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Liposomal Daunorubicin: Reduced Cardiotoxicity in the Face of Unaltered Antitumor Activity in Swiss Mice Bearing Fibrosarcoma

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Liposomes were prepared by the versatile pH gradient method with an aim to modify drug disposition and increase the therapeutic efficacy of the drug, daunorubicin. The preparation were then characterized with respect to size and its distribution, entrapment efficiency, *in vitro* drug release profile and its stability under specified conditions of storage. Antitumor efficacy bearing solid tumor namely fibrosarcoma and organ toxicity studies in swiss albino mice were conducted with the liposomes administered by intravenous route at a dose of 5 and 10 mg kg^{-1} body weight. Liposomes were found to be spherical, multilamellar in nature with mean diameter being 5 μm. The entrapment efficiency of drug was found to be 82.5±1.28%, with the drug being incorporated into the aqueous layer of the vesicles. Prepared liposomes were stable under refrigeration (4°) as evident by less drug leakage and released the loaded drug in a controlled fashion. Antitumor studies indicate an insignificant difference ($P>0.05$) in volume doubling time between the free drug and daunorubicin liposomes at both doses but the animals treated with drug formulation exhibited dramatic decrease in cardiac toxicity. The result revealed that encapsulation of daunorubicin in liposomes are a potential tool for the delivery of drug, as this formulation significantly decreases the inherent cardiac toxicity of daunorubicin.

The anthracycline antibiotics play a prominent role in the treatment of leukemia and solid tumors in humans[1,2]. Among this class of compounds, daunorubicin (adriamycin) is one of the most important antitumour agents and has demonstrated activity for a wide range of human cancers including lymphomas[3,4], leukemia[5] and solid tumors[6]. The mechanism of action is the formation of a complex with nuclear DNA by intercalating between base pairs thus causing steric obstruction to DNA-dependent RNA synthesis[7]. In addition, a number of observations have implicated a direct interaction of the anthracycline drug with cell membranes[8]. Daunorubicin produces acute toxicity in the form of bone marrow depression, alopecia and oral ulceration[9]. Chronic cardiotoxicity manifested in the form of refractory congestive heart failure, however, has greatly limited the clinical uses of this drug in humans[10]. Although the mechanism of daunorubicin induced cardiotoxicity is unexplained, one probable pathway is the peroxidation of cardiac lipids, as has been recently demonstrated[11]. Pharmacological studies have shown that daunorubicin is avidly taken up by cardiac tissue[12].

In an attempt to alter the biodistribution, drug disposition and to increase the therapeutic efficacy of this drug, alternative dosage forms have been suggested, including many macromolecular drug carriers such as liposomes, niosomes, microspheres and nanoparticles for intravenous administration. Among these, liposomes, the phospholipid vesicles, which form spontaneously when certain lipids are hydrated in aqueous media[13] have attracted a considerable amount of interest for potential use as a drug delivery

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system due to their suitable characteristics. Liposomes are composed of relatively biocompatible and biodegradable material, and are formed due to entrapment of an aqueous phase by one or more bilayers of natural and/or synthetic lipids. They may be expected in some cases to target the drug to the desired site of action and to control its release[14,15]. Drugs with widely varying lipophilicities can also be encapsulated in liposomes, either in the phospholipid bilayer, in the entrapped aqueous volume or at the bilayer interface. Liposomes have been investigated as carriers of various pharmacologically active agents such as antineoplastic and antimicrobial drugs, chelating agents, steroids, vaccines and genetic material.

In the present investigation, an attempt has been made to prepare liposomes with daunorubicin using the pH gradient method. Various *in vitro* studies including size and its distribution, entrapment efficiency, release and stability at different storage conditions were conducted. The prepared formulation were then evaluated for antitumor activity in a well established solid tumor model, filbrosarcoma along with associated organ toxicities in swiss mice.

MATERIALS AND METHODS

Egg phosphatidylcholine, cholesterol and dicetyl phosphate were obtained from Sigma Chemicals Co., USA. Daunorubicin hydrochloride was a gift sample from Rhone Poulenc, Paris, France. Chloroform, analytical grade was purchased from Merck, Darmstadt, Germany. All other reagents were of analytical grade and used without further purification.

