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**EFFECT OF LIPOSOME COMPOSITION AND CHOLESTEROL ON
THE CELLULAR UPTAKE OF STAVUDINE BY HUMAN
MONOCYTE/MACROPHAGES**

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Abstract: The objective of this study was to determine the cellular uptake of stavudine (an approved drug for AIDS treatment) by human monocyte/macrophages (U 937). The effect of lipid used, cholesterol concentration and the presence of charge on the liposome bilayer, on the cellular uptake by monocyte/macrophages was investigated. Liposomes employed in the study were prepared by reverse phase evaporation. The lipids egg PC, DMPC, DPPC, DSPC, DMPG and sphingomyelin were employed in this study. The effect of cholesterol on cellular uptake was studied by using liposomes containing a constant amount of lipid and varying amounts of cholesterol. Stearylamine or dicetylphosphate (10 mol%) was used to induce positive or negative charge on the bilayer. The cells were separated from liposomes by centrifugation in membrane filters and the amount of stavudine taken up by macrophages was estimated using tritium labeled drug as a marker. Stavudine uptake was found to be the maximum (approximately 950 ng/million cells) in liposomes containing dipalmitoyl phosphatidylcholine (DPPC). The presence of sphingomyelin, which increases bilayer rigidity decreased cellular uptake of stavudine and the presence of negative charge on the bilayer, enhanced the uptake of stavudine compared to positive charge. There is no apparent difference in uptake when varying amounts of cholesterol was added to liposomal formulations. The present study shows that the sensitivity of macrophages to different charge and lipid type can be used to either decrease or increase cellular uptake as desired.

Key Words: Liposomes, Cellular Uptake, Stavudine, Monocyte/Macrophages, AIDS

INTRODUCTION

Liposomes have been developed and evaluated as carriers to deliver encapsulated molecules to mammalian cells *in vitro* and *in vivo*. Recent investigations of liposomes have led to the development of their applications to medicine and many drugs have been encapsulated in lipid vesicles, and such encapsulated drugs have pharmacodynamic and pharmacokinetic properties, which are radically different from that of free drug [1]. Once administered in the body, liposomes of conventional formulations are avidly removed from circulation by the macrophages of the reticuloendothelial system or mononuclear phagocyte system (MPS) [2]. This apparent propensity of liposomes to be taken up by macrophages can be advantageous in the treatment of diseases such as AIDS where macrophages themselves harbor infectious HIV [3].

Stavudine or d4T is one of the latest of drugs, belonging to the class of reverse transcriptase inhibitors approved by the FDA for the treatment of AIDS. The advantage of stavudine *vis-a-vis*, other reverse transcriptase inhibitors is that it has greater bioavailability (88-99%), has a lower dosage regimen and is effective against strains resistant to AZT and didanosine (ddI). However, long term administration of stavudine over a period of 6 months result in adverse side effects such as a dose limiting peripheral neuropathy. Less significant but potentially limiting side effects include anemia, insomnia and malaise [4]. A liposomal drug delivery system may be ideal in the case of stavudine in that, it can alleviate drug toxicity and also deliver the drug directly to the macrophages in a passive manner. Such a reduction in toxicity has been demonstrated in the case of drugs like Doxorubicin and Amphotericin B [5-8]. Cellular uptake of the drug by macrophages assumes significance since it is required to monitor, and is representative of the fraction of input drug delivered to the desired site of action.

Previous studies have indicated that a wide variety factors such as, liposome composition, cholesterol content of liposomes, presence of charge on the bilayer, and liposome size dictate the uptake of liposomes by macrophages [9]. In the present study we systematically observed the cellular uptake of stavudine (d4T) as a function of lipid type, cholesterol concentration of liposomes and charge on liposome surface. The results obtained are significant in the design and formulation of liposomal delivery systems in the treatment of AIDS.

MATERIALS AND METHODS

The lipids, egg phosphatidylcholine (PC), dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC), dimyristoyl phosphatidylglycerol (DMPG) and sphingomyelin were purchased from Avanti Polar Lipids (Alabaster, AL).

