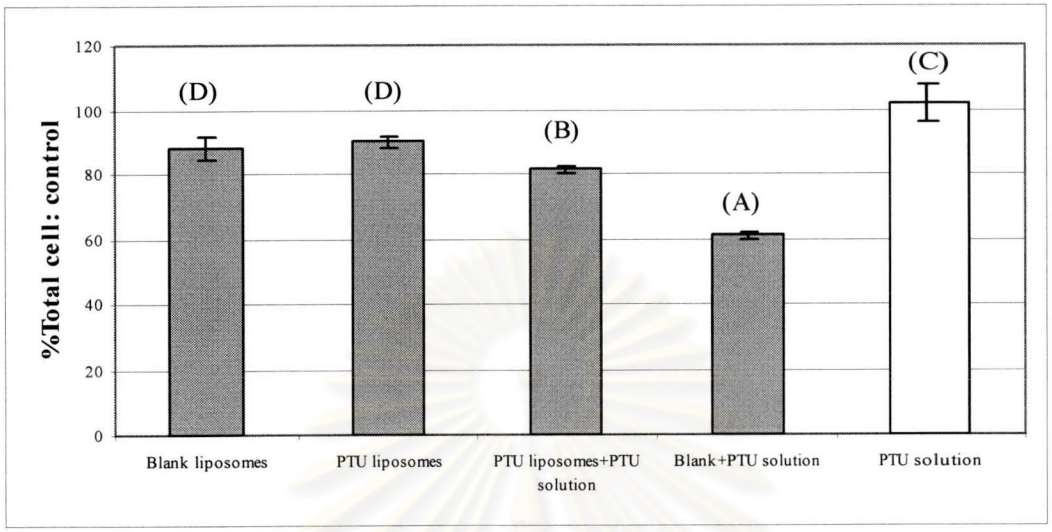


Figure 13: Antiproliferative effect of PC/DCP liposomes at lipid concentration of 0.075 mg/mL. Each bar represents mean ± S.D. (n = 4). Bars with the same alphabet belong to the same group by ANOVA and Tukey's test.

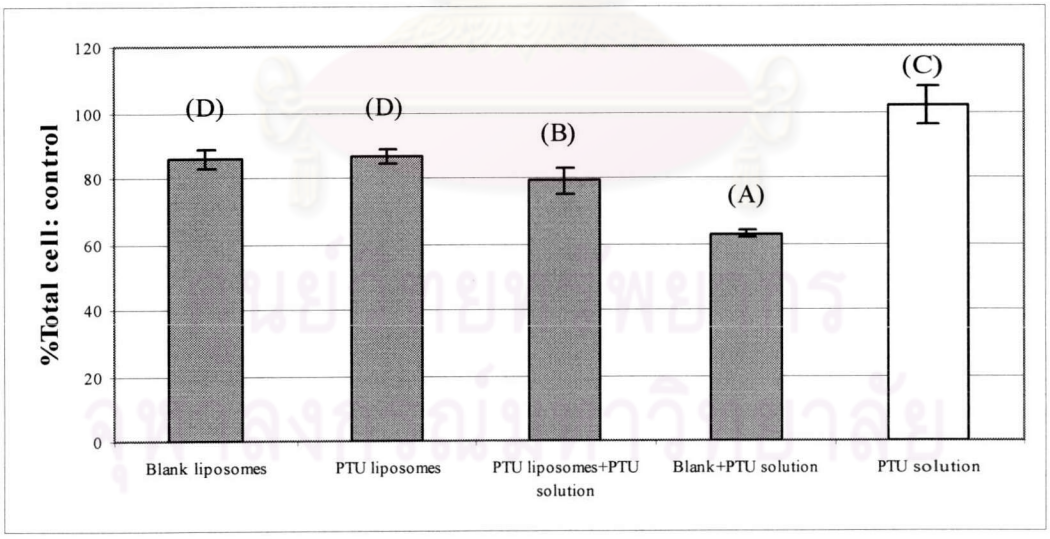


Figure 14: Antiproliferative effect of PC/DCP/CH liposomes at lipid concentration of 0.075 mg/mL. Each bar represents mean ± S.D. (n = 4). Bars with the same alphabet belong to the same group by ANOVA and Tukey's test.

4.1.4 Antiproliferative effect of negatively charged liposomes containing PC and PS without cholesterol

Figure 15 shows the antiproliferative effect of PC/PS liposomes under various treatment conditions. The effect of blank PC/PS liposomes was significantly different from that of blank PC liposomes ($p < 0.05$). This indicates that inclusion of PS might facilitate uptake of liposomes into the cells. Specific receptors for PS are known to present on the cell surface of macrophages (Allen et al., 1988; Balasubramanian and Schroit, 1998; Veldhuizen et al., 1998). Thus, endocytosis/phagocytosis via specific receptors might be the underlying mechanism. Many authors reported similar results where PS enhanced liposome uptake into macrophages with different cell lines (Allen et al., 1991; Bakker-Woudenberg, Lokerse, and Roerdink, 1988; Hsu and Juliano, 1982; Lee, Hong, and Papahadjopoulos, 1992). These include one study in the U-937 cell line (Katragadda et al., 2000). In this study, however, inclusion of PS did not increase the effect of PTU liposomes. In fact, the effect of PTU PC/PS liposomes was significanly less than that of blank PC/PS liposomes ($p < 0.05$). It should be possible that presence of PTU molecules in the bilayer of liposomal vesicles would affect molecular distribution of PS in the bilayer. This could lead to difference in charge density or spacing of the negative charges. Thus, binding between PS on the liposomal bilayer with the PS receptor on the macrophage cell might also change. If this was the case, then manipulation of PS amount on liposomes should show some supporting evidence. Thus, this point should be further explored.

Due to the high entrapment efficiency of these formulations, only a small amount of PTU could be added to keep the total concentration of 5.5 mg/mL. Thus, the effect of added PTU was not seen between PTU liposomes and PTU liposomes with additional PTU solution ($p > 0.05$). Similarly, the synergistic antiproliferative effect between blank liposomes and PTU solution was not seen ($p > 0.05$). The effect from blank PS liposomes could obscure the effect from PTU solution since the former was already abundant.

