Journal of PharmaSciTech
ISSN: 2231 3788 (Print)
2321 4376 (Online)
Research Article

Enhanced Tumor Targeting and Antitumor Activity of Gemcitabine Encapsulated Stealth Liposomes

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Abstract

The purpose of this study was to prepare conventional and stealth liposomes containing Gemcitabine in an attempt to improve cytotoxic effect against various tumor, both Stealth and Conventional liposomes has been investigated by taking various molar ratios of lipids like DPPC, Cholesterol, DSPE-MPEG 2-K, and prepared Active pH gradient method, SUV were obtained by extrusion through sonicator and characterized for vesicle size range as (130±1 to 265±2.4 nm) and Polydispersity index was found (0.11 to 0.625) indicating stable formulation.Entrapment efficiency of gemcitabine was improved by active pH gradient method results shown that stealth liposomes (SL-5) contain DPPC : Cholesterol: DSPE-MPEG 2-K with molar ratio (6:2:0.2) improved up to 75.3 %.Zeta potential was obtained for various formulations as the range of (-9.3±2.8 to -37.3±2.3). Pharmacokinetic profile of optimized formulation of SL-5 compared with CL-5 and Pure drug and parameter like AUC,AUMC, Cmax, Vd, t1/2 value conformed SL-5 are superior than others. The in-vivo anticancer study revealed that SL-5 formulation shows better anticancer activity based on Tumor volume (1±0.12 cm3) after 30 days and weight (1.7±0.31 gm) compared with pure drug and CL-5. Tissue distribution study indicate that SL-5 formulation appeared more in Tumor site (12.45±0.5μg/ml) and CL-5 only (2.4±1.2 μg/ml) after 48 h similarly in Plasma profile i.e. (16.66±0.3 μg/ml) concentration of SL-5 and (1.8±0.45 μg/ml) for CL-5. RES uptake of pure drug and CL-5 are more than SL-5.Incorporation of Gemcitabine into bilayers was determined by phase transition behaviour in DSC thermogram as well as FTIR study.

Keywords: Gemcitabine, stealth liposome, pH gradient, zeta potential, pharmacokinetics

Introduction

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Cancer is not just one disease but many diseases. There are more than 200 different types of cancer [1-4]. For instance, although there are numerous anticancer agents that are highly cytotoxic to tumor cells in vitro, the lack of selective antitumor effect in vivo precludes their use in clinic. One of the major limitations of antineoplastic drugs is their low therapeutic index (TI), i.e. the dose required to produce anti-tumor effect is toxic to normal tissues.

Liposomes are spherical vesicles composed of lipid bilayers arranged around a central aqueous core. The particle size of liposomes ranges from 20 nm to 10 μm in diameter. They can be composed of natural constituents such as phospholipids and may mimic naturally occurring cell membranes. Liposomes have the ability to incorporate lipophilic and hydrophilic drugs within their phospholipid membrane or they can encapsulate hydrophilic compounds within the aqueous core [6].

Gemcitabine is new cytotoxic drug but some of limitations while its use likes it suppress the activity of bone marrow i.e. effect on blood forming cells. Higher water solubility needs to improve encapsulation efficiency for better therapeutics effect. Stealth liposomes by pH gradient technology lower half life- 7-18 min, unable to deliver by oral and other route. Higher dose-1000-1250 mg/m2 require against malignancies are effective against various solid tumor like colon, lungs, breast etc [7, 8]. Sterically stabilized liposomes can be formulated by incorporating hydrophilic long-chain polymers (PEG) in the bilayer which can form a coat on the liposome surface and repel opsonin penetration and adsorption. Reduction in 'marking' by opsonins leads to slower uptake of these liposomes (LCL) by the cells of reticuloendothelial system (RES) [9]. In present investigation focuses on to perform innovative research work is to avoid the problem associated with gemcitabine use and effective against solid tumor with minimum toxic effect by incorporating it in stealth liposomes.

Materials and Methods

Materials

Gemcitabine was obtained as gift sample from Sun Pharma Pvt Ltd, Vadodara, (DPPC) 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine, Soya PC, (DSPE-MPEG-2000) 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-methyl-polyethyleneglycol conjugate-2000 Na+ salt, Cholesterol was obtained from Lipoid GmbH, Ludwigshafen, Germany, Chloroform, Methanol, and other chemical was purchased from Loba Chemicals, Mumbai. All other solvent and reagents were of analytical grade.