Stavudine was a gift from Bristol-Myers Squibb (Princeton, NJ) and radiolabeled (^3H) stavudine was obtained from Moravek Biochemicals (Brea, CA). Stearylamine (SA), dicetylphosphate (DCP), Triton X-100, RPMI 1640 medium were purchased from Sigma Chemical Company (St. Louis, MO). Fisher Scientific (Fairlawn, NJ) supplied the solvents, ethyl ether and chloroform. Polycarbonate membrane filters, and scintillation cocktail were purchased from Millipore Corporation (Bedford, MA) and Packard Instrument Company (Meriden, CT) respectively. Becton-Dickinson Labware (Lincoln Park, NJ) was the manufacturer of six well cell culture plates.

Preparation of liposomes

Unilamellar liposomes were prepared by reverse phase evaporation (REV) method described by Szoka and Papahadjopoulos [10]. Accurately weighed quantities of the respective lipid and cholesterol were transferred to a 50 ml round bottom flask and the contents dissolved using ethyl ether. Most of the lipids were soluble in ethyl ether and where solubility was the rate-limiting step, minute quantities of chloroform was used as a co-solvent. The aqueous phase containing 1 mg/ml of stavudine spiked with traces of tritium (^3H) labeled stavudine was added to the lipid in organic solvent and a w/o emulsion was subsequently formed by high energy transfer provided by a probe sonicator (Heat Systems-Ultrasonics Inc., NY) for 3-4 min. A thorough emulsion was deemed formed when the polar and non-polar components remained homogenous for at least 30 minutes. The organic solvent was then removed from the emulsion in a rotary evaporator, the water bath maintained at 30°C , until a smooth suspension of unilamellar liposomes was formed. The suspension was then exposed to a stream of nitrogen gas for a period of 1 hour to expel traces of organic solvent. The liposomes were formulated to contain $66\ \mu\text{mol}$ of lipid and the amount of organic solvent used was three times the quantity of aqueous phase for optimum drug entrapment. To achieve a homogeneous population of unilamellar liposomes, the preparations were passed through an extruder (Lipex Biomembrane, Vancouver, Canada) with a stack of two polycarbonate filters of pore diameter $0.2\ \mu\text{m}$. 10 mol% of stearylamine or dicetylphosphate were used to impart positive or negative charge respectively.

Cellular uptake studies

Cultured human macrophages (U 937) were used to determine the extent of cellular drug uptake from liposomal formulations. Cells were grown in RPMI 1640 medium containing 10% v/v of fetal calf serum (FCS), benzyl penicillin (100 U/ml) and streptomycin ($10\ \mu\text{g/ml}$). Cells in the log phase of growth were utilized in the investigation.

About 3 ml of the cell suspension, corresponding to a seeding density of 5×10^6 cells/well, was transferred to six well culture plates and 10 μ l of the appropriate liposomal suspension was then added to each of the six wells containing macrophages. As a control 10 μ l of 1 mg/ml stavudine solution spiked with traces of tritiated stavudine was added to one of the wells. The plates were incubated in a controlled environment at a temperature of $37 \pm 1^\circ \text{C}$ for a period of 48 hours.

Estimation of cellular uptake

During incubation of the plates, at appropriate time points of 0, 1, 4, 8, 20 and 48 hours the entire cell suspension in each well was transferred to polycarbonate filters (poresize 0.45 μ m). The wells of the cell culture plates were rinsed with 1 ml of phosphate buffered saline (PBS), pH 7.4 and the washings subsequently transferred to the filters. The cells were separated from the medium, in the form of a pellet by centrifuging the filters at 4000 rpm for 15 minutes. About 0.5 ml of Triton X-100 was added to the pellet to rupture the cells and the mixture were incubated at room temperature for 5-6 hours. Subsequently, 2 ml of PBS, pH 7.4 was added to the cells, the filter vortex mixed and the cell suspension transferred to scintillation vials. About 3 ml of scintillation cocktail was added to the vials and the associated radioactivity was determined in a liquid scintillation counter (Beckman LS 5000 TD, Beckman Instruments Inc., Fullerton, CA).

Data analysis

Data analysis was performed using independent group ANOVA. Newman-Keuls multiple comparison tests has been performed for the data sets and statistical significance has been determined at 95% confidence level.