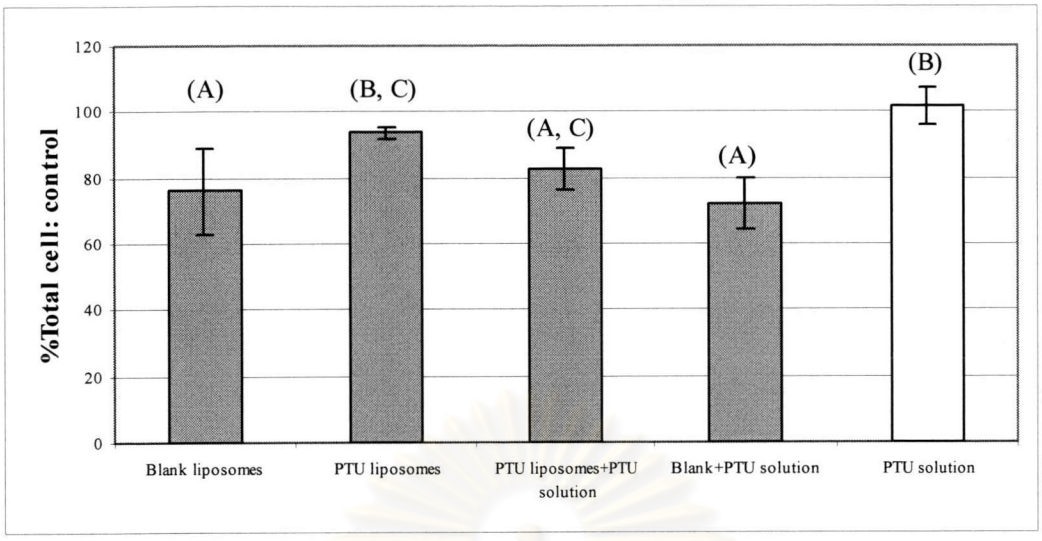
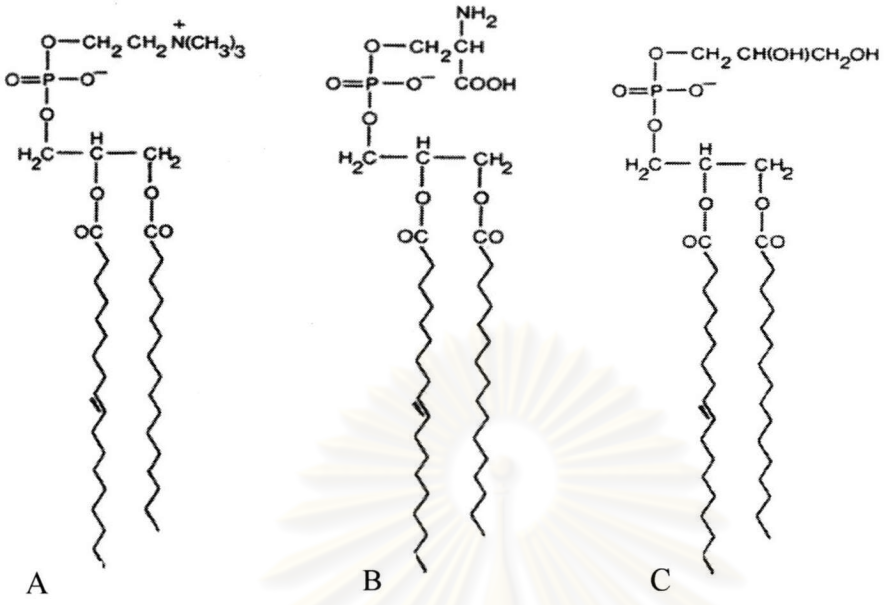


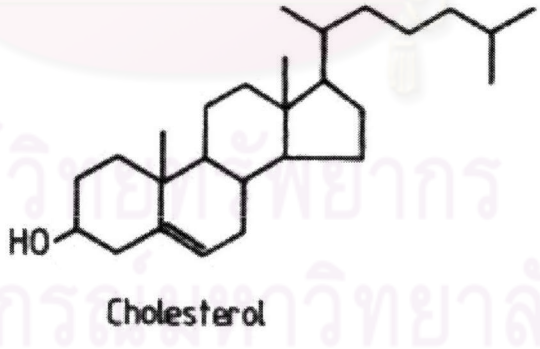
Figure 15: Antiproliferative effect of PC/PS liposomes at lipid concentration of 0.075 mg/mL. Each bar represents mean ± S.D. (n = 4). Bars with the same alphabet belong to the same group by ANOVA and Tukey's test.

4.1.5 Antiproliferative effect of negatively charged liposomes containing PC and PS with cholesterol

The results of negatively charged liposomes containing PC and PS with cholesterol (PC/PS/CH) are shown in Figure 16. When CH was included in the liposomes, the dramatic effect of blank liposomes was absent. In fact, the antiproliferation was completely lacking. Addition of PTU solution did not restore the synergistic effect seen in previous formulations. Unexpectedly, slight antiproliferation was seen with PTU liposomes regardless of additional PTU solution ($p < 0.05$). In this present study, CH was included at 30 mole%. Presence of this relatively large amount of CH in the bilayer would definitely affect the spacing of PS molecules on the bilayer. Apparently, this spacing was not suitable for binding of PS on the liposomal membrane with PS receptors on the cells. In addition, the improper spaced PS might cause charge repulsion between liposomal surface and the negatively charged cell surface. CH is also known to modify bilayer fluidity, which may affect liposome-cell interaction (Allen et al., 1991). Many studies have also shown that inclusion of CH



Molecular structure of phospholipids. A, phosphatidylcholine (PC); B, phosphatidylserine (PS); C, phosphatidylglycerol (PG). (From Graham and Higgins,1997)



Molecular structure of cholesterol (From New, 1997)

APPENDIX E

Phospholipid assay: Preparation of reagents

(New, 1997)

Lipid assay

Preparation of reagents

Ammonium molybdate-sulfonic acid reagent:

The solution was prepared by mixing of 5 ml of 5 M sulfuric acid with approximately 50 ml of double-distilled water and then adding of 0.44 g. of ammonium molybdate to the acid solution. The solution was mixed until ammonium molybdate dissolved completely, and the volume of this solution was adjusted to 200 ml with double-distilled water.