Swiss mice, 6-8 w old (22-30 g) of either sex were obtained from National Institute of Nutrition, Hyderabad, India. The animals were maintained under controlled conditions of temperature and humidity in polypropylene cages filled with sterile paddy husk. Food and water were freely available.

Preparation of liposomes:

For preparation, vesicle exhibiting transmembrane pH gradient (acidic inside) method was employed[16,17]. The detailed composition of the formulation is given in Table 1. In brief, egg phosphatidylcholine (EPC), cholesterol and dicetyl phosphate were mixed with 6 ml of chloroform in a 100 ml round bottom flask which was connected to a rotatory flash evaporator (Remi Equipments Pvt. Ltd., Mumbai) and the chloroform was removed under vaccum at a temperature of 37°. As the chloroform evaporated, a film of lipid layer resulted on the rotating flask. To ensure complete evaporation of the chloroform, the rotating flash evaporator was maintained at vaccum and 37° for an additional 10 min after complete removal of organic solvent as indicated by visual observations. Vesicles was prepared by hydrating the lipid film in the presence of 300 mM citric acid (pH 4.0). The multilamellar vesicles (MLVs) formed were then frozen and thawed five times[16]. Then these vesicles were diluted two fold with sterile saline and the exterior pH of these vesicles was then adjusted to 7.8 with 1 M NaOH, thus creating a pH gradient (acidic inside) across the vesicles. This vesicle solution and powdered daunorubicin hydrochloride were then heated at 60° for 10 min with intermittent vortex mixing.

TABLE 1: COMPOSITION OF DAUNORUBICIN HYDROCHLORIDE LIPOSOMES.

Egg phosphatidylcholine (EPC)	14.5 mg
Cholesterol	5.7 mg
Dicetyl phosphate (DCP)	2.4 mg
Daunorubicin hydrochloride	5.0 mg

Drug:lipid-1:4 (wt:wt), EPC:Cholesterol-55:45 (mol:mol)

Entrapment efficiency of the vesicular carriers was determined by column chromatography using Sephadex G 50. One milliliter of prepared liposomal suspension was placed on the top of the column and elution was carried out using phosphate buffered saline (PBS) (pH 7.4). The amount of free daunorubicin hydrochloride was determined using UV spectrophotometer (UV-240 Graphicord, Shimadzu, Japan) at λ_{max} 233 nm after suitable dilution.

In vitro drug release from liposomes:

The *in vitro* release of the drug from the liposomal formulation was dertermined using the membrane diffusion technique. Briefly 1.5 ml of liposomal suspension containing known amount of drug was placed in a sac of semi permeable membrane (previously soaked in distilled water for 24 h). The sac was placed in 25 ml of PBS (pH 7.4), maintained at 37° and stirred with a magnetic stirrer (Remi Equipments Pvt. Ltd., Mumbai). Aliquots of the release medium were withdrawn every half an hour and the sample was replaced with fresh PBS (pH 7.4) to maintain a constant volume. The absorbance of the samples was measured at λ_{max} 233 nm after suitable dilution, if necessary, using appropriate blank.

Drug leakage from the vesicles and stability evaluation:

Vesicle stability with respect to drug leakage and degradation upon storage was studied at 37°, room temperature and under refrigeration for one month. Liposomes containing a known quantity of drug were stored in amber-colored vials under specified conditions of storage. Samples were withdrawn weekly and the entrapment efficiency was determined as described above.

Tumor model:

The mouse fibrosarcoma cell lines were obtained from Deparment of Radiobiology, Kasturba Medical College, Manipal and were propagated by serial transplantation in to the skin of mice. Solid tumors were obtained by intradermal innoculation of 5 x 10^5 viable tumour cells on the dorsal skin. Once the tumour became palpable, diameters in three perpendicular planes (D_1, D_2, D_3) were measured on alternate days with plastic vernier callipers[18]. The tumor volume (V) was calculated from the formula: $V = \pi/6 (D_1 \times D_2 \times D_3)$ mm^3.