Table 1. Composition of conventional liposomes

Batch Code	Gem Citabine (mg)	Soya PC (Molar Conc.)	DPPC (Molar Conc.)	Cholesterol (Molar Conc.)	Chloroform (ml)	Methanol (ml)	Distil Water (ml)	Drug : Lipid Ratio
CL-1	10	5	-	1	5	5	10	1:6
CL-2	10	6	-	2	5	5	10	1:8
CL-3	10	7	-	3	5	5	10	1:10
CL-4	10	-	5	1	5	5	10	1:6
CL-5	10	-	6	2	5	5	10	1:8
CL-6	10	-	7	3	5	5	10	1:10

Preparation of CL and SL by pH Gradient Method

Both CL and SL are composed of lipid and cholesterol with different molar ratio is shown in Table 1 and 2. DPPC: Cholesterol were taken in different molar ratios and dissolved in Chloroform-Methanol solvent system (3:1). Film is hydrated in Rota Evaporator (Equitron Roteva) at controlled pressure, 60 rpm, 60°C under nitrogen purging. Film was formed. Film was kept overnight under vaccum to remove any trace solvents. Hydration of film is done by (250 mM) of Ammonium sulphate solution for 15 min. to establish pH gradient inside and outside the vesicle to promote encapsulation of hydrophilic drug like gemcitabine. Multi Lamellar Vesicles (MLV) were formed then ubmitted for 5 cycles (3 min each) of freezing and thawing at 30° C in swater bath. Untapped ammonium sulphate was removed by centrifugation (lab centrifuge- Sigma 3,K 30) at 14000 rpm for 30 min. Pellet is resuspended in an isotonic solution of Gemcitabine at room temp for 3 h. This will be subjected to Probe sonication (Sonics Vibra cell) for 5 minutes (15 seconds sonic on, 5 seconds off) at 20 kHz at 4° C. MLV will get converted to Small Unilamellar vesicles. Mannitol was dissolved in the solution (30 to 40 mg per ml of solution) and subjected for Freeze drying. A dry solid powder of the product will finally be obtained at end of lyophilization which can be reconstituted with sterile water for injection and infused. Stealth liposomes were prepared similarly by use of additional lipid such as DSPE-MPEG-2K and process is similar mentioned above.

Table 2. Composition of stealth liposomes

Batch Code	Gem-citabine (mg)	Soya PC (Molar Conc.)	DPPC (Molar Conc.)	DSPE-MPEG (Molar Conc.)	Cholesterol (Molar Conc.)	Chloroform (ml)	Methanol (ml)	Distil Water (ml)	Drug : Lipid Ratio
SL-1	10	5	-	0.1	1	5	5	10	1:6
SL-2	10	6	-	0.2	2	5	5	10	1:8
SL-3	10	7	-	0.3	3	5	5	10	1:10
SL-4	10	-	5	0.1	1	5	5	10	1:6
SL-5	10	-	6	0.2	2	5	5	10	1:8
SL-6	10	-	7	0.3	3	5	5	10	1:10

Evaluation of Liposomes

Entrapment Efficiency [12]

Gemcitabine entrapped within the liposomes was estimated after removing the unentrapped drug by centrifugation at 10,000 rpm in refrigerated centrifuge. Supernatant contains unentrapped free drug which is analyzed by UV Spectroscopy at 268 nm. The pellet formed will be lysed by 1 ml of methanol:ether (50:50 v/v). This solution is than diluted and analyzed in UV Spectroscopy. The values obtained were same.

$$\%EE = 1 - \frac{\text{Untrapped drug}}{\text{Entrapped drug}} \times 100$$

Vesicle Size and Size Distribution (PDI) [13]

The mean particle size and particle size distribution of the trial batch was obtained by Zeta sizer 1 mL of liposome suspension was diluted to 100 times with the deionized water. The sample was analyzed using Zeta sizer (Nano ZS, Malvern).

Zeta Potential [14]

Zeta potential of formulation was determined using Zetasizer (Nano ZS, Malvern). 1 mL of liposome suspension was diluted up to 100 ml using deionised water and sample was placed placed in clear zeta cells and results were recorded. Before putting the fresh sample, cuvettes were washed with the deionised water and rinsed using the sample to be measured before each experiment.

In Vitro Release Study [15, 16]

Sigma dialysis membrane (Molecular weight cut-off of 10,000) was hydrated with the receptor medium (pH 7.4 phosphate buffer) for 12 h before being fastened between the donor and receptor compartments. The diffusion cell apparatus consists of a glass tube with an inner diameter of 2.5cm, open at both ends, one end of the tube is tied with Sigma dialysis membrane. Liposome equivalent to 10 mg of Gemcitabine was taken in a dialysis tube and placed in 100 ml of PBS (pH 7.4) The medium was stirred by using the magnetic stirrer at 150 rpm and the temperature was maintained at 35 ± 0.50 C Periodically 5 ml of samples were withdrawn and after each withdrawn same volume of medium was placed All samples were analyzed for Gemcitabine content at 268 nm. The experiment was done in triplicate for 24-48 h.