1-Amino 2-naphthyl4-sulfonic acid reagent:

The solution was prepared by weighing of 0.8 g of Fiske-Subbarrow reducer and then dissolving it in 5 ml of double-distilled water. This solution was freshly prepared on the day of use.

VITA

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and sphingomyelin (SM) decreasesd the uptake of liposomes by macrophages both in vitro and in vivo (Allen et al., 1991; Foong and Green, 1988; Moghimi and Patel, 1992). On the contrary, PTU liposomes displayed some antiproliferative effect. Association of PTU with the bilayer might, in one way or another, modify surface property of liposomes. The modification then resulted in some uptake of liposomes.

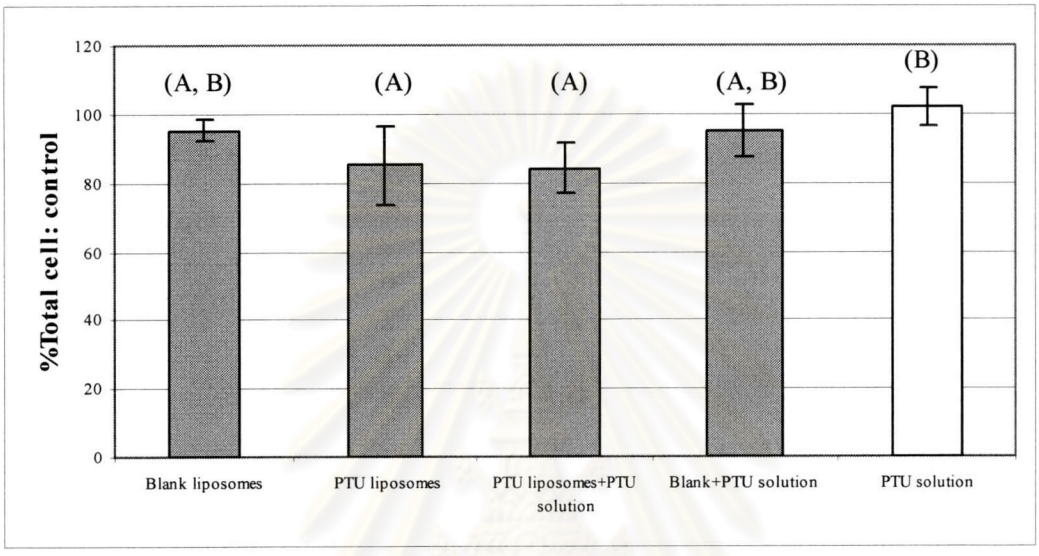

Figure 16: Antiproliferative effect of PC/PS/CH liposomes at lipid concentration of 0.075 mg/mL. Each bar represents mean ± S.D. (n = 4). Bars with the same alphabet belong to the same group by ANOVA and Tukey's test.

4.1.6 Antiproliferative effect of negatively charged liposomes containing PC and PG without and with cholesterol

The antiproliferative effects of the negatively charged liposomes containing PC and PG with and without cholesterol are depicted in Figures 17 and 18. The effects of PC/PG liposomes were not different from those of PC/PS liposomes. However, CH gave no further effects in the antiproliferation of these negatively charged liposomes.

Similar to what was seen with PS, blank PC/PG liposomes displayed stronger antiproliferation than that seen with blank PC liposomes. The same result was

seen when CH was also included. Many studies reported that the negative surface charge can be recognized by receptors found on a variety of cells, including macrophages (Allen et al., 1991; Allen et al., 1990; Allen et al., 1988; Lee, Hong, and Papahadjopoulos, 1992). In addition, Fidler (1988) found that inclusion of negatively charged phospholipids such as PS and PG in multilamellar vesicles (MLV) consisting of PC greatly enhanced their binding to and phagocytosis by macrophages. The antiproliferation seen here was an indirect evidence of enhanced uptake of these liposomes by the macrophages. Thus, the same scenario to that of PS was seen here. However, changes due to CH were not evident.

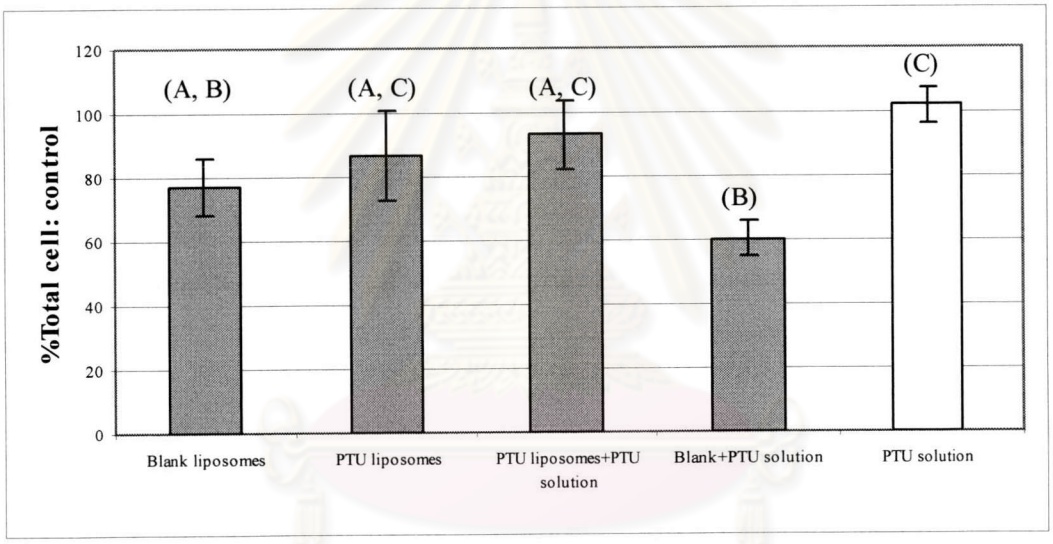

Figure 17: Antiproliferative effect of PC/PG liposomes at lipid concentration of 0.075 mg/mL. Each bar represents mean ± S.D. (n = 4). Bars with the same alphabet belong to the same group by ANOVA and Tukey's test.

