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Enhancement of Anti-metastatic Activity of Pentoxifylline by Encapsulation in Conventional Liposomes and Sterically Stabilized Liposomes in Murine Experimental B16F10 Melanoma Model

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Abstract

Pentoxifylline has been shown to exhibit anti-metastatic activity by inhibiting homing of B16F10 melanoma cells in the murine experimental metastasis model. In this study, the effect of encapsulation of pentoxifylline in conventional and sterically stabilized liposomes on its anti-metastatic activity in the murine experimental metastasis model was investigated.

After a single intravenous dose (10, 20 or 40 mg kg^{-1}), pentoxifylline solution, as well as conventional pentoxifylline liposomes, significantly reduced the number of pulmonary nodules compared with the untreated control group. Conventional pentoxifylline liposomes showed significantly higher inhibition (69%) of pulmonary tumour nodule formation at a dose of 20 mg kg^{-1} as compared with pentoxifylline solution (49%) at the same dose. Encapsulation of pentoxifylline in sterically stabilized liposomes prepared by incorporation of monomethoxypolyethyleneglycol (5000)–cholesteryl ester further enhanced the inhibition of pulmonary nodule formation (77%) at a dose of 20 mg kg^{-1} as compared with conventional pentoxifylline liposomes.

Overall, the results suggest that encapsulation of pentoxifylline in conventional liposomes enhanced its anti-metastatic activity. Steric stabilization of pentoxifylline liposomes also resulted in a two-fold increase in anti-metastatic activity (at dose of 10 mg kg^{-1}) as compared with conventional liposomes.

The ability of malignant cells to metastasize and form tumours at secondary foci is one of the major hurdles in achieving complete regression of tumours. Use of chemotherapeutic agents in cancer treatment is associated with severe side-effects because of damage to other normal rapidly dividing cells. Attempts have been made to use relatively less toxic drugs like aspirin (Gasic et al 1972) or warfarin (Zacharski et al 1981) to affect the essential steps in the metastatic spread of the tumour. Pentoxifylline, a methyl xanthine derivative, has been extensively used in conditions involving defective regional microcirculation. Pentoxifylline reduces the viscosity of blood by increasing deformability of red cells and by inhibiting platelet aggregation (Ward & Clissold 1987). It also improves tumour blood flow (Song et al 1994) and tumour radiosensitivity in mice (Honness et al 1993). Gude et al (1996) have reported that pentoxifylline mediated inhibition of homing of B16F10 melanoma cells in a murine experimental metastasis model.

Liposomes have been widely used as carriers for drug and vaccine delivery (Gregoriadis 1995) because of their ability to encapsulate drugs of varying solubility in their aqueous or lipid phase. The use of liposomes for drug delivery has unique advantages such as improved stability of the drug

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in the biological environment and potential site-specific delivery. One of the major obstacles in the use of conventional liposomes for drug delivery is their rapid detection and uptake by cells of the mononuclear phagocytic system (MPS), particularly that of the liver and spleen (Woodle 1995), thereby restricting their use to conditions involving the MPS system. The shortcomings of conventional liposomes have been overcome to some extent by developing sterically stabilized liposomes involving surface modifications using gangliosides or polymers such as polyethylene glycol (Allen et al 1995).

This study investigates liposomes as a delivery system for pentoxifylline with a view to enhance its anti-metastatic activity in the murine experimental B16F10 melanoma model. It also compares the anti-metastatic activity of pentoxifylline when encapsulated in conventional pentoxifylline liposomes with sterically stabilized liposomes, in the murine experimental B16F10 melanoma model.

Materials and Methods

Materials

Phospholipon 90 (PL90; soya phosphatidylcholine) and Phospholipon 90H (PL90H; hydrogenated soya phosphatidylcholine) were a generous gift from Nattermann GmbH, Germany. Cholesterol and stearyl amine were purchased from Loba Chemie Pvt. Ltd, Mumbai and Sigma, USA, respectively. Monomethoxypolyethyleneglycol(5000) carboxylic acid was synthesized according to the method of Lele & Kulkarni (1998) and conjugated with cholesterol to obtain monomethoxypolyethyleneglycol(5000)–cholesteryl ester (PEG-CH). Pentoxifylline B.P. was obtained as a gift sample from Sun Pharmaceuticals Ltd, Baroda, India. Iscove's Modified Dulbecco's Medium (IMDM) was reconstituted from powdered medium obtained from GIBCO, India and foetal calf serum (FCS) was from GIBCO, BRL, USA. Benzyl penicillin and streptomycin were purchased from Alembic Chemicals Ltd, India and Sarabhai Chemicals, India respectively. Chloroform (Analytical Reagent) was obtained from s. d. fine-chem. Ltd, Mumbai. All aqueous solutions and reagents were prepared in double distilled water.