In-Vivo Study of Optimized Conventional and Stealth Liposomes

Pharmacokinetic Studies [17,18]

Pharmacokinetic studies were performed using either sex mice (100–200 g). The protocol in prescribed proforma B for animal studies was submitted to IAFC of Parul institute of pharmacy ,Vadodara . Albino mice of either sex were fasted overnight and divided into four groups each containing three mice. The group under treatment was designed as follows

Group I: Tumor control

Group II: Pure gemcitabine

Group III: Conventional liposomes

Group IV: Stealth liposomes

The group I received normal saline buffer solution through tail vein of mice similarly group II,III,IV also received 10mg/kg dose of pure drug solution in saline buffer, conventional liposomes, stealth liposomes respectively after 7 days of tumor implantation (MCF-7) when solid tumor sufficiently grows with specific volume .The blood sample were withdrawn at an interval 1,6,12,24,and 48 h. Distribution profile of gemcitabine in the various organs including plasma were measured by HPLC analysis in which stationary phase C18G (250×4.6 mm, 5µm) and mobile phase was Acetonitrile: methanol (55:45) with flow-rate: 1.0 ml/min, Injection volume : 20 µL and detection wavelength were 268 nm. Estimation of gemcitabine was carried out using standard curve and solution was injected and the chromatogram was recorded.

Tissue Distribution Study [19, 20]

To assess distribution pattern of gemcitabine in biological organs which assure for either localization of drug towards desired tumor site via prolong circulation or its uptake by RES rich organs like spleen ,liver which prevent the desire localization hence distribution profile of gemcitabine containing both conventional and stealth liposomes were check out by using tumor bearing animal model. Experiment were carried in similar manner like pharmacokinetic section by receiving out by 10mg/kg dose of pure drug solution in saline buffer, conventional liposomes, stealth liposomes respectively after 7 days of tumor implantation when solid tumor sufficiently grows with specific volume. The mice were sacrificed and major organs like lungs, spleen, liver, kidney, tumor were removed, washed with

normal saline solution and subjected for centrifugation at 25000 rpm for 10 min. The aliquots were analyzed by HPLC to estimate gemcitabine content in various organs with respect to the time by using standard curve of gemcitabine

In-Vivo Antitumor Activity

The anticancer activity of gemcitabine was evaluated by measuring its cytotoxic effect on tumor by measuring its dimension in suitable animal model based on Tumor volume and Weight parameter.

Effect on Solid Tumor Volume [21]

The human breast cancer cell line i.e. MCF-7 was cultured as described previously (tissue distribution study). This cell line diluted with phosphate buffer solution and injected subcutaneously into the right flank of the mice and tumor were allowed to develop. The free gemcitabine, conventional liposomes and stealth liposomes were injected into the tumor bearing mice via tail vein at 10mg/kg of dose. The size of the tumor and weight of each mouse was monitor thereafter. The anticancer effect of gemcitabine based formulation was evaluated on the basis of the changes in tumor volume and weight at selected time interval i.e. when the tumor acquired specific size after implantation of MCF-7 cell line (at 10th day) and administration of sample itself. At the specific days interval mice were sacrificed and tumor was harvested for determine the volume of tumor, two bisecting diameter of each tumor were measured with slide caliper to determine tumor volume and calculation were performed using the formula as $V = 0.5 \times ab^2$ where a = largest diameter of tumor (mm) and b = smallest diameter of tumor (mm).

Effect of Solid Tumor Weight [22]

At the end of study the weight profile of tumor treated with different form of gemcitabine as pure gemcitabine, optimized conventional and stealth liposomal formulation were comparatively evaluated by measuring tumor weight which implicate the possible anticancer activity of gemcitabine.

Results and Discussion

Determination of Entrapment Efficiency

Hydrophilic nature of drug unable to load inside the vesicle efficiently but the results obtained from pH gradient methods indicate that encapsulation of drug in the liposomes is not significantly enhanced supported by value about 47.4% ±3.2 to 55.0±4.1 %. The use synthetic lipid (DPPC) with different molar conc. of cholesterol both CL and SL using pH gradient methods in which ammonium sulphate establish pH gradient either side of liposomes promote the encapsulation of gemcitabine inside the vesicle without back diffusion due to precipitation and formation of gel and the value obtained after study was increased as 70.2 ±2.12 % to 75.3±4.11% hence encapsulation of gemcitabine depends on concentration of lipid and cholesterol as the concentration of cholesterol increases % EE was decreases this may due to increase the rigidity of liposomal structure by the insertion of cholesterol and pH gradient methods as well as synthetic lipid DPPC is suitable for further study than Soya PC (Table 3).