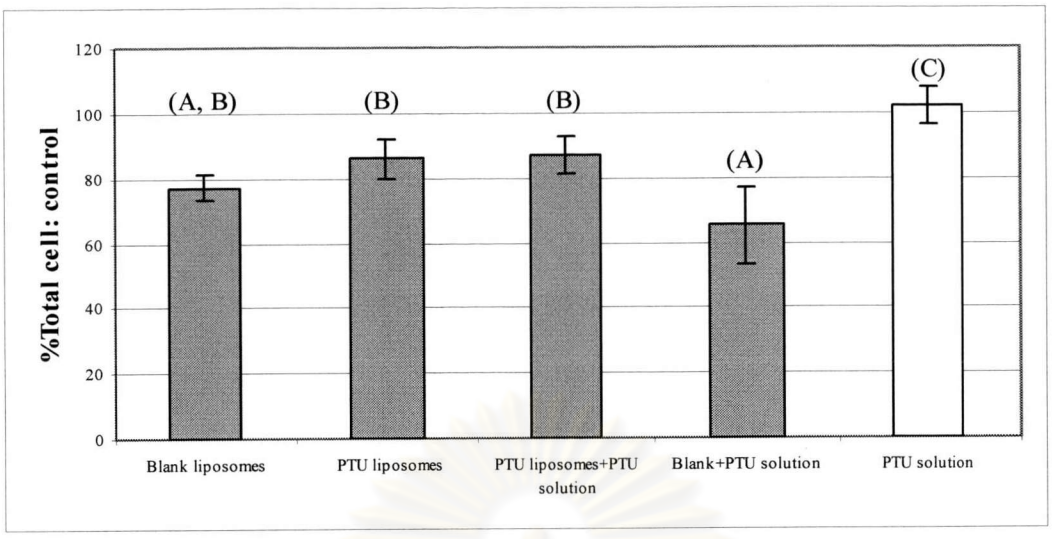

Figure 18: Antiproliferative effect of PC/PG/CH liposomes at lipid concentration of 0.075 mg/mL. Each bar represents mean ± S.D. (n = 4). Bars with the same alphabet belong to the same group by ANOVA and Tukey's test.

4.2 Effects seen at lipid concentration of 0.15 mg/mL

Since the effects of the entrapped PTU were not clearly seen with the previous experiments due to the low entrapment efficiency, the following experiments were carried out. In these experiments, the lipid concentration was increased in order to increase PTU entrapped in the liposomes. The lipid concentration of 0.15 mg/mL was used in these experiments because it had low toxicity and low antiprolifertive effect (see Figures 9 and 10).

4.2.1 Antiproliferative effect of neutral liposomes without and with cholesterol

From Figures 19 and 20, the trend of antiproliferation of neutral liposomes, both with and without cholesterol, was similar to the results of neutral liposomes with and without cholesterol at the lipid concentration of 0.075 mg/mL (see Figures 11 and 12). The antiproliferative effects were not significantly different ($p > 0.05$), except for those of blank PC/CH liposomes and blank PC/CH liposomes with

additional PTU solution. The difference seen with blank liposomes was minimal, which might not have any physical consequences. Higher lipid concentrations seemed to enhance the synergism seen between blank liposomes and PTU solution ($p < 0.05$). This result was still in agreement with the argument made earlier that blank liposomes might modify either drug uptake or drug susceptibility of the cells. Apparently, this modification was dose dependent.

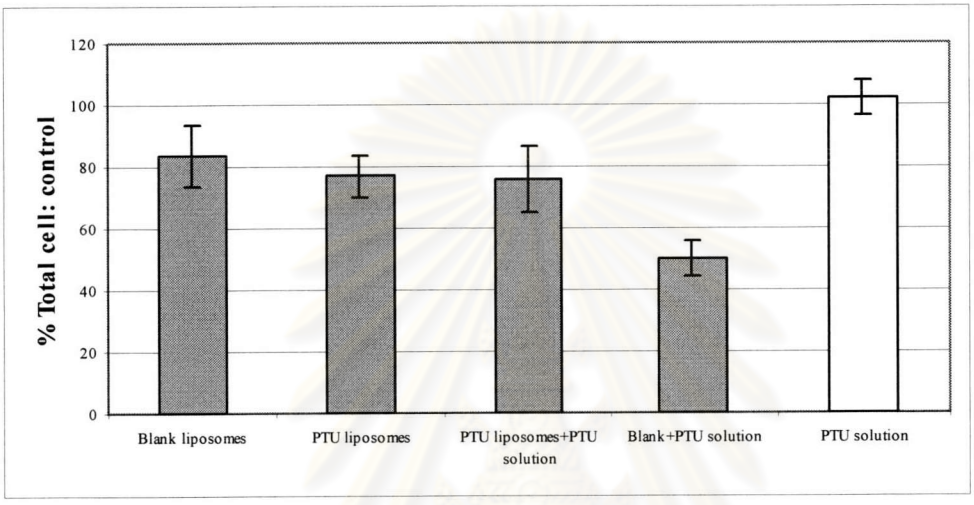

Figure 19: Antiproliferative effect of PC liposomes at lipid concentration of 0.15 mg/mL. Each bar represents means ± S.D. (n = 4).