Drug administration:

Mice were divided into different groups of 12 animals each. These animals were injected intravenously with plain drug and liposomal formulation at a dose of 5 and 10 mg kg^{-1} keeping one group as control (no treatment). The treatment was given in single dose modalities once the tumor size reached 100±10 mm^3. The injections were given using a 26-gauge needle fitted to sterile syringe. The volume doubling time was the time required to double the tumor volume from 100 to 200 mm^3 and was calculated using the formula: $\log (T_1-T_0)/\log V_1-\log V_0$, V_0 is the volume of the tumor at time T_0 and V_1 is the volume at time V_1.

Organ toxicity studies:

For evaluating long term toxicity effect of the daunorubicin formulation, multiple dosing schedule in healthy swiss mice for a period of three months was followed. Mice, 6-8 w old, both male and female, weighting abount 25 g were taken for the study. The free daunorubicin hydrochloride and its liposomal formulation were injected at a dose level of 5 and 10 mg kg^{-1} once a week. All the treatments were given as intravenous injection. Animals were sacrificed at the end of three months and its vital organs (heart, liver and kidney) were isolated. Histopathological investigations were carried out on the isolated tissue samples and any abnormal changes in the tissues were reported.

RESULTS

The liposomes formed by the versatile pH gradient method were found to be multilamellar vesicles having the size range of 3.3 to 11.2 µm with the mean diameter being 5 µm. Moreover, liposomes formed were spherical in shape with constant distance between two consecutive lamellae when seen under light microscope. The liposomes encapsulated about 82.5±1.28 % of the total daunorubicin hydrochloride. *In vitro* study on the release of drug from the prepared vesicles are shown in fig. 1. It was observed that it exhibited maximum release of the drug from the formulation as 43.2 ± 3.47 % in the specified time in a controlled fashion. Initially, there was high rate of drug release (upto 4 h) followed by a relatively slow release pattern. As the storage temperature increased, the degree of leaching also increased (Table 2). Storage under refrigerated condition showed promising results with 81.3% of drug entrapment after one month. Whereas at room temperature and 37°, the percent drug entrapment after one month was 72.5% and 66.4, respectively. The volume doubling time (VDT) for control and treated group is given in Table 3. There was a significant ($P<0.05$) increase in VDT for free daunorubicin hydrochloride as well as its liposomal preparation at both doses (single dose) against control. Results also revealed that, there was insignificant difference of liposomal preparation at both doses in terms of VDT prolongation against free drug. The formulation also exhibited dramatic decrease in cardiac toxicity with unaltered liver or kidney toxicity when

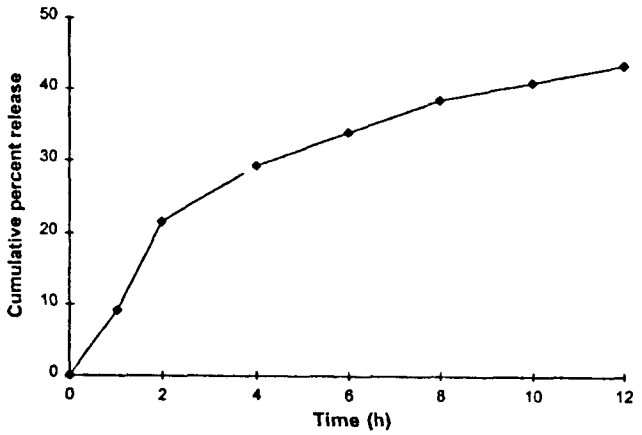

Fig. 1: *In vitro* release profile of liposomal daunorubicin hydrochloride.

***In vitro* release studies were conducted using 25 ml of phosphate buffered saline pH 7.4 and samples were withdrawn at regular intervals of time and analysed spectrophotometrically for the drug content.**

TABLE 2: EFFECT OF TEMPERATURE ON VESICLE STABILITY OF LIPOSOMAL DAUNORUBICIN HYDROCHLORIDE.