Table 3. Percent drug entrapment efficiency of liposomal formulations

Batch Code	Soya PC (Molar Conc.)	DPPC (Molar Conc.)	DPPC (Molar Conc.)	Cholesterol (Molar Conc.)	% EE pH gradient method
CL-1	5	-	-	1	50±0.98
CL-2	6	-	-	2	55.0±4.1
CL-3	7	-	-	3	47.4±3.2
CL-4	-	1	-	1	63.8±2.30
CL-5	-	2	-	2	66.5±1.72
CL-6	-	3	-	3	62.4± 4.31
SL-1	5	-	0.1	1	66.0±2.34
SL-2	6	-	0.2	2	63.5±5.11
SL-3	7	-	0.3	3	60.4±4.13
SL-4	-	5	0.1	1	70.2±2.12
SL-5	-	6	0.2	2	75.3±4.11
SL-6	-	7	0.3	3	65.3±5.41

Determination of Vesicle Size, Polydispersity Index (PDI) and zeta potential for CL

Vesicle size of CL was reported in Table 4 and results confirm that size range of CL composed by DPPC was satisfactory (132.5±0.17 to 145.5±1.33) and assure for long circulation and EPR effect at tumor site while PDI of liposomal formulation indicate for uniformity in size of vesicle (monosize). The zeta potential governs the physical stability of liposomes. Zeta potential result of formulation depends on the nature of lipid (natural Soya PC) and synthetic lipid (DPPC, DPSE-MPEG 2K) with its molar concentration tried for initial batches reveled that Soya PC containing CL possess the zeta potential value between -9.3±0.3 to -10.9±0.5 for natural lipid whereas for synthetic lipids (DPPC) containing CL vale of zeta potential are in acceptable range i.e.-16.3±2.2 to -32.5±0.8.

Determination of vesicle size, PDI and zeta potential for SL

Vesicle size of stealth liposome is affected by lipid level with cholesterol from the data shown in following table concludes that as the concentration of cholesterol increases the rigidity and size of liposomes was decreases. PDI of stealth liposomes suggest that size distribution are uniform and monosize for all vesicles without aggregation. Similarly for SL synthetic lipids such as DPPC, DSPE-MPEG 2K was incorporated into the liposomes and zeta potential becomes more negative due to PEG residue i.e. -25.3±1.7 to -37.3±0.8 that indicates electrostatic repulsion between two particles. DLVO theory states that electric double layer repulsion will stabilize liposomal formulation and aggregation is not expected to take place, due to the highly negative charge of particles (Table 5). The relative vesicle size and zeta potential of CL and SL were presented in Figure1 and Figure 2, respectively.

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Table 4. Vesicle size, PDI and zeta potential of conventional liposomes

Batch code	Vesicle size (nm)	PDI	Zeta Potential
CL-1	209.0±1.20	0.49	-9.3
CL-2	265.2±2.40	0.37	-10.3
CL-3	168.9±2.10	0.16	-10.9
CL-4	132.5±0.17	0.21	-16.3
CL-5	145.5±1.33	0.21	-30.6
CL-6	140.2±1.98	0.36	-32.5

Table 5. Vesicle size, PDI and zeta potential of stealth liposomes

Batch code	Vesicle size (nm)	PDI	Zeta Potential
SL-1	155.0±4.12	0.26	-17.4
SL-2	145.0±3.78	0.52	-24.7
SL-3	150.0±3.12	0.22	-25.0
SL-4	145.0±2.50	0.34	-25.3
SL-5	130.6±1.41	0.21	-35.8
SL-6	130.0±1.00	0.12	-37.3

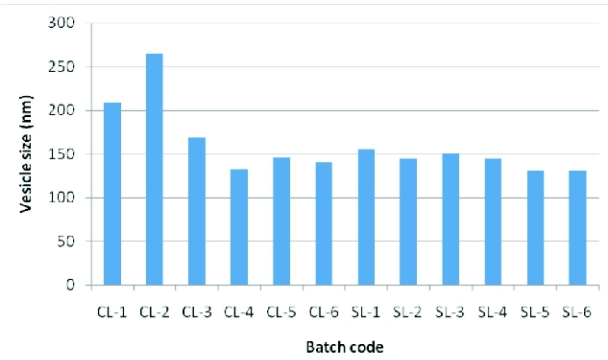

Figure 1. Comparative vesicle size (nm) of CL and SL

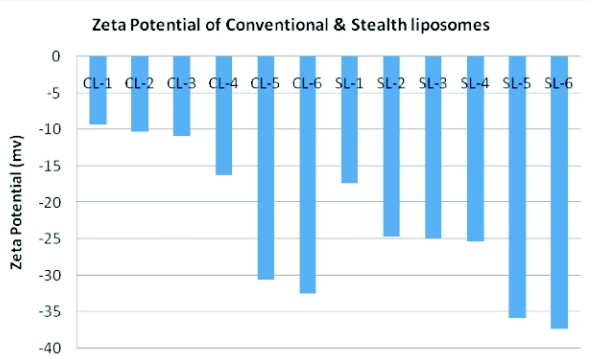

Figure 2. Comparative zeta potential of CL and SL

In Vitro Release Study (CL and SL)