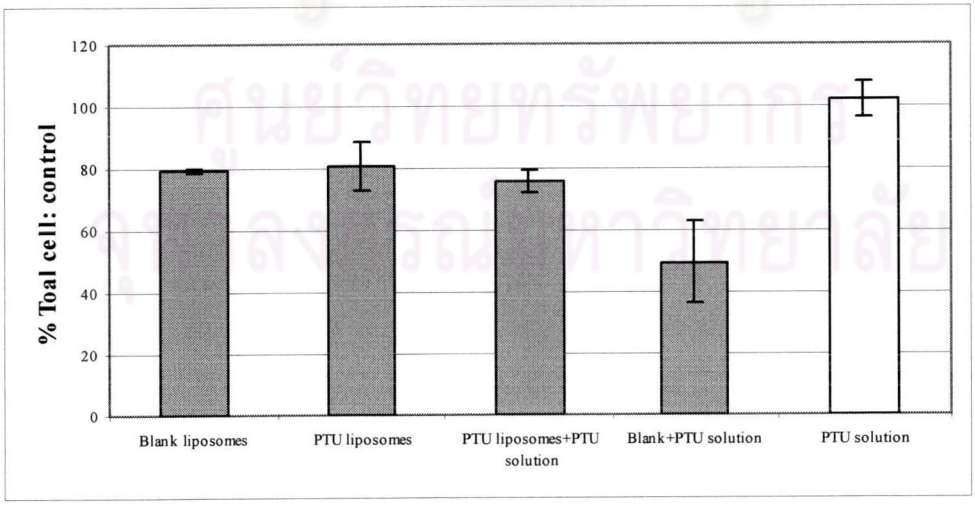

Figure 20: Antiproliferative effect of PC/CH liposomes at lipid concentration of 0.15 mg/mL. Each bar represents mean ± S.D. (n = 4).

4.2.2 Antiproliferative effect of negatively charged liposomes containing PC and DCP without and with cholesterol

The results in Figures 21 and 22 show antiproliferative effects of PC/DCP liposomes with and without CH. These results were basically the same as those seen with PC liposomes in Section 4.2.1.

In conclusion, increase in lipid concentration had the same effects for PC and PC/DCP liposomes with and without CH. These formulations were those with blank liposomes inserting relatively low effects on U-937 proliferation.

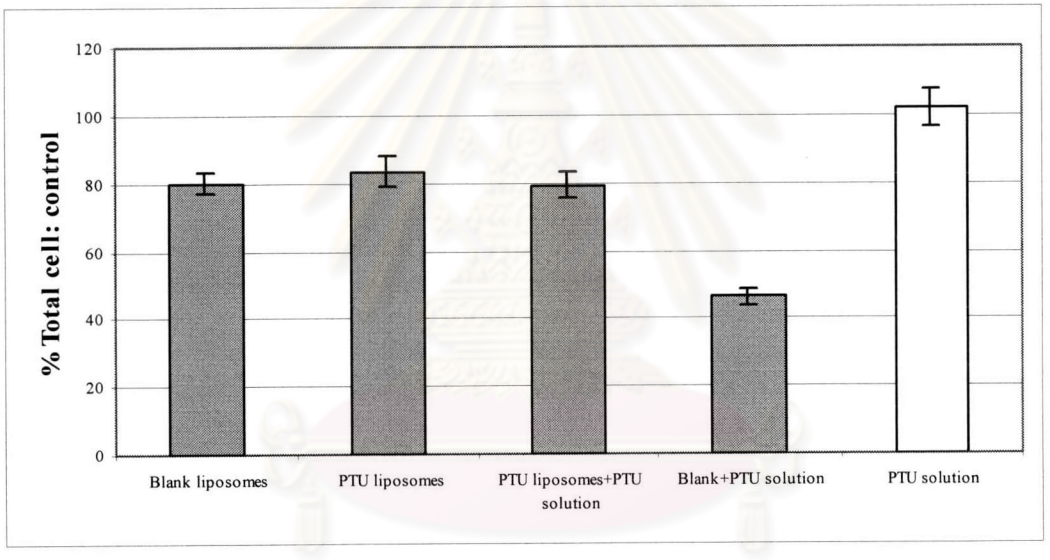

Figure 21: Antiproliferative effect of PC/DCP liposomes at lipid concentration of 0.15 mg/mL. Each bar represents mean ± S.D. (n = 4).

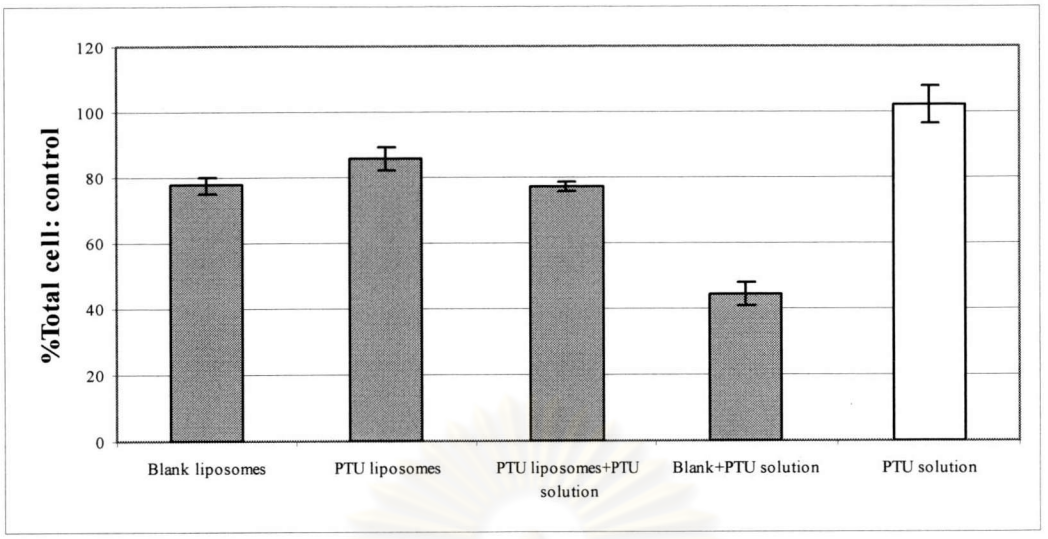

Figure 22: Antiproliferative effect of PC/DCP/CH liposomes at lipid concentration of 0.15 mg/mL. Each bar represents mean ± S.D. (n = 4).

4.2.3 Antiproliferative effect of negatively charged liposomes containing PC and PS without and with cholesterol

On the contrary, when liposomes were composed of PS, increase in lipid concentration did not affect the antiproliferation of U-937 in all cases ($p > 0.05$). This is also in agreement with the argument that PS rendered liposomes uptake via PS receptors on the cell surface. If saturation took place at 0.075 mg/mL of total lipid, these results would be expected.