Cell culture

The B16F10 melanoma cell line was generated at the Cancer Research Institute, Parel, Mumbai from B16F1 melanoma cell line as in the procedure described by Gude et al (1996). The cells were routinely maintained at 37°C in a humidified atmosphere of 5% CO_2 in IMDM with 10% FCS and antibiotics (100 IU mL^{-1} penicillin and 0·1 mg mL^{-1} streptomycin). Cells were harvested from flasks for subculturing with saline–EDTA.

Animals

In-bred C57BL/6 pathogen-free male and female mice, 6–8 weeks old, were obtained from Animal House, Cancer Research Institute, Mumbai, India.

Preparation of liposomes

Empty and pentoxifylline-loaded liposomes were prepared by slight modification of the thin-film hydration method described by Gabizon & Papahadjopoulos (1988). Briefly, PL90, PL90H, cholesterol and stearyl amine were placed in a 250-mL round-bottom flask in a molar ratio of PL90–PL90H–cholesterol–stearyl amine, 5·5 : 5·5 : 11 : 0·22 and dissolved in chloroform. Glass beads were added to increase the surface area available for film formation. Chloroform was evaporated under reduced pressure on a rotary evaporator (Superfit, India) at 40°C to form a thin film on the inner surface of the flask. The lipid film was hydrated by phosphate buffered saline pH 7·4 (PBS) or pentoxifylline solution (20 mg mL^{-1}) in PBS above the gel-to-liquid-crystalline phase transition temperature of the phospholipids. The round-bottom flask was hand-shaken vigorously for 5 min followed by heating to 60°C for 5 min to anneal liposomes. The flask was then shaken on a horizontal shaker bath (Expo, India) for 6 h with intermittent sonication (30 s) in a bath sonicator (Expo, India) after each hour to reduce the vesicle size. Sterically stabilized pentoxifylline liposomes were prepared by following the same procedure wherein PEG-CH was included at 10 mol% concentration in the phospholipid mixture.

Characterization of liposomes

Determination of entrapment efficiency. Liposomes were diluted with PBS and centrifuged (Remi, India) at 21 000 *g* for 30 min. The supernatant was appropriately diluted and absorbance was measured at 274 nm on a Shimadzu 160A UV-Visible Spectrophotometer with respect to similarly treated empty liposomes as the blank. Entrapment efficiency was calculated from the difference between the initial amount of pentoxifylline added and that present in the supernatant and was expressed as percentage of total amount of pentoxifylline added.

Particle size distribution. The particle size distribution of uncentrifuged and resuspended conventional, as well as sterically stabilized, pentoxifylline liposomes was evaluated by laser light scattering on a Malvern Mastersizer MS 3 (Malvern Instruments, USA).

Effect of conventional pentoxifylline liposomes on homing of B16F10 melanoma in C57BL/6 mice

C57BL/6 mice were divided into eight groups each containing five animals. B16F10 melanoma cells (1×10^5 cells/0·1 mL PBS) were injected intravenously via the lateral tail vein of mice on day zero. Empty and conventional pentoxifylline liposomes were prepared under aseptic conditions. The animals were treated with pentoxifylline solution (10, 20 or 40 mg kg^{-1}), conventional pentoxifylline liposomes (equivalent to 10 mg, 20 or 40 mg kg^{-1} of pentoxifylline) and empty liposomes intravenously on day one. Mice were killed on day 21, their lungs excised and the number of pulmonary metastatic nodules on the surface were counted under a dissecting microscope after fixing overnight in Bouin's fluid. The percent inhibition due to pentoxifylline treatment was calculated with respect to the number of nodules present in the untreated control group.

Enhancement of anti-metastatic activity of pentoxifylline encapsulated in sterically stabilized liposomes

Eight groups of C57BL/6 mice were injected with B16F10 melanoma cell line as mentioned above. On day one, mice were treated intravenously with pentoxifylline solution, conventional pentoxifylline liposomes and sterically stabilized pentoxifylline liposomes at doses of 10 mg kg^{-1} and 20 mg kg^{-1}, while one group was treated with sterically stabilized empty liposomes. Mice were killed on day 21 and pulmonary metastatic nodules were counted as reported above.