The conventional liposomes released about 100% gemcitabine within 24, 32 and 36 h, respectively for the batches as CL-1, CL-2, and CL-3. Therefore, it concluded that natural lipid like soya PC is not effective retardant for gemcitabine release. When the liposomes contains the synthetic lipid (DPPC) release rate was decreased and about 82.23±3.2, 73.23±2.1 and 68±1 for CL-4, CL-5 and CL-6 respectively indicate that release rate highly affected by concentration of lipid and cholesterol at higher concentration of DPPC:DSPE-MPEG 2-K :Cholesterol (7:0.3:3) release rate was decreased upto 68% after 36 h. Hence based on release profile CL-5 has been optimized formulation for further studies due to prolong release rate for 36 h (Figure 3).

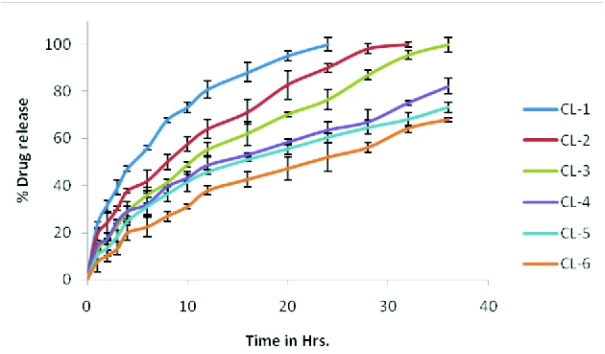

Figure 3. In vitro release profile of CL-1 to CL-6

Stealth liposomes composed with different molar concentration of Soya PC: DSPE-MPEG 2K as 5:0.1, 6:0.2 and 7:0.3 for the batches SL-1, SL-2 and SL-3 release of gemcitabine is almost 100% within 28, 32, and 36 h. As shown in Fig.4, burst effect was observed in the gemcitabine release profile of all liposomal formulation during first 2 h. This finding was probably due to rapid desorption of gemcitabine from liposomal bilayers. Dissolution profile of stealth liposomes i.e. SL-4, SL-5 and SL-6 composed by synthetic lipid with different molar concentration of DPPC : Cholesterol : DSPE-MPEG 2K as 5:1:0.3, 6:2:0.2 and 7:3:0.3 evaluated comparatively and release rate was obtained 73.13±2.3%, 70.23±3.2% and 65±2% respectively the release rate of gemcitabine from the vesicle is sustained or controlled manner upto 36 h. for stealth liposomes all batches give the assurance for prolong release of content with improvement of circulation half life of gemcitabine for achieving of the maximum therapeutic drug concentration at tumor site with less toxic effect and SL-5 were optimized due to release rate of gemcitabine in controlled manner for longer period of time.