Time (d)	Drug entrapment (%)		
	4°	Room temp.	37°
7	79.43±2.41	74.13±2.85	71.29±3.24
14	75.85±2.28	69.18±1.49	65.97±2.25
21	70.79±3.12	61.65±2.21	57.34±2.71
28	66.06±2.37	53.70±3.10	49.48±2.96

Vesicle Stability with respect to drug leakage was studied for a period of one month. Results are expressed as mean ± s.d. of three trials.

histopathological studies were performed on the tissues (Table 4) after multiple dosing schedule in healthy swiss mice for a period of three months. The animals belonging to group, treated with free daunorubicin hydrochloride showed toxic reactions in the heart at both the doses. The animals experienced severe cardiac damage as compared to control. Cellular edema and cell necrosis was evident. The animal treated with liposomal formulations developed limited cardiac edema and no cell necrosis.

DISCUSSION

Several methods have been developed for the preparation of multilamellar vesicles (MLVs) including lipid hydration, solvent injection, detergent dialysis, calcium induced fusion, and reverse phase evaporation (REV) techniques. For preparation of daunorubicin liposomes, a versatile pH gradient or active trapping technique[19] have been employed for encapsulating the drugs into preformed liposomes with high entrapment efficiency. In this method, the pH in the liposome interior is such that the unionized drug which enter the liposome by passive diffusion is ionized inside the liposome, and ionized drug molecules accumulate in the liposome interior in high concentrations due to their inability to diffuse out through lipid bilayer. The use of pH gradient-driven uptake to encapsulate in preformed vesicles systems circumvents certain complications such as poor entrapment efficiency and non-uniform size distribution. In addition, the simplicity of the △pH active entrapment procedure allows daunorubicin to be encapsulated into preformed vesicles immediately prior to use and does not require the removal of free drug. Such an active trapping protocol alleviates the possible stability problems related to chemical integrity of the drug and drug retention in the vesicles that are inherent in passive trapping procedures. Repeated freezing and thawing of multilamellar vesicles produces physical disruption of the liposomal phospholipid bilayers, probably due to ice crystal formed during the freezing process. This also serves to break apart the closely spaced lamellar of the vesicles thereby raising the trapping efficiency by increasing the ratio of aqueous solute to lipid. Dilution of these vesicles was necessary to avoid death on injection caused by high citric acid concentration at intravenous doses of liposomal daunorubicin.

TABLE 3: ANTITUMOUR EFFICACY OF LIPOSOMAL DAUNORUBICIN HYDROCHLORIDE.

Formulation	Dose (mg/kg)	Volume Doubling Time (VDT)	Samble size (n)
Control (no treatment)		3.61±0.68	12
Empty liposomes		3.78±0.46	12
Free Daunorubicin hydrochloride			
	5	6.20±0.43*	12
	10	9.05±0.72*	12
Daunorubicin hydrochloride liposomes	5	8.14±0.78*	12
	10	10.11±1.22*	12

Antitumor studies of liposomal daunorubicin hydrochloride with two doses (5 and 10 mg/kg) were conducted in swiss mice bearing fibrosarcoma. Tumor volume doubling time was recorded. Results are expressed as mean ± s.d. *$P < 0.05$, significantly different from control.

The behavior of liposomes *in vivo* is strongly dependent upon vesicle size. Although the size distribution of these vesicles tends to be fairly wide, this can be modified by altering the hydration time and rate of stirring. Attempts can be made to prepare small liposomes by sonication, extrusion or micro-fluidization of MLVs, so that it can correlated with increased accumulation in tumor tissues as smaller liposomes can extravasate by passive convective transport through the tumor capillaries much more easily than its larger counterparts. The high percent entrapment of hydrophilic drug may by attributed to the multilamellar nature of the vesicles where aqueous spaces are present between the membranes in addition to the spaces at the center of the vesicles. Also the liposomes are negatively charged due to the presence of dicetyl phosphate which increases the electrostatic repulsion between bilayers, resulting in swelling of the vesicles. One more reason can be given is the presence of non leaky bilayers resulted by the addition of cholesterol which increases the viscosity of the microenvironment and rigidity of the bilayers.