Statistical analysis

All data are reported as mean ± s.d. and differences between the groups were tested using Student's *t*-test at the level of significance of $P < 0{\cdot}05$.

Results and Discussion

The liposomal formulations used in this investigation utilized a combination of saturated (PL90H) and unsaturated (PL90) phospholipids to impart stability (Huang et al 1998). An equimolar proportion of cholesterol was included in the formulation since cholesterol reduces the permeability of bilayers and increases the stability of liposomes in the presence of biological fluids (Vemuri & Rhodes 1995). Stearyl amine was added to impart a positive charge to the bilayer, which increases the interlamellar distance due to repulsive forces. This therefore increases the the entrapment of water-soluble drugs (Vemuri & Rhodes 1995).

The entrapment efficiency of conventional pentoxifylline liposomes was found to be $10{\cdot}02 \pm 0{\cdot}99\%$ (n = 3) and that of sterically stabilized pentoxifylline liposomes was found to be $4{\cdot}52 \pm 0{\cdot}35\%$ (n = 5). Pentoxifylline is freely soluble in water and hence its entrapment efficiency is low. It is reported that the encapsulation efficiency of water-soluble compounds in liposomes prepared by the thin-film hydration method is generally low (Szoka & Papahadjopoulos 1978). Incorporation of PEG-CH led to a decrease in the entrapment efficiency of pentoxifylline. The results are in agreement with previous reports by Schneider et al (1996) who observed a sharp concentration-dependent reduction in the encapsulation efficiencies of water-soluble contrast agents in liposomes containing different concentrations of cholesteryl hemisuccinate monomethoxypolyethylene glycol and distearoylphosphatidylethanolamine monomethoxypolyethylene glycol. This observation can be explained by a reduction in internal vesicular volume due to bulky PEG chains covering both inner and outer monolayers of sterically stabilized liposomes.

Laser light scattering measurements showed unimodal distributions for both conventional and sterically stabilized pentoxifylline liposomes. To determine the effect of centrifugation on the aggregation of liposomes, particle size distributions of both uncentrifuged and resuspended conventional pentoxifylline liposomes was studied. The mean volume diameter and range of size for both conventional and sterically stabilized pentoxifylline liposomes are shown in Table 1. The mean diameter of conventional pentoxifylline liposomes, as well as their mean size range, increased slightly during the process of centrifugation and resuspension in PBS, suggesting little aggregation of vesicles. Incorporation of PEG-CH in liposomes led to a decrease in the mean volume diameter.

Fidler (1973) reported that the B16F10 subline has higher metastatic efficacy than the B16F1 cell line as observed by variation in the number of pulmonary tumour nodules. Studies with enrichment of B16F10 have shown a six-fold increase in lung homing as compared with B16F1 cells by in-

Table 1. Particle size distribution of conventional and sterically stabilized pentoxifylline liposomes.

	Conventional pentoxifylline liposomes		Sterically stabilized pentoxifylline liposomes
	Uncentrifuged	Resuspended	Uncentrifuged
Mean volume diameter (μm)	2·33	2·47	1·63
Range (μm)	0·67–6·63	0·13–7·72	0·05–6·63

vitro and in-vivo passages (Fidler 1973; Gude et al 1996). The B16F10 experimental metastasis model gave reproducible and satisfactory results in determining the anti-metastatic activity of pentoxifylline (Gude et al 1996, 1999). Hence, this animal model was selected for evaluating the effect of encapsulation of pentoxifylline in liposomes on its anti-metastatic activity and to compare the activity of pentoxifylline entrapped in conventional and sterically stabilized liposomes.