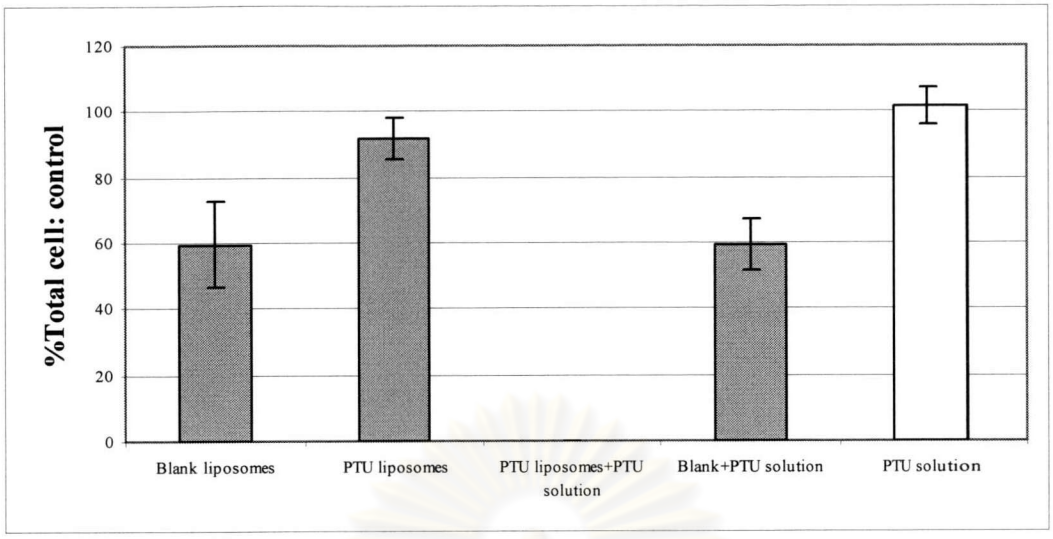

Figure 23 : Antiproliferative effect of PC/PS liposomes at lipid concentration of 0.15 mg/mL. Each bar mean ± S.D. (n = 4).

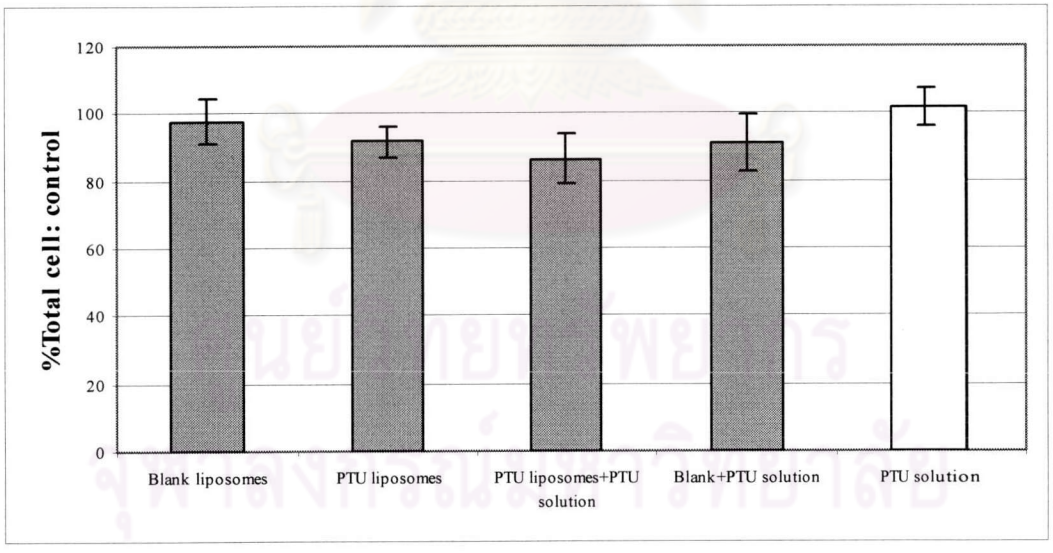

Figure 24: Antiproliferative effect of PC/PS/CH liposomes at lipid concentration of 0.15 mg/mL. Each bar represents mean ± S.D. (n = 4).

4.2.4 Antiproliferative effect of negatively charged liposomes containing PC and PG without cholesterol

Antiproliferative effects were increased when lipid concentration was increased to 0.15 mg/mL in all cases for PC/PG lipsomes without CH (Figure 25, $p < 0.05$). These results suggest that the uptake saturation had not been reached with 0.075 mg/mL lipids. This scenario was possible since PG-containing liposomes can also be actively taken by receptors on the cell surface.

4.2.5 Antiproliferative effect of negatively charged liposomes containing PC and PG with cholesterol

The results seen with PC/PG/CH liposomes were the same at 0.075 and 0.15 mg/mL total lipid (Figure 26, $p > 0.05$). Inclusion of CH per se did not exert any effects on antiproliferation of PC/PG liposomes in all treatments at 0.075 mg/mL lipid (see Section 4.1.4). Thus, it was expected that comparable results to those of PC/PG would have been seen. The results seen here suggest that the stoichiometry of binding/uptake between PC/PG liposomes and PC/PG/CH liposomes might not be the same. Thus, saturation of receptors was not seen at the same lipid concentration.

It is worth noting that, from all the results under section 4.1 and 4.2, no conclusions could be drawn regarding the biological consequences of entrapping PTU within liposomal vesicles. Further study should be carried out to clarify this point.

