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Gemcitabine loaded vesicular drug delivery system for targeting

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ABSTRACT

Gemcitabine is a potent anticancer drug approved for the treatment of pancreatic, non-small-cell lung, breast, and ovarian cancers. Gemcitabine is a highly reactive molecule and binds extensively to plasma and tissue proteins leading to a fast inactivation of a large part of the administered dose. Thus, the clinical use of Gemcitabine faces two major problems, serious dose-limiting toxicities and rapid inactivation of the drug in the circulation. Both problems could possibly be prevented by shielding of the drug from the extracellular environment by means of a lipid coating. In the present investigation Gemcitabine loaded long circulating liposomes were prepared by thin film hydration method. By using different concentrations of PEGylated phospholipids, from the preliminary in vivo work, it was observed that stealth liposome's formulation was better than free Gemcitabine. Stealth liposome's formulation decreased the volume of solid tumor as well as ascites volume, decreased average body weight and increased the life span. In vivo pharmacokinetic studies were carried out in lymphoma bearing mice and the drug was detected in plasma even after 24 hours. This reveals that the stealth liposome's formulation had improved stability in the biological fluids. Tissue distribution studies done in the drug loaded long circulating liposome's showed preferential drug targeting to liver followed by spleen, lungs, and kidneys. Higher concentration of drug was targeted to the organs after administering the dose in the form of long circulating liposomes except in the heart. Drug levels in the heart are closely related to the inherent cardiac toxicity of Gemcitabine. Therefore using liposomal Gemcitabine formulation could reduce the cardiac toxicity of Gemcitabine.

Keywords: Gemcitabine, Liposomes, PEGylated phospholipids, Cancer.

INTRODUCTION

The poor efficacy of anticancer drugs is often related to their poor selectivity towards tumor tissue and to their toxicity. Use of innovative drug delivery systems can optimize their therapeutic features, protecting the drug against metabolic inactivation, increasing its plasma half-life, and improving both the therapeutic index and the anticancer efficacy of the drug [1-3]. As a deoxycytidine analogue that interferes with DNA synthesis, gemcitabine (2',2'-difluorocytidine,dFdC) is a potent anticancer drug against an unusually broad spectrum of solid tumors. [4] It is an FDA-approved first-line therapy for advanced or metastatic pancreatic cancer as a single agent, a first-line therapy for advanced or metastatic nonsmall-cell lung cancer in combination with cisplatin, a first-line therapy for metastatic breast cancer in combination with paclitaxel, and a second-line therapy for advanced ovarian cancer in combination with carboplatin. In addition, a large number of gemcitabine-based therapies combined with cytotoxins or molecularly targeted agents are currently being evaluated in clinical trials for the treatment of many common cancer types. [5-9]

The major deficiencies of gemcitabine therapy, however, are its rapid metabolic inactivation and therapeutic window. The standard narrow gemcitabine regimen is to administer the drug via 30 min intravenous infusion at a weekly dose of $1000-1250 \text{ mg/m}^2$. During circulation, gemcitabine is extensively deaminated to the inactive metabolite 2',2'-difluorouridine (dFdU) by cytidine deaminase, which is abundantly expressed in leukocytes and normal tissues [7]. The rapidly declining gemcitabine concentration in plasma necessitates the administration of large doses of the drug in cancer patients. However, the clinical benefits of gemcitabine are limited and short-lived with the median survival extended merely for a few months.

[10- 14] This is largely attributable to insufficient drug accumulation and activation in the tumor cells. On the other hand, the very high initial gemcitabine concentration in plasma immediately following intravenous administration commonly causes severe myelosuppression and toxicities in well-perfused organs including liver, lung, and kidney, which prohibit more frequent administration of the drug than once-weekly dosing in cancer patients.

Liposomes are biocompatible and biodegradable self-assembled vesicles, characterized by a supramolecular lipidic organization which is the same as that found in the natural membranes of living cells (Figure 1). [15] This is an advantage from the standpoint of biocompatibility and biodegradability because it induces neither side effects nor accumulation. Of all the possible nanomedicine platforms, liposomal formulations are the ones that have been approved by the US Food and Drug Administration for the treatment of cancer. [16, 17] It has been well demonstrated that use of liposomes in the treatment of solid tumors in particular protects the incorporated molecule from being inactivated following intravenous administration, which reduces accumulation of the anticancer drug in healthy tissues before it reaches the desired site of action.



Figure 1: Schematic representation of a liposomal structure with a characteristic microenvironment and possible drug encapsulation as a function of its physicochemical features.