In a previous study (Gude et al 1996), intravenous administration of 40 mg kg^{-1} of pentoxifylline on day 1 and day 3 had shown significant inhibition of pulmonary metastatic nodules as compared with the untreated control group. Based on that report, we selected 3 doses of pentoxifylline, 10, 20 and 40 mg kg^{-1}, for intravenous treatment on day 1. The objective of this experiment was to investigate the effect of pentoxifylline encapsulation in conventional liposomes on the inhibition of pulmonary tumour nodule formation. The number of pulmonary metastatic nodules present after treatment with pentoxifylline solution and conventional pentoxifylline liposomes at 10, 20 or 40 mg kg^{-1} are shown in Figure 1. It was observed that all doses of pentoxifylline solution, as well as conventional pentoxifylline liposomes, resulted in significant ($P < 0{\cdot}001$) decreases in pulmonary nodule formation as compared with the untreated control group, whereas empty liposomes did not cause any significant reduction in lung nodules. Pentoxifylline solution and conventional pentoxifylline liposomes caused a dose-dependent increase in inhibition of nodule formation, with a maximum 77% inhibition by 40 mg kg^{-1} pentoxifylline in conventional liposomes. Reductions in lung nodule formation by pentoxifylline solution and conventional pentoxifylline liposomes were not significantly different at a dose of 10 mg kg^{-1}. However, at higher doses (20 and 40 mg kg^{-1}), there was a significant ($P < 0{\cdot}05$) decrease in pulmonary nodule formation by conventional pentoxifylline liposomes as compared with pentoxifylline solution. At a dose of 20 mg kg^{-1}, pentoxifylline solution caused 49% inhibition while conventional pentoxifylline liposomes inhibited nodule formation to the extent of

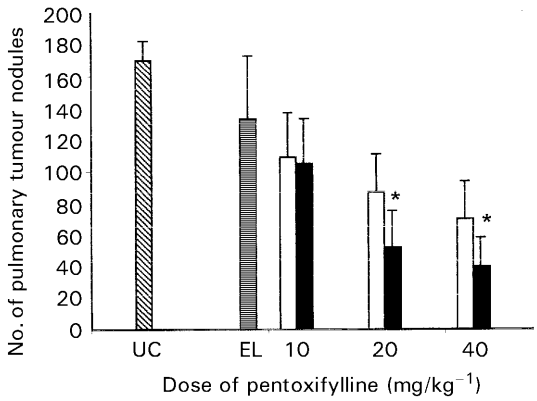

Figure 1. Effect of conventional pentoxifylline liposomes on pulmonary tumour nodule formation in C57BL/6 mice. Error bars indicate mean ± s.d., n = 5. UC, untreated control; EL, empty liposomes; □, pentoxifylline solution; ■, conventional pentoxifylline liposomes. *$P < 0{\cdot}05$ compared with respective dose of pentoxifylline solution.

69%. Also, administration of pentoxifylline solution (40 mg kg^{-1}) resulted in 59% inhibition while conventional pentoxifylline liposomes (40 mg kg^{-1}) caused 77% inhibition. These results indicate that encapsulation of pentoxifylline in liposomes leads to a significant increase in its anti-metastatic activity in this experimental metastasis model. This is probably achieved by an increased accumulation of pentoxifylline by conventional pentoxifylline liposomes in lung tumour nodules as compared with pentoxifylline solution. It is also possible that encapsulation of pentoxifylline in liposomes might protect it from metabolic enzymes thereby enhancing its duration of action, as, normally, pentoxifylline is rapidly metabolized with a half-life of 0·89 h (Ward & Clissold 1987).

Conventional liposomes containing phospholipids and cholesterol are rapidly taken up by cells of the MPS system. This is a drawback if the drug needs to be delivered to other organs of the body. Recently, sterically stabilized liposomes containing phosphatidylethanolamine derivatives of polyethylene glycols have showed prolonged blood circulation time and reduced MPS uptake (Allen & Hansen 1991; Woodle & Lasic 1992). In another report (Schneider et al 1996), cholesterylhemisuccinate monomethoxypolyethyleneglycol was

successfully incorporated into liposomes and affected physical properties such as encapsulation efficiency, particle size and zeta potential. Further, Ishiwata et al (1995) described the effect of incorporating another cholesterol derivative, poly (oxyethylene) cholesteryl ether on the biodistribution of liposomes. These liposomes exhibited longer circulation times in the blood after intravenous administration to rats. Also, their accumulation in the liver and spleen was significantly reduced compared with that of conventional liposomes. Based on these reports, it is conceivable that such steric stabilization of pentoxifylline liposomes may further enhance the anti-metastatic activity of pentoxifylline. To investigate this possibility, a separate study comparing the anti-metastatic activity of conventional pentoxifylline liposomes with sterically stabilized pentoxifylline liposomes was carried out. A new derivative of cholesterol, the cholesteryl ester of monomethoxypolyethyleneglycol-5000, was synthesized and incorporated into the lipid phase during liposome preparation to obtain sterically stabilized liposomes.

