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COMMUNICATION

High-Entrapment Liposomes for 6-Mercaptopurine—A Prodrug Approach

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ABSTRACT

Low entrapment of drugs into liposomes is a serious challenge in their commercial application. 6-Mercaptopurine (6-MP), an antineoplastic agent, is such a drug with low entrapment efficiency (EE). We devised their lipophilic derivatization as a means of enhancing EE by covalently coupling 6-MP with glyceryl monostearate (GMS) via a succinic anhydride spacer. This prodrug had an improved partition coefficient value of 25.16 compared to 1.22 for free drug, confirming higher lipophilicity. A hydrolysis rate study of prodrug indicated 2.90%, 12.5%, 24.1%, and 25.1% hydrolysis in phosphate buffered saline (PBS) (pH 7.4) and 10%, 20%, and 30% serum, respectively. Liposomes of phosphatidylcholine (PC)/sphingomyelin, cholesterol, and dicetyl phosphate bearing drug or prodrug were prepared by shaking by hand and sonication methods. The EE was found to increase from 1.92% for free drug to 91.8% for drug-conjugate. An in vitro cell line toxicity study on L1210 leukemia cells showed improved performance of liposome-encapsulated drug-conjugate compared to free drug. The plasma drug level profile following administration of free drug and the liposomal formulation containing prodrug (HE liposome) manifested a higher sustained level of the latter, which was further improved in case of sphingomyelin-containing liposomes (STHE liposome). The pharmacokinetic parameters revealed an increase in half-life, from 61 min to 120 min for the HE liposomes and 296 min for the STHE liposomes. Therefore, increased entrapment was made possible through lipophilic derivatization, and it was subsequently tested in vivo.

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Key Words: *In vitro cell line study; In vivo study; Lipophilic derivatization; Stealth liposomes.*

INTRODUCTION

Liposomes, widely reported as carriers for drugs (1), should encapsulate the maximum possible quantity of drug to achieve optimum efficacy and cost effectiveness. When a large amount of phospholipid is required for the encapsulation of a low dose of the drug, then the system is not able to be administered and is not economic. Furthermore, if the drug encapsulation is very low, unencapsulated drug may remain in the continuous aqueous phase in either dissolved or crystalline form (in the case of a drug that has low solubility in water). Recovery of such unencapsulated drug is necessary from both the therapeutic (to avoid undesirable effect) and commercial (to reduce cost) points of view. Hence, an ideal liposomal system would have nearly 100% encapsulated drug (2).

6-Mercaptopurine (6-MP) is a very powerful antineoplastic (3) with severe side effects and short biological half-life (4). To improve its therapeutic effectiveness, this drug was encapsulated in liposomes, but due to its limiting solubility in both polar and nonpolar phases, it showed low entrapment efficiency (EE) (5). Based on the high entrapment properties of lipophilic drugs in liposomes, we synthesized a bioreversible lipophilic prodrug by covalently linking 6-MP to glyceryl monostearate (GMS) using a spacer to enhance lipophilicity.

On intravenous administration, the liposomes are cleared primarily by the reticuloendothelial system (RES) (6); however, liposomes containing sphingomyelin (SPG) tend to evade RES encapture by attracting serum dysopsonin and inhibiting their phagocytosis by liver cells (7). Therefore, the present study aimed to formulate high-entrapment liposomes bearing 6-MP by lipid derivatization and including SPG in the lipid composition to make it long circulating in plasma.

EXPERIMENTAL

Materials

Phosphatidylcholine (PC), SPG, dicetyl phosphate (DCP), and cholesterol (CH) were from Sigma (St. Louis, MO). Other chemicals and solvents used were synthetic or pharmacopoeial grade.

Synthesis and Characterization of Prodrug

The drug-conjugate (DC) was synthesized by a previously reported method (8). Briefly, 6-MP was linked with a succinic acid spacer (by reacting with succinic anhydride) through the mercaptan group. After converting the free carboxyl group of the succinic acid spacer into acyl chloride (using thionyl chloride), it was reacted with glyceryl monostearate, obtaining the prodrug. The melting point was and values of elemental analysis were within $\pm 0.5\%$ of the theoretical values. Intrared and nuclear magnetic resonance (NMR) spectroscopy confirmed the structure of the prodrug.