MATERIALS AND METHODS

Gemcitabine purchased from Nap rod life sciences Pvt. Ltd, Thane., Methoxy polyethylene phosphoethanolamine 2000-distearoyl (DSPE, Di Sodium salt), palmitoyel glycerol 3 phosphocholine (DPPC), Di steryl phosphotidyl choline (DSPC) purchased from Genzyme, Switzerland, Genzyme, Switzerland, Di sodium hydrogen phosphate, Potassium dihydrogen phosphate, Chloroform, Sucrose purchased from S.D. Fine-Chem. Ltd., Mumbai.

Animals

Swiss albino mice of either sex weighing between 20 to 25 gms, were used for experimental purpose. The animals maintained under standard environmental conditions (25 to 300, 12hours dark / light cycle) and fed with standard rodent, feed and water adlibitum. All the experiments performed in this present study reviewed and accepted by the Institutional Animal Ethics Committee with no: P22/VCP/IAEC/2013/01/AE6/Mice.

Tumor cells

Ehrlich ascites carcinoma (EAC) cells obtained from Amala Cancer Research Institute, Thirussor, Kerala, India.

Statistical analysis

All the values expressed as mean \pm SEM. The data statistically analyzed by one-way ANOVA followed by Dunnett test. P values < 0.05 considered significant. *In vivo* evaluation study carried out for the best formulation conventional liposome formulation and stealth liposome's formulation, which has shown better performance than other formulations.

Pharmacokinetic studies

The Pharmacokinetic studies of conventional liposome's and stealth liposome's were carried out in Swiss albino mice bearing Ehrlich Ascites Carcinoma cells, mice of male weighing from 20-25 g were selected for this study and they were fed with a standard pellet diet and water ad libitum. The animals divided into 4 groups each group containing 8 animals.

The groups under treatment designed as follows

Group I: Tumor Control

Group II: Free Gemcitabine drug solution (30mg/kg) **Group III:** Stealth liposome formulation (30mg/kg) The drug treatment of was given i.v on 7th day of tumor transplantation. The mice from each group sacrificed at predetermined time intervals and blood samples collected. The collected blood centrifuged in a cooling centrifuge and blood plasma separated. To 1ml of plasma, Trichloroacetic acid added until precipitate forms. Then filtered and the filtrate was analysed by HPLC.

Anti tumor Activity

Effect on Median Survival Time and Average Body Weight Change

Animals were inoculated at 1×10^{6} cells/mouse on day 0, and treatment with Gemcitabine conventional and stealth liposome started 24 h after inoculation, at doses of 30 mg/kg. The control group treated with the same volume of 0.9% sodium chloride solution. All the treatments given out for 9 day, Median survival time (MST) of each group, containing 6 mice, was noted. The smear was prepared from ascites fluid on 14th day and stained with Geimsa staining techniques. Average body weight was calculated. The antitumor efficacy of Gemcitabine conventional and stealth liposome compared with that pure sample of Gemcitabine. MSTs of treated groups compared with those of control groups

Effect on Solid Tumor Volume

Mice divided into four groups and each group containing 6 animals Tumor cells $(1X10^6 \text{ cells/mice})$ injected into the right hind limb of all the animals intramuscularly.

Group I: Tumor Control

Group II: Free Gemcitabine drug solution.

Group III: Stealth Liposome formulation.

The drug treatment of 30mg / kg was given orally for 5 alternate days. Tumor mass was measured from 11th day of tumor induction and was repeated every 5th day for a period of 30 days.

Assay for Proliferation Studies

MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5diphenyltetrazolium bromide) method

0.1ml of the cell suspension (containing 1x105cells) and 0.1ml of the test solution (6.25-100 mg/ml) in DMSO were added to the 96 well plates kept in carbon dioxide incubator with 5% CO2, at 370 C for 72 hours, Blank contains only cell

suspension and control wells contain 1% DMSO and cell suspension. After 72 hours, 20ml of MTT added and kept in carbon dioxide incubator for 2 hours followed by 80ml of lysis buffer. The plate covered with aluminum foil to protect it from light. Then the 96 well plates kept in rotary shaker for 8 hours. After 8 hours, the 96 well plates processed on ELISA reader for absorption at 562nm. The readings were averaged and viability of the test samples were compared with DMSO control