In vitro release study reveals that initially, there was high rate of drug release, which may be due to the presence of free or unentrapped drug. This is necessary to give an initial burst to initiate therapy. The most likely explanation for sustained release thereafter being slower diffusion of the drug through the bilayer as presence of cholesterol in the formulation effects the membrane fluidity by making it more rigid[20]. These confirm the stability of the product and also establish that introduction of cholesterol in liposomes decreases permeability. An important feature of any liposomal drug delivery system to be employed as a pharmaceutical is that is exhibits a constant level of entrapped drug for extended periods. One of the major problem limiting the widespread use of liposomes is its stability, both physical and chemical. Liposome stability is thought to be

TABLE 4: HISTOPATHOLOGICAL STUDIES AFTER TREATMENT WITH LIPOSOMAL DAUNORUBICIN HYDROCHLORIDE IN SWISS MICE.

Formulation	Dose (mg/kg)	D	N	I	C&E
Free Daunorubicin hydrochloride					
Heart	5	0	+	0	+
	10	0	++	0	+
Liver	5	0	0	+	+
	10	+	0	+	++
Kidney	5	0	0	+	+
	10	0	0	+	+
Daunorubicin hydrochloride liposomes					
Heart	5	0	0	0	0
	10	0	0	0	+
Liver	5	0	0	+	+
	10	0	0	+	+
Kidney	5	0	0	0	0
	10	0	0	+	+

Histopathological studies were done after administration of free daunorubicin hydrochloride and liposomal daunorubicin hydrochloride by i.v. injection. Degeneration (D), necrosis (N), inflammation (I) and congestion and edema (C&E) of the tissues studied were graded as nil (0), mild (+), moderate (++) and severe (+++). Twenty number of animals were kept in each group.

affected by lipid composition and type of liposome. The assessment of the degree of leakage of daunorubicin hydrochloride from the phospholipid vesicles was used as the parameter to ascertain the stability of liposomes at various temperatures. The temperature selection to study the stability of liposomes was done on the basis of likely temperatures with which the phospholipid vesicles would come in contact either during storage or on administration. A direct relationship between the percent leaching of the drug from the vesicles and temperature was observed. This may be attributed to the fact that at low temperatures, although there was no inhibition of peroxide formation, peroxide decay, i.e. fatty acid breakdown decreases. At higher temprerature oxidation of phospholipid fatty acids might have taken place resulting in higher drug leakage due to destabilization of bilayer structure. Moreover, the transition temperature of prepared liposomes are around 30-37° thus making the liposomal bilayer membrane more fluid and more leaky at the physiological temperature.

The antitumour activity of daunorubicin hydrochloride in the free form as well as in the delivery system was assessed in well-established transplantable mouse solid tumor model, namely fibrosarcoma. We chose solid tumors as models for evaluation since majority of the complex problems encountered in cancer chemotherapy are in treating solid tumors. In the present study, daunorubicin produced comparable antitumor activity, whether the drug was given in the free of liposomally encapsulated form. With free daunorubicin hydrochloride, as the dose was immediately available for cytotoxic action, it retarded the tumour growth at initial stages itself. Upon formulating as liposomes with EPC/cholesterol, they have relatively short blood circulation time. When administered intravenously, they are rapidly removed from the circulation by reticuloendothelial system via phagocytosis. The results obtained above can also be explained, based on the liposomal size. The prepared liposomes are multilamellar in nature which are opsonized rapidly to a greater extent compared to small liposomes and therefore, the rate of liposome uptake by RES increases with the size of the vesicles. We assessed daunorubicin's organ toxicity with the qualitative histological examination of tissue section taken from swiss mice given equal doses of free and liposomal daunorubicin and demonstrated lower cardiotoxic potential for the liposomally encapsulated form. Such difference in cardiac toxicity can be attributed to decreased cardiac uptake of daunorubicin by this delivery system.

Finally, it is evident that liposomal encapsulation of daunorubicin greatly reduces the drug toxicities without impeding its antitumor efficacy but further indepth studies in areas such as pharmacokinetics, myelosuppression and tumor specificity may be expected to lead to a more detailed understanding of the mechanism of action of liposomal daunorubicin and development of optimum preparations appropriate to particular applications.

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