The partition coefficients of drug and prodrug were determined in a *n*-octanol/phosphate buffered saline (PBS; pH 7.4) system and analyzed in aqueous phase by spectrophotometry on a Shimadzu UV-160A spectrophotometer (Kyoto, Japan) at 325 nm. The hydrolysis study of the prodrug was performed in the presence of different concentrations of serum and in plain PBS (pH 7.4). Prodrug (10 mg) was dissolved in 250 ml PBS (pH 7.4) with the aid of 2 ml DMSO. A 10-ml aliquot from this solution was transferred in bottles containing 10%, 20%, and 30% serum (in PBS; pH 7.4) and plain PBS kept at $37°C \pm 1°C$. The samples were withdrawn up to 5 hr, filtered, and after suitable dilution, the 6-MP content of each sample was determined spectrophotometrically at 325 nm.

Liposome Preparation

For liposome preparation by the cast-film method, a weighed amount of lipids (i.e., PC or SPG) with or without cholesterol and dicetyl phosphate (DCP), as indicated, were dissolved in chloroform : methanol (3 : 1) containing drug or prodrug in a round-bottom flask affixed to a rotary evaporator and solvent evaporated to deposit a thin film of lipid. The dried lipid film was hydrated in 100 ml of PBS (pH 7.4) by constant shaking for 2 hr. These liposomes prepared by the cast-film method were ultrasonicated (Soniweld, Mumbai, India) for 10 min at a 50% cycle to obtain a sonicated liposomal dispersion.

Liposome Characterization

The liposomes were observed microscopically under a phase contrast microscope (Biomed, Leitz, Germany)

at a magnification of 1000× to determine the size distribution. For determining EE, the unentrapped drug was removed by centrifugation at 15,000 rpm (Remi, Mumbai, India). The sediment containing the liposomes was washed four times with excess PBS (pH 7.4) and lysed with 1% w/v solution of Triton X-100, drug was estimated spectrophotometrically.

The in vitro release study was performed by separately placing 2 ml of formulation or drug with similar 6-MP contents in dialysis tubes with 0.4 ml of serum and immersing them vertically in 200 ml PBS (pH 7.4) placed on a magnetic stirrer. At regular time intervals, 5-ml samples were withdrawn (replaced with a similar volume of fresh buffer) and were analyzed spectrophotometrically.

In Vitro Cell Line Toxicity Study

This study was performed on the leukemia L1210 cell line (NCCS, Pune, India), revived by keeping in an incubator at 37°C for 18 hr. The 24-well (6 × 4) sterile tissue culture plates (Corning plastic) were seeded with 10^5 cells each. Dulbecco's modified Eagle medium (DMEM) (1 ml) containing 10% fetal calf serum was added to each well. Various concentrations of plain drug solution and selected liposomal formulations equivalent to 1 ng, 10 ng, and 100 ng of 6-MP were added to each well in triplicate; in the remaining 6 wells, plain PBS solution was added. The plates were incubated at 37°C and 95% relative humidity (RH) in 5% carbon dioxide environment (using Kipps apparatus, $CaCO_3$ and dilute HCl). Cell viability was measured by the trypan blue exclusion method (9). Percentage inhibition was determined as percentage of control.

In Vivo Study

HE (high-entrapment) liposomes and STHE (stealth high-entrapment) liposomes prepared by the sonication method were evaluated for in vivo efficacy. Albino rats of either sex weighing 200–250 g were assigned randomly to three groups (of 6 animals each) and fasted overnight prior to study; then, the preparations were administered as a bolus injection via caudal vein at dose level of 2.5 mg/kg body weight.

The blood (50 µl) was withdrawn from the retroorbital plexus of a rat from each group at indicated time intervals and diluted with the dextrose isotonic solution (Core Parenteral Ltd., Ahmedabad, India, L-1470) up to 1 ml, and the content was centrifuged at 5000 rpm for 10 min. Plasma was separated and deproteinized with trichloroacetic acid. Phosphate buffer (pH 7.4) and methanol in 2:1 ratio was used for extraction. After extraction, the 6-MP contents of the samples were determined polarographically using potassium chloride as the supporting electrolyte. Diffusion current was measured for the sample before and after adding a standard solution of 6-MP. Drug contents of the samples were determined by the ratio of sample to standard solution diffusion current.