Tissue distribution studies

Male Swiss-albino Mice (Mahaveera Enterprises, Hyderabad, India) weighing between 24 and 26 g were used in this study. The animals kept in cages with free access to water and standard diet ad libitum. The animals acclimatized to the environmental conditions. The protocol for the animal experiments reviewed and approved by Animal ethical committee of the institution. The mice were fasted overnight with free access to water ad libitum before the day of the experiment. On the day of the experiment, the mice were anaesthetized with ether and Gemcitabine 0.54mg dissolved in a saline-propylene glycol-ethanol vehicle (5:4:1, v/v/v; 1mg/ml) was injected intravenously to one group of mice through the tail vein. The animals remained unrestrained during the entire drug administration and sampling time. At 0.25, 0.5, 1, 2, 4 and 6 h following the Gemcitabine dose 0.54mg, the animals anesthetized by ether. The blood sample with drawn from retro-orbital sinus vein into pre-heparinized polypropylene tubes. The animals then immediately decapitated and the tissue samples including whole brain, lungs, heart, liver, kidney and spleen removed. The serum separated by centrifuging the blood samples at 4000rpm for 30 min. The tissue samples washed with normal saline. The serum and tissue samples kept frozen at -80 °C until drug analysis. A total number of 36 mice used to collect three replicate biological samples at six time points from two study groups (i.e., 18 mice in each group).

This study carried out to compare the targeting efficiency of drug- loaded liposome's with that of free drug in term of percentage decrease in targeting to various organs of reticuloendothelial system (RES) like liver, lungs, spleen and kidneys.

Distribution of Gemcitabine in tumors

were killed 10-15 Animals min post Gemcitabine injection and tumors were excised and placed immediately in optimum cutting temperature compound, frozen in liquid nitrogen, and stored at -70 °C prior to sectioning and immune histo chemical staining. Two 10µm-thick cryostat sections cut from each tumor (sections $\sim 50 \ \mu m$ apart), mounted on glass slides and air-dried. Gemcitabine fluorescence (which might include a component from fluorescent metabolites) detected using an Olympus Upright BX50 microscope with a Photometrics Coolsnap HQ2 camera and a 100 W HBO mercury light source equipped with 530-560 nm excitation and 573-647 nm, emission wavelength filter sets.

Tissue sections tiled using a motorized stage. Blood vessels in tissue sections recognized by expression of CD31 on endothelial cells, after imaging for Gemcitabine, tissue sections fixed in acetone, washed in PBS, and blocked with a protein-blocking reagent (ID Labs, Inc., London, ON, Canada). Tissue sections then stained with a rat anti-CD31 (1/100) antibody for one hour in a humidified chamber washed in PBS and stained with a Cy3 conjugated goat anti-rat IgG secondary antibody (1/400). CD31-stained sections were reimaged using the same method used to capture Gemcitabine fluorescence. Composite images generated by overlaying those for Gemcitabine and blood vessels using Media Cybernetics Image Pro plus Software (Version 6.0). Gemcitabine staining converted to an 8-bit grey-scale with fluorescence intensities ranging from 1-254, while blood vessels stained with anti-CD31 represented by an intensity of 255. Regions for data analysis selected by excluding artefact, fluorescence, and necrosis, and objects <5µm in diameter were removed. Readings from regions without nuclear staining provided average background fluorescence for each tumor section. The pixel area was 0.4 µm2 and customized algorithms measured the distance to the nearest blood vessel for each pixel within a selected area of interest (AOI), Gemcitabine intensity (I) relative to background averaged over all pixels at a given distance (L) from the nearest blood vessel and plotted as a function of that distance. Gemcitabine distribution in each AOI was determined by calculation of the area under the intensity vs. distance graph and differences between cells lines and treatments were assessed using a t-test (p<0.05 was considered statistically significant).

Estimation of pharmacokinetic parameters and statistical significance

The pharmacokinetic parameters such as $c_{max},$ $t_{max},\ AUC_{(0-6\ h)},\ t_{1/2},\ MRT$ and the rapeutic

RESULTS AND DISCUSSION

availability (TA) were calculated by using the Kinetica software (version 5.0). The values expressed as mean \pm SD. The two samples comparisons done with unpaired student t-test and P < 0.05 considered as statistically significant.

Table 1: Pharmacokinetic Studies of Gemcitabine Pure Drug and Gemcitabine Stealth Liposome's				
Formulation				

r of mulation.			
Parameter	For Pure Drug	Gemcitabine Stealth	
	(Gemcitabine)µg hr/ml	Liposome's Formulation.	
C _{max}	26.39 µg hr /ml	49.54µg hr/ml	
T _{max}	0.5hrs	4 hrs	
AUC	62.07µghr/ml	731.56µg hr/ml	
AUMC	98.54 μghr ² /ml	7080