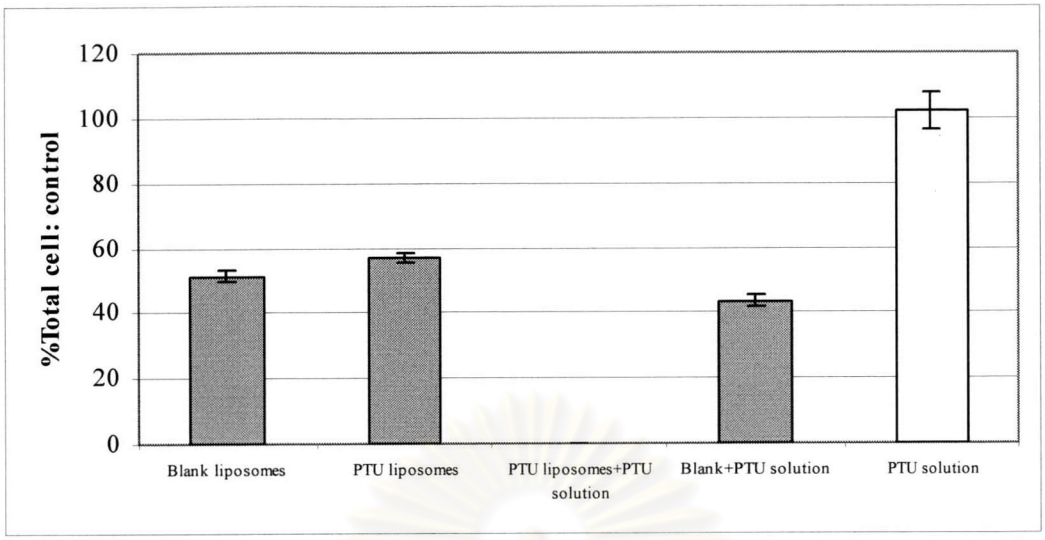

Figure 25: Antiproliferative effect of PC/PG liposomes at lipid concentration of 0.15 mg/mL. Each bar represents mean ± S.D. (n = 4).

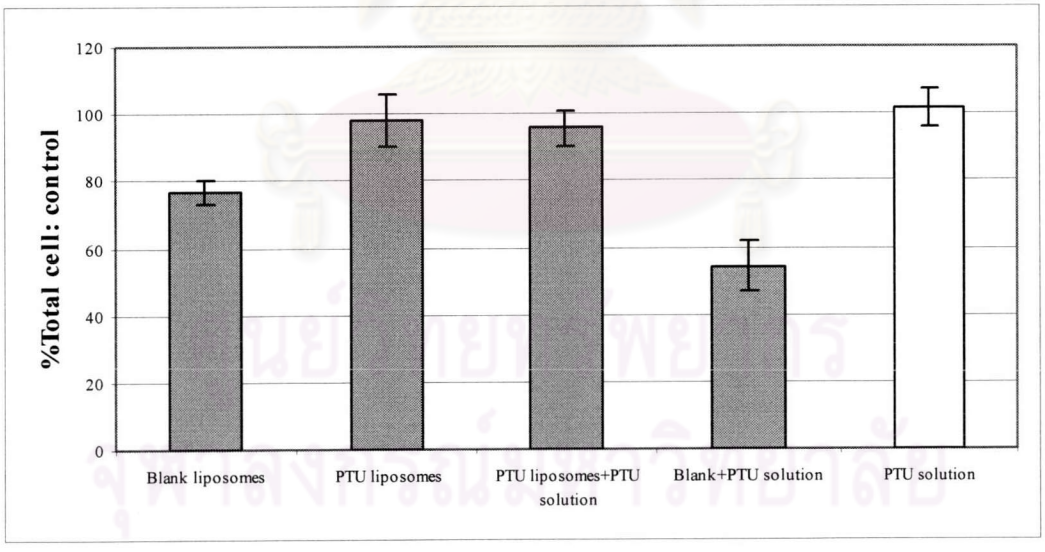

Figure 26: Antiproliferative effect of PC/PG/CH liposomes at lipid concentration of 0.15 mg/mL. Each bar represents mean ± S.D. (n = 4).

5. Uptake of calcein-loaded PC liposomes by the monocyte/macrophage U-937 and the fibroblast BALB/c 3T3 cell lines.

The difference in the experimental results between the U-937 and the BALB/c 3T3 cell lines was not seen at early time points (5 and 12 hours). Figures 27 and 28 show the photographs taken under a fluorescent microscope after the cells were incubated with calcein-loaded liposomes for 24 hours. The results seen with the BALB/c 3T3 cell line was in good agreement with fusion of liposomes with the cells (Papahadjopoulos et al., 1973). Fluorescence was seen confined within the fibroblasts (Figure 27). Treatment with calcein solution did not show any fluorescence (Figure 28). Thus, binding of calcein to the cell surface could be ruled out. On the contrary, no fluorescence was seen within the U-937 cells. These preliminary results show no evidence that calcein-loaded PC liposomes underwent fusion with U-937 cells. Since calcein is highly hydrophilic, the uptake via endocytosis and/or phagocytosis was still compatible with these results. It is possible that hydrophilic content of liposomes will not be released from endosomes or phagosomes into the cytoplasm during the process (Straubinger et al., 1983). However, other processes of liposome-cell interaction could not be totally ruled out. These include adsorption and modification of the cell surface by liposomes. Besides, only one type of liposomes was studied. Thus, a series of more-refined experiments would be necessary to elucidate the mechanism of PTU liposomes uptake by the U-937 cells.