RESULTS AND DISCUSSION

The chemical and instrumental analysis of synthesized prodrug and intermediate confirmed the prodrug formation. The drug was slightly soluble in both polar and nonpolar solvents; however, prodrug was freely soluble in nonpolar solvents. Similarly, the partition coefficient in *n*-octanol/PBS system increased from 1.22 for 6-MP to 25.16 for prodrug, thus indicating that the synthesized derivative was highly lipophilic in nature. Figure 1 shows the hydrolysis rate profile of prodrug in the presence of 10%, 20%, and 30% serum and in plain PBS (pH 7.4) solution. In PBS, about 2.9% drug had hydrolysed in 5 hr, and half of this amount had released within the first half hour. The presence of serum enhanced the hydrolysis rate depending on its concentration; that is, the hydrolysis rate was enhanced with the increase in concentration of serum used: 10% serum hydrolyzed 12.5% prodrug in 5 hr, 20% serum hydrolyzed 24.1%, and 30% serum hydrolyzed 25.1% in the same time compared to 2.9% in plain PBS.

Mean vesicle size, polydispersity index (PDI), and EE of various liposomal formulations are compiled in Table 1. The mean vesicle size of the unsonicated formulation was in the range 3.0–3.5 µm, which on sonication (Soniweld) was reduced to the size range 1.5–2.0 µm. Inclusion of DCP in the lipidic composition significantly reduced the vesicle size, and comparatively uniform distribution of vesicle size was obtained. EE data indicate

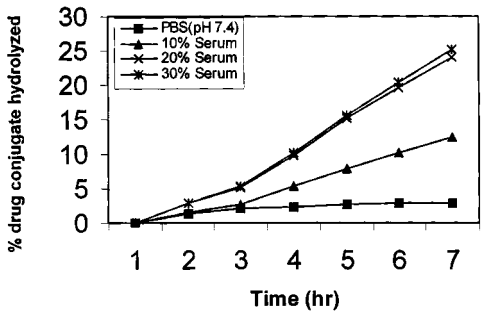

Figure 1. Hydrolysis profile of drug-conjugate.

Table 1

Effect of Lipid Composition on Mean Vesicle Size and Entrapment Efficiency of Liposomes (n = 3)

Sonicated Liposome No.	Code	Composition	Mean Diameter (μm) (PDI)		EE (%)	
			NS	S	NS	S
1	HE-1	PC:CH:DC (7:3:3)	3.49 (0.38)	1.73 (0.60)	94.0 ± 3.2	92.5 ± 4.1
2	HE-2	PC:DC (10:3)	3.23 (0.41)	1.73 (0.59)	90.4 ± 3.5	91.2 ± 4.8
3	HE-3	PC:CH:DC:DCP (7:3:3:2)	2.84 (0.47)	1.66 (0.51)	92.1 ± 2.8	91.8 ± 3.9
4	STHE	SPG:CH:DC:DCP (7:3:3:2)	—	1.65 (0.42)	—	92.1 ± 5.2
5	MP-1	PC:CH:6-MP (7:3:0.6)	3.55 (0.39)	1.69 (0.60)	1.83 ± 0.3	1.88 ± 0.3
6	MP-2	PC:6-MP (10:0.67)	3.13 (0.42)	1.66 (0.60)	1.70 ± 0.4	1.70 ± 0.5
7	MP-2	PC:CH:DCP:6-MP (7:3:2:0.67)	2.83 (0.49)	1.66 (0.55)	1.88 + 0.2	1.92 ± 0.3

NS = nonsonicated liposomes; PDI = polydispersity index; S = sonicated liposomes.

a dramatic increase in the EE of free drug, from approximately 1.5–2% to 90–95% of conjugate. The tendency of a drug to interact by various forces, such as polar or nonpolar forces and/or electrostatic interactions with the bilayer, determines whether it would be incorporated into the aqueous compartments (like nonbilayer-interacting water-soluble drugs, for which entrapment depends on the encapsulated aqueous volume) or into the lipid bilayer structures (like bilayer-interacting hydrophobic drugs that are bound inside the hydrophobic region of the bilayers) (11) or whether it would be associated firmly with the polar head groups of the bilayers via electrostatic interactions. The drugs that are not water soluble or bilayer bound or associated exhibit very low encapsulation efficiency. Chemical modification to improve hydrophobicity has been employed for such steroidal drugs as hydrocortisone (12), triamcinolone (13), and the like by derivatizing into a fatty acid for improving entrapment efficiency. Lipidic derivatization of the anticancer drug 6-MP by GMS via a succinic acid spacer is supposed to have provided sufficient lipidic anchoring to make it interact with bilayer thoroughly enough that a huge improvement in entrapment efficiency is observed.