The effect of sterically stabilized pentoxifylline liposomes was compared with that of conventional pentoxifylline liposomes at 2 doses, 10 and 20 mg kg^{-1}, in the same murine experimental metastasis model. From Figure 2, it is evident that both doses of pentoxifylline solution, conventional pentoxifylline liposomes and sterically stabilized pentoxifylline liposomes lead to significant ($P < 0{\cdot}001$) decreases in pulmonary nodules as compared with the untreated control group, while the empty sterically stabilized liposomes did not show any significant effect. The percentage inhibition of pulmonary nodules was significantly increased ($P < 0{\cdot}05$) when the mice were administered 10 mg kg^{-1} or 20 mg kg^{-1} of pentoxifylline as sterically stabilized pentoxifylline liposomes as compared with conventional pentoxifylline liposomes at the same doses. Figure 3 indicates a two-fold decrease in the dose of pentoxifylline required to achieve comparable anti-metastatic activity when pentoxifylline was encapsulated in sterically stabilized liposomes as compared with conventional liposomes. The results suggest that incorporation of PEG-CH in pentoxifylline liposomes increases their anti-metastatic activity as compared with pentoxifylline solution and conventional pentoxifylline liposomes. Incorporation of PEG-CH sterically stabilized the liposomes, thus reducing their uptake by the MPS system, resulting in longer circulation times of the sterically stabilized liposomes in the blood compared with the conventional pentoxifylline liposomes, thus enhancing their probability of reaching distant target organs such as the lungs.

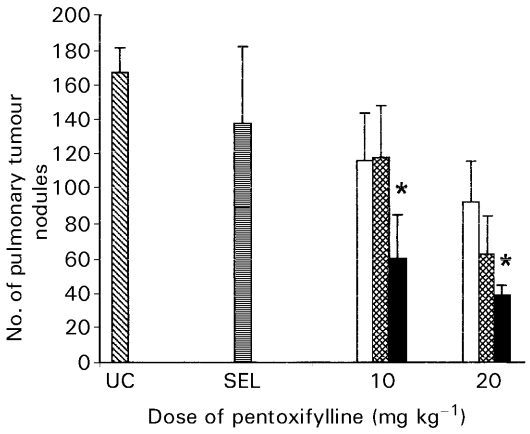

Figure 2. Enhancement of anti-metastatic activity of pentoxifylline by sterically stabilized liposomes. Error bars indicate mean ± s.d., n = 5. UC, untreated control; SEL, sterically stabilized empty liposomes; □, pentoxifylline solution, ▨, conventional pentoxifylline liposomes; ■, sterically stabilized pentoxifylline liposomes. *$P < 0{\cdot}05$ compared with respective dose of conventional pentoxifylline liposomes.

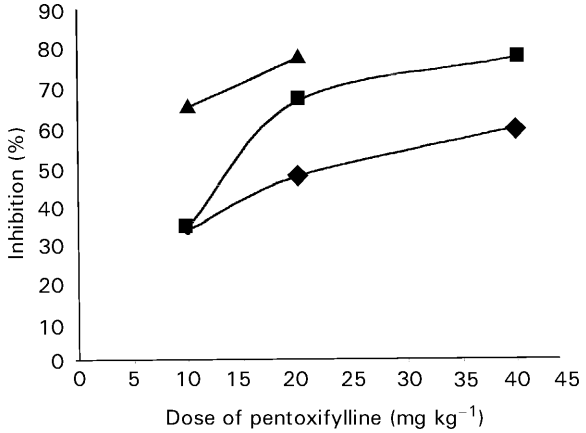

Figure 3. Improvement in the percentage inhibition of pulmonary tumour nodule formation by encapsulation of pentoxifylline in conventional pentoxifylline and sterically stabilized pentoxifylline liposomes. ◆, Pentoxifylline solution; ■, conventional pentoxifylline liposomes; ▲, sterically stabilized pentoxifylline liposomes.

It can be concluded that encapsulation of pentoxifylline in liposomes significantly increases its anti-metastatic activity, which is further improved by incorporation of PEG-CH in the liposomes. The use of sterically stabilized liposomes may therefore reduce the dose of pentoxifylline required thereby improving its clinical utility.

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