RESULTS AND DISCUSSION

The encapsulation efficiency of stavudine in liposomes ranged from 35-50% of total input drug and the particle size of liposomes prepared by reverse phase evaporation ranged from 0.6 to 1.4 μ m. The formulations were extruded through polycarbonate membranes of pore size 0.2 μ m in order to prepare a homogeneous population of liposomes. Although similar amounts of lipid are taken up when the macrophages are exposed to either multilamellar, unilamellar or reverse phase evaporated liposomes, that actually corresponds to different numbers of particles and to greatly different uptakes of trapped volume. Previous studies have indicated that in terms of particle number, uptake of small unilamellar vesicles exceeded uptake of large reverse phase evaporated vesicles by 100 times and in terms of uptake of trapped volume reverse phase liposomes exceeded the uptake of unilamellar liposomes by a factor of 100 [11]. Hence,

extrusion of reverse phase evaporated vesicles offers dual advantage both in terms of uptake of the numbers of particles and also the uptake of trapped volumes.

The liposomes were separated from the cells by centrifugation. The basis for using filters to estimate encapsulation is that liposomes which had a particle size of approximately 0.2 μm readily passed through the filter during centrifugation whereas the macrophages which are bigger than the liposomes are retained on the filter along with the stavudine they have taken up. Figures 1-2 show the uptake of stavudine from liposomal formulations. It can be observed that drug uptake was higher in all the cases except when sphingomyelin liposomes were used, where the apparent uptake was slower than that of the free drug. Maximum uptake was observed when DPPC liposomes were used, which is 3 fold compared to the free drug. While DSPC and Egg PC showed a linear increase in uptake with time, the profile of DPPC and DMPC resulted in enhanced uptake within 10 hours followed by a plateau. The extent of drug uptake from liposomal formulations is an important determinant of the dosage of the formulation. It is highly desirable that drug uptake from liposomal formulations be maximal so that a smaller dosage of the formulation would suffice to achieve an optimal therapeutic effect. Moreover, small doses are convenient to the patient in terms of parenteral administration.

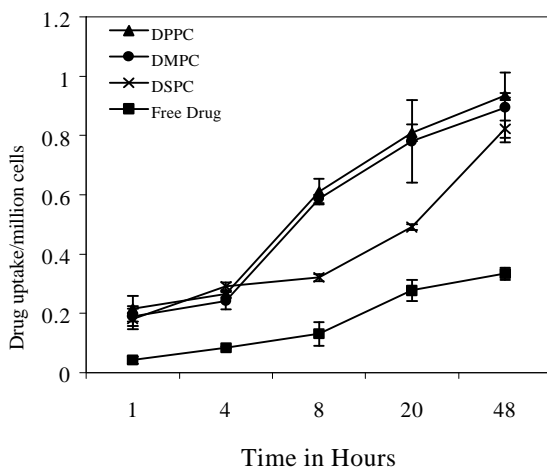


Fig.1. Cellular uptake of stavudine ($\mu\text{g}/\text{million cells}$) as a function of time from liposomal formulations composed of DMPC, DPPC, DSPC. 1 mg/ml free drug was used as control. All formulations contained lipid:cholesterol in the ratio 2:1. Data expressed as mean \pm s.e.m for n=3 samples.

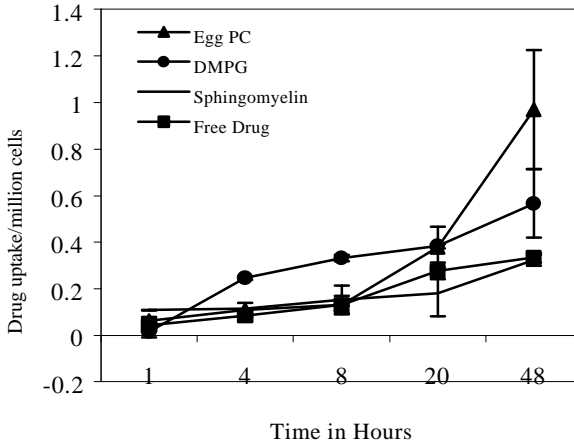


Fig. 2. Cellular uptake of stavudine ($\mu\text{g}/\text{million cells}$) for a period of 48 hrs from liposomal formulations composed of Egg PC, DMPG and Sphingomyelin. 1 mg/ml free stavudine was used as control. All formulations contain lipid:cholesterol in the ratio 2:1. Data expressed as mean \pm s.e.m for n=3 samples.