The in vitro release profiles of drug from the liposomal formulation is depicted in Fig. 2. The study was performed in the presence of 20% serum to simulate the in vivo fate of HE liposome and STHE liposome and to compare it with release of free drug from its solution. It was found that pure drug solution released 95% drug in 4 hr, while the drug release from HE liposomes and STHE liposomes was 17.2% and 16.8% after 6 hr, respectively.

The results of in vitro cell line study are given in Table 2. The liposomal formulation manifested significantly stronger inhibition compared to the free drug solution.

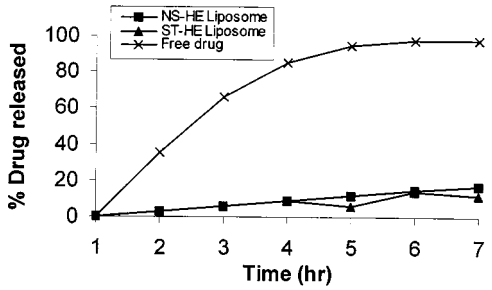

Figure 2. In vitro release profile of 6-mercaptopurine in the presence of 20% serum.

This clearly suggests that, in a liposomal formulation, drug is more effective than the plain drug in the same concentration, possibly due to easy endocytosis of the liposomes by the cells. Moreover, in the liposome formulation, drug is present as prodrug, which is highly lipophilic in nature, thus allowing easy permeation through

Table 2

Percentage Cell Inhibition by Different Formulations

Sonicated Liposome No.	Formulation	Drug Concentration	Percentage Inhibition (after 4 hr)
1	PBS solution	—	0
2	6-MP solution	1 ng	20.5
		10 ng	62.7
		100 ng	90.3
3	ST-HE liposome	1 ng	34.4
		10 ng	85.2
		100 ng	96.1

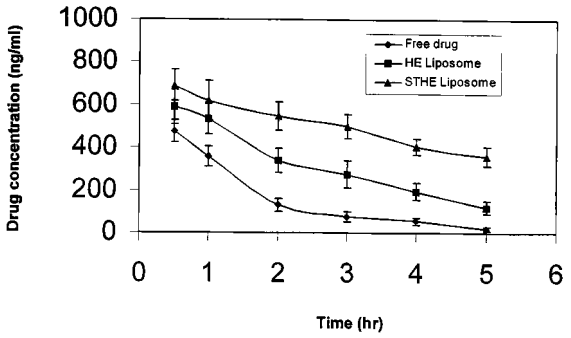

Figure 3. Blood-level profile of 6-mercaptopurine following intravenous administration of formulation and free drug in albino rats ($n = 3$). The data are statistically significant at $p < .05$.

the cell wall. It has been reporteod that negatively charged liposomes associate more with the cells than plain liposomes; thus, the presence of DCP can be one factor for the better performance of the system (14).

The plasma level profiles of 6-MP following administration of free drug, HE liposomes, and STHE liposomes were determined polarographically and are presented graphically in Fig. 3. The pharmacokinetic parameters derived from the plasma drug concentration profile using reported formulas (15) revealed that encapsulation of 6-MP in HE liposomes increased its half-life from 61 min to 120 min, which further increased up to 296 min in the case of STHE liposomes. The area under the curve (AUC) increased from 925 ng · hr/ml of free 6-MP to 1736 ng · hr/ml for HE liposomes and 2623 ng · hr/ml for stealth HE liposomes. These parameters establish the prolonged residence time of liposome-encapsulated drug, and the significant increase in AUC proves the higher bioavailability of the drug. Thus, these studies indicate that the therapeutic index of 6-MP in the proposed HE liposomes has been increased, and it is further accentuated in stealth HE liposomes.

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