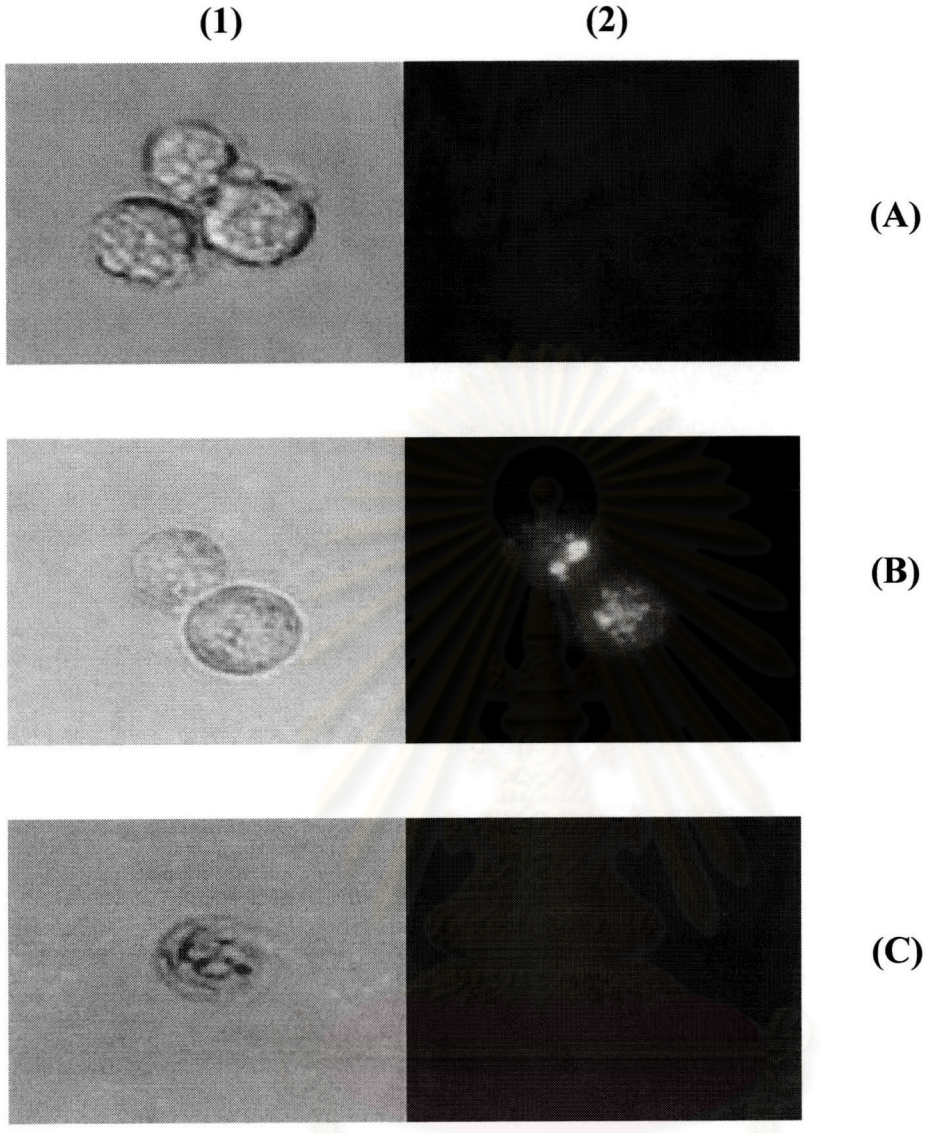

Figure 27: Photographs from light (1) and fluorescence (2) microscopes of BALB/c 3T3 fibroblasts (X400). The cells were incubated with calcein-loaded liposomes for 24 hours: (A) control, (B) cells incubated with liposomes and (C) cells incubated with calcein solution.

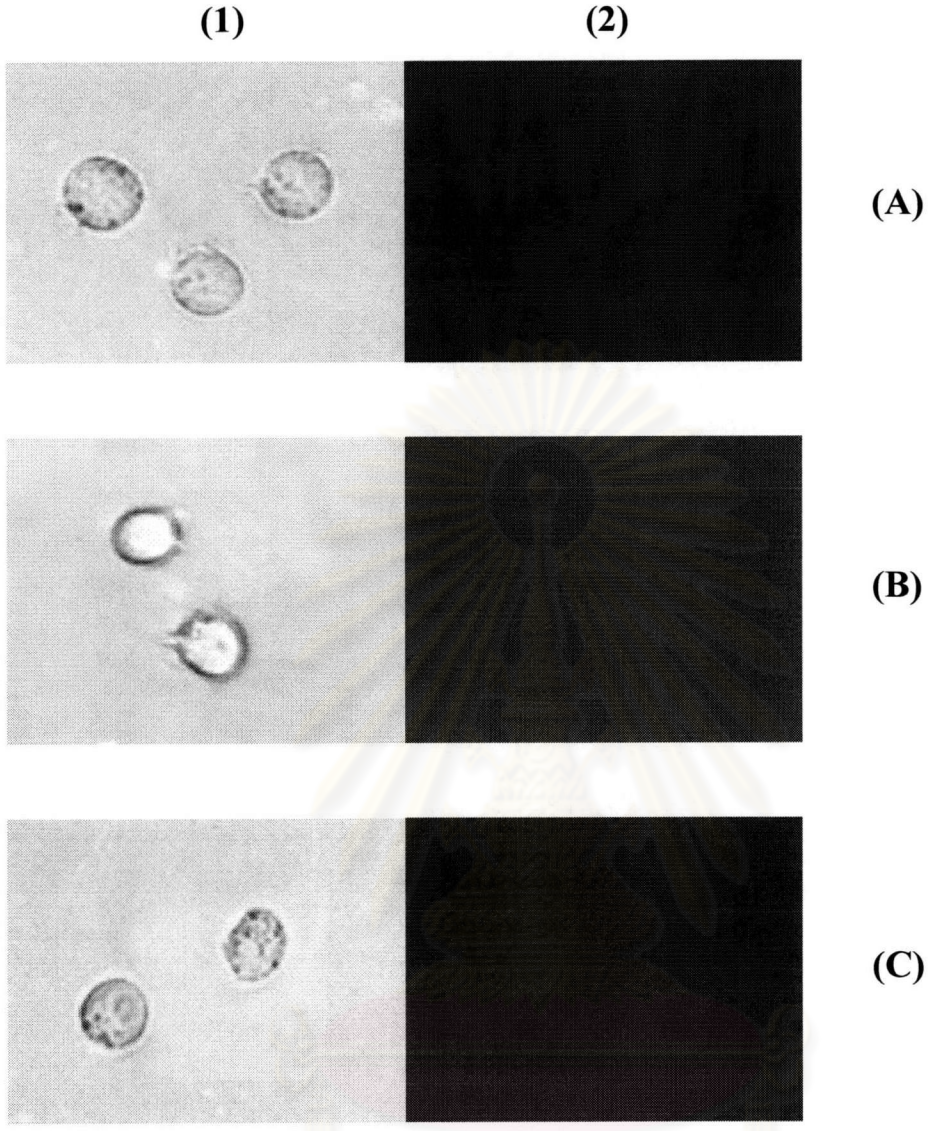

Figure 28: Photographs from light (1) and fluorescence (2) microscopes of U-937 macrophages (X200). The cells were incubated with calcein-loaded liposomes for 24 hours: (A) control, (B) cells incubated with liposomes and (C) cells incubated with calcein solution.