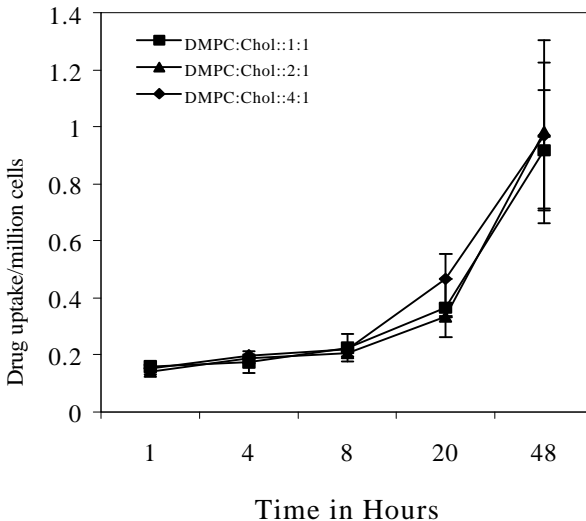


Fig. 3. Effect of cholesterol concentration of DMPC liposomes on the cellular uptake of stavudine ($\mu\text{g}/\text{million cells}$) by monocyte/macrophages. Formulations containing lipid:cholesterol in the ratio of 1:1, 2:1 and 4:1 have been compared. Data expressed as mean \pm s.e.m for n=3 samples.

The release of liposome encapsulated drug into tissues is based on the following mechanisms. The initial event in liposome cell interaction is thought to be adsorption of intact vesicles to the cell surface followed by endocytosis [12]. Endocytosis is an energy dependent process which, is saturable and which is inhibited at low temperatures. Hence liposome uptake represents a combination of vesicle adsorption to the cell surface and active cellular endocytosis. Other possible mechanisms include enzymatic degradation of the lipid bilayer and subsequent leakage of drug into the immediate vicinity of the cells, lipid exchange with the cells and non-facilitated diffusion of the drug across the lipid bilayer [13]. Diffusion is of more significance in the case of hydrophobic drugs since they are intercalated in the lipid bilayer due to their high oil/water partition coefficient as compared to hydrophilic drugs that are encapsulated in the internal aqueous compartment.

Figures 3-4 show the effect of cholesterol composition of liposomes on the uptake of stavudine by macrophages. Formulations containing lipid and cholesterol showed no difference in uptake in the case of DSPC liposomes. Also, the uptake of liposomes that contained lipid and cholesterol in the ratio of 4:1 was found to be higher, though insignificant, than those of 2:1 proportion in DMPC liposomes. There is no significant difference in the uptake of DPPC:Chol liposomes at all the ratios used in this study. Hence, from these results it is not clear as to how cholesterol composition of liposomes affects cellular uptake.

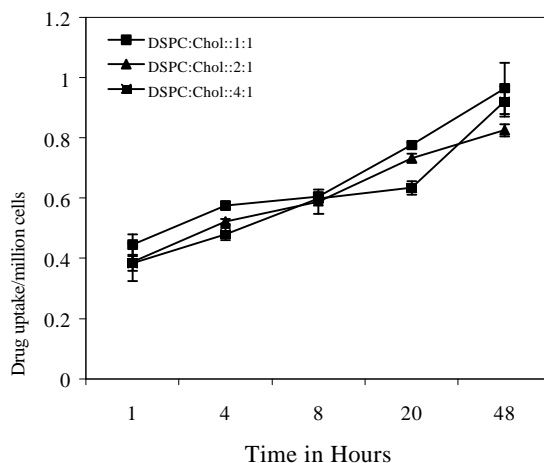


Fig. 4. Effect of cholesterol concentration of DSPC liposomes on the cellular uptake of stavudine ($\mu\text{g}/\text{million cells}$) by monocyte/macrophages. Formulations contain lipid and cholesterol in the ratio of 1:1, 2:1 and 4:1. Data expressed as mean \pm s.e.m for $n=3$ samples.