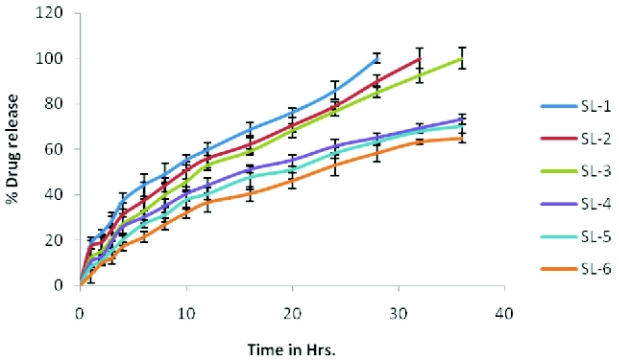

Figure 4. In vitro release profile of SL-1 to SL-6

Pharmacokinetic study

To assess the pharmacokinetic behavior of gemcitabine loaded optimized CL and SL with dose as 10 mg/kg was administered by I.V route to the MCF-7 tumor bearing animal like mice. The pharmacokinetic parameters were presented in Table 6. From this result, the free gemcitabine solution was quickly removed from the circulation at 1 h after I.V. injection with negligible blood concentration showing biphasic pattern with rapid elimination phase with half life (t1/2) 1.33±0.27 h. The volume of distribution (Vd) 5.24±0.29 ml was very low, further the value of AUC, AUMC, MRT of free gemcitabine significantly lower then CL-5 and SL-5 suggested by ANOVA ($p<0.005$) .The pharmacokinetic of CL-5 were studied and the value of t1/2 ,MRT, AUC was 5.28±1.62 h, 4.15±1.0 h and 15.22±0.02 μg/ml h respectively and from this it was clear that in vivo circulation behavior of CL-5 was significantly much better than pure (free) gemcitabine solution. When the SL-5 was compared with pure gemcitabine, CL-5 based on pharmacokinetic profile after I.V injection to animal model and the AUC, AUMC, MRT and t1/2 significantly greater than pure drug and CL-5. In contrast blood level of SL remained high for long period of time than CL-5. The area under curve (AUC) of SL was 19.37±0.09 μg/ml h much higher than the CL-5 as 15.22±0.02 μg/ml h and very less for free drug 8.37±0.04 μg/ml h. The small volume of distribution of SL-5 as 2.4±0.24 ml and for the CL-5 was 3.1±0.17 ml conform that SL-5 restricted to the systemic circulation whereas the pure gemcitabine have a large volume of distribution 5.24±0.29 ml means distribution of pure gemcitabine in the various tissue rather than blood. The t1/2 of SL-5 increased 8.6 fold and MRT 12 fold increased than CL-5 proves that prolong circulation half-life of SL-5 reduce the chances of rapid uptake by element of Mononuclear Phagocytic system (MPS) and plasma opsonin due to steric barrier produce by incorporation of PEG residue on the vesicle which make liposomal formulation as more hydrophilic and physiologically stable.

Table 6. Comparative pharmacokinetic profile of pure gemcitabine, CL-5 and SL-5

S.N.	Pharmacokinetic parameters	Units	Free Gemcitabine	Conventional liposomes (CL-5)	Stealth liposomes (SL-5)
1	AUC	μg /ml h	8.37±0.04	15.22±0.02	19.37±0.09
2	AUMC	μg /ml h	9.65±0.12	62.55±0.13	230±0.11
3	C_{max}	μg/ml	18.8±0.98	23.3±2.1	36.2±1.85
4	V_d	ml	5.24±0.29	3.1±0.17	2.4±0.24
5	$t_{1/2}$	H	1.33±0.27	5.28±1.62	11.48±0.21
6	K_e	h-1	0.11±0.01	0.33±0.02	0.01±0.025
7	Cl	ml/min	2.173±0.05	0.66±0.86	0.012±0.11
8	MRT	H	1.02±0.11	4.15±1.0	12.10±0.44

Tissue Distribution Study

The tissue distribution of pure drug, CL-5 and SL-5 was examined by inoculating human breast tumor cell culture (MCF-7) into the mice.

Biodistribution profile of Gemcitabine in Spleen

To evaluate real potency of optimized stealth liposomes (SL-5) against certain solid tumor by measuring it distribution in various RES rich organs such as spleen and result are shown in Figure 5.

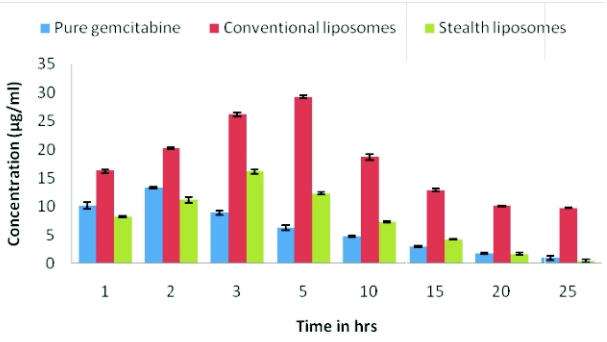

Figure 5. Biodistribution of pure gemcitabine, CL-5, and SL-5 in spleen

In case of pure gemcitabine maximum concentration achieved in spleen was (13.32±0.21 μg/gm of spleen) after 2 h i.e. Initial phase and decline rapidly and almost negligible at 24 h (0.98±0.4 μg/gm of spleen). However, conventional liposome's (CL-5) showing massive and prolonged presence in spleen with rapid uptake due to lipid and cholesterol which is unable to prevent accumulation of liposomes in the spleen. Spleen is a major RES enrich organs so far conventional liposomes quickly cleared by blood pool and enter in the spleen and the maximum concentration of CL-5 in spleen was (29.23±0.23 μg/gm of spleen) observed after 5 h and detected after 24 h with higher concentration (9.76±0.16 μg/gm) . The distribution pattern of stealth liposomes (SL-5) to the spleen was drastically altered due to steric stabilization by inclusion of PEG grafting avoids the uptake by spleen and only (16.11±0.4 μg/gm of spleen) concentration was found at 3 h and almost disappeared after 24 h.

Biodistribution profile of Gemcitabine in Liver

The high concentration of conventional liposomes (CL-5) obtained from the liver (16.8±0.57 μg/gm of liver at 5 h), followed by pure gemcitabine administration (9.1±0.9 μg/gm of liver at 1 h) is also the result of an extensive uptake, which is a reflection of its higher value. This finding can be explained by the high affinity binding of conventional liposomes to liver. The reduction of the CL-5 uptake by the liver at 15 h and pure gemcitabine 4 h, respectively, may be due to the saturation of the mononuclear phagocytic system. After these time periods, elimination over the entire experiment time period. However, stealth liposomes (SL-5) were found in liver with maximum concentration (10 ±0.21 μg/gm) at 5 h study and almost removed from liver after 20 h (Figure 6).

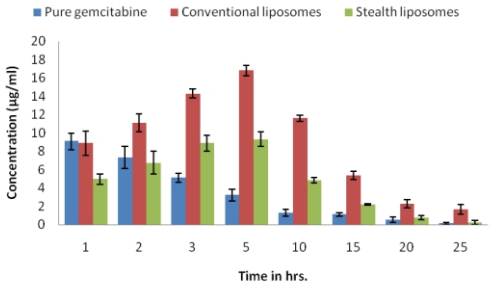

Figure 6. Biodistribution of pure gemcitabine, CL-5, and SL-5 in liver

Biodistribution of Gemcitabine in Lung

Specificity of liposomal formulation along with pure drug towards lungs was negligible and maximum drug appeared within 1 h and rapid decline phase has been started later time period CL-5 shows around (10.43±0.12 μg/gm) concentration in lungs slightly higher than pure drug (8.7±0.28 μg/gm) and SL-5 (5.15±0.2 μg/gm.) All the formulation diapered from lungs after 24 h study (Figure 7).

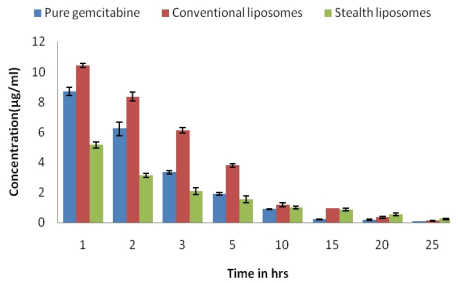

Figure 7. Biodistribution of pure gemcitabine, CL-5, and SL-5 in lungs

Biodistribution of Gemcitabine in Kidney

In fact in the case of free gemcitabine, it was interesting to note its rapid appearance in kidney after 1-2 h and concentration observed as (18.9±0.12 μg/gm of kidney) while as significant decreased in conventional liposomes with concentration as (7.21±0.78 μg/gm of kidney at 3 h) and stealth liposomes (6.42±0.1 μg/gm of kidney). This phenomenon is probably due to metabolism of gemcitabine and rapid elimination through urine but entrapment of drug inside the vesicle gives the protection against metabolism with little appearance in kidney. Almost all the formulation was detected in kidney prior to 24 h study (Figure 8).

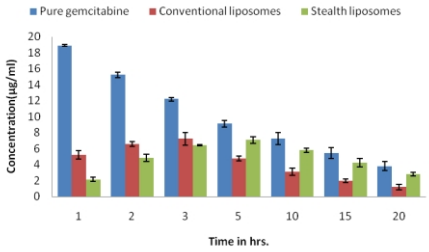

Figure 8. Biodistribution of pure gemcitabine, CL-5, and SL-5 in kidney

Biodistribution of Gemcitabine in Tumor

As the time increased, the accumulation in tumor was found to increase, reaching a peak at 24 h; after 48 h, the concentration in tumor was decreased. The PEG grafting on stealth liposomal formulation most promising to avoid uptake of gemcitabine in RES rich organs and enhance the circulation half life of gemcitabine and smaller vesicular size promote Enhanced permeability retention (EPR) effect for maximum localization of drug in tumor cells around (12.45±0.5 μg/gm) concentration of gemcitabine achieved after 24 h while as CL-5 shows as only (2.4±1.2 μg/gm) concentration and pure drug was (0.3±0.87 μg/gm) appeared at tumor site it may due to distribution of pure drug and CL-5 towards various organs rather than tumor (Figure 9).

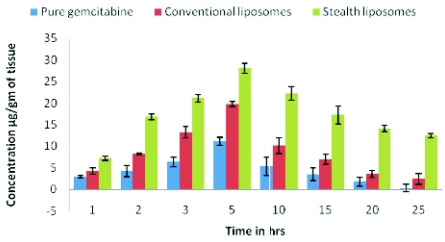

Figure 9. Biodistribution of pure gemcitabine, CL-5, and SL-5 in tumor

Biodistribution of Gemcitabine in Blood Plasma

In particular, by observing the distribution in mice of gemcitabine in it was found that gemcitabine in free form rapidly appeared in blood within 1 h after I.V injection of tumor bearing mice with maximum concentration as (18.87±0.1 μg/ml) but as the time increase the decline phase was started it might due to rapid metabolism of drug in blood and form inactive compound. when gemcitabine encapsulated in conventional liposomes (CL-5) the plasma level after 1 h was found to be (50.23±0.7 μg/ml) i.e. around 2.8 fold increased compare with free drug and further it remain into the blood after 24 h. As expected drug incorporated in stealth liposomes (SL-5)showed a remarkable enhancement of blood concentration than free drug and conventional liposomes and was still present in circulation after 24 h (16.66±0.3 μg/ml) i.e. around 16 fold higher than the CL-5 (Figure 10) .

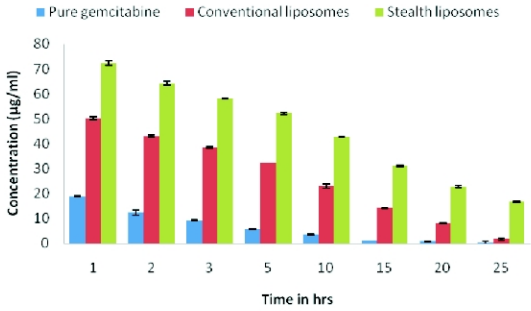

Figure 10. Biodistribution of pure gemcitabine, CL-5, and SL-5 in blood plasma

Tumor localization of liposomes

Figure 9 shows that distribution of gemcitabine in tumor at various time intervals after injection. The accumulation of gemcitabine in tumor was decreased when the drug was entrapped into the CL-5 and free form. However, a marked increase in accumulation in tumor was found for SL-5 supported by the concentration as (18 μg/gm) after 24 h. Smaller size of SL-5 and steric stabilization by PEG improve enhanced permeability and retention effect (EPR) by promoting SL-5 into tumor interstitial space and extravasation.

In Vivo Antitumor Activity

Effect on Tumor Volume

Mice bearing MCF-7 tumor were injected with free gemcitabine, conventional liposome's and stealth liposome's with 10 mg/kg dose, mice were given saline solution as control. Tumor growth inhibition curve in terms of mean tumor size (mm) were presented in Fig. 11 and Table 7.

The pure gemcitabine was not much effective for prevention of tumor growth compared to conventional liposomes (CL-5) treatment with CL -5 as it displayed strong tumor inhibition than pure gemcitabine. When the tumor treated with stealth liposomes (SL-5) provide cellular advantages in terms of tumor accumulation of gemcitabine due to PEG coating. In this case tumor cell distribution of SL-5 could be combined with fusogenic property of PEG to induce interaction with tumor cell membrane and consequently to promote an efficient delivery of drug which reduce the tumor volume. This feature is of particular importance for suppression of tumor growth with increased local concentration at tumor site via EPR effect.

Effect on Tumor Weight

When optimized stealth liposomal formulation treated tumor weight was compared with control group it clearly indicate that tumor weight

Table 7. Effect of pure gemcitabine, CL-5 and SL-5 tumor volume

Treatment	Tumor volume (cm3)					
	Dose	10 Day	15 Day	20 Day	25 Day	30 Day
Saline solution	10mg/kg	0.9±0.12	1.5±0.23	1.9±0.32	2.5±0.1	3.1±0.14
Pure Gemcitabine	10mg/kg	0.4±0.13	0.8±0.21	1.4±0.24	1.7±0.12	2.1±0.15
CL-5	10mg/kg	0.3±0.21	0.7±0.11	1.1±0.2	1.4±0.23	1.7±0.11
SL-5	10mg/kg	0.1±0.15	0.3±0.12	0.5±0.19	0.8±0.27	1±0.12

was about 3 times less than (1.7±0.31gm) than control group as (6.5±0.23gm) hence growth of tumor were retarded upto 30 days of study. Similarly the effect of pure gemcitabine and optimized conventional liposomal formulation on tumor weight was (5.1±0.12gm to 3.8±0.41gm) respectively hence above comparison with respect to tumor weight was helpful for stealth liposomal formulation is effective against solid tumor with maximum cytotoxic effect (Table 8).

Table 8. Effect of pure gemcitabine, CL-5 and SL-5 on tumor weight

Treatment	Dose	Days	Tumor weight (gm)
Saline solution	10mg/kg	30	6.5±0.23
Pure Gemcitabine	10mg/kg	30	5.1±0.12
CL-5	10mg/kg	30	3.8±0.41
SL-5	10mg/kg	30	1.7±0.31

Acknowledgements

The authors are thankful to Lipoid GmbH, Ludwigshafen Germany, for providing the gift samples of phospholipids.

Conflicts of interest

The author declares no competing interests.

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