Also, these results do not conform to previous literature reports, which suggest that addition of increasing amounts of cholesterol to the formulations decrease their uptake by macrophages [14,15]. It is thought that addition of cholesterol to liposomes decreases the fluidity of the lipid bilayer and inhibits the release of drug molecules from the liposomes. Also, it can be observed from Figure 1 that, in the case of sphingomyelin, another lipid that increases the rigidity of the bilayer, the macrophage uptake of stavudine is lower than that of free drug. However, *in vitro* release testing of these liposomal formulations yielded release profiles such that the extent of drug released from liposomes containing lipid and cholesterol in the ratio of 1:1, 2:1 and 4:1 is not statistically significant [16]. Hence, it can be concluded that the apparent inability of cholesterol to reduce uptake is due to the fact that there is no difference in the extent of drug released from liposomes in the presence of varying amounts of cholesterol.

Effect of liposomal charge on cellular uptake

The effect of bilayer charge on the cellular uptake of liposomes is a matter of considerable debate. There are wide discrepancies in literature reports as to how charge effects the uptake of liposomally encapsulated molecules. Some reports have concluded that negatively charged liposomes are more effective than either neutral or positive vesicles where as other reports have found that positively charged liposomes are more effective [11,17-19].

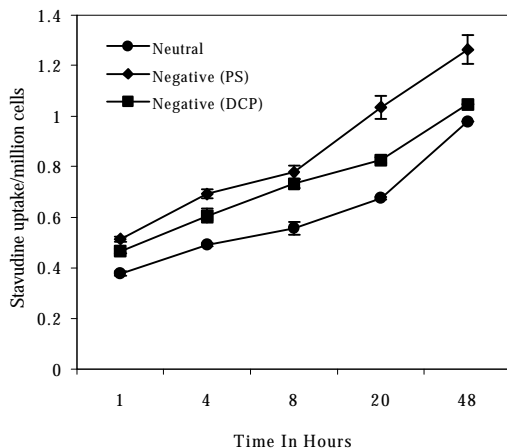


Fig. 5. Effect of charge on the uptake of stavudine ($\mu\text{g}/\text{million cells}$) from DPPC liposomes by monocyte/macrophages. Neutral, positive, and negative liposomes contained DPPC and cholesterol in the ratio of 2:1. Data expressed as mean \pm s.e.m for $n=3$ samples.

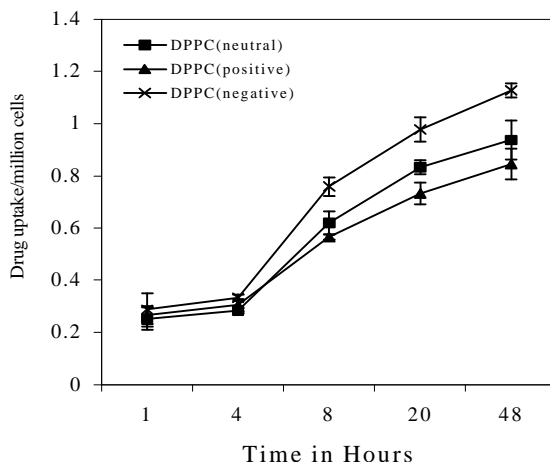


Fig. 6. Cellular uptake of stavudine ($\mu\text{g}/\text{million cells}$) from neutral and negatively charged DPPC liposomes containing 10 mol% of phosphatidyl serine and dicetylphosphate. Liposomes contained DPPC and cholesterol in the ratio of 2:1. Data expressed as mean \pm s.e.m for $n=3$ samples.

There may be variations in serum protein adsorption onto vesicles caused by surface charge, which should affect vesicle intake. Under our experimental conditions, negatively charged liposomes containing phosphatidylserine and dicetylphosphate were capable of delivering greater amount of stavudine into the macrophages than either neutral or positive liposomes. As can be observed from Figure 5 there is greater uptake in the case of negative liposomes. Among the negatively charged liposomes the uptake of liposomes containing phosphatidylserine (PS) is higher than liposomes prepared using dicetyl phosphate (DCP) and this is due to efficient fusion property of phosphatidylserine (Figure 6). Though it appears from Figure 5 that positively charged liposomes were taken up to a lesser extent than neutral liposomes there is no statistically significant difference ($p<0.05$). The apparent difference in uptake of stavudine in the presence of charge may be due in part to the extent of interaction between the charged bilayer and cells, the extent of interaction being greater in the case of negatively charged liposomes.

In summary, our results suggest that the specific lipid selected for formulation and the presence of charge on the bilayer has a bearing on the extent of uptake of encapsulated drug by macrophages. This study was not able to conclusively establish the effect cholesterol on cellular uptake and further studies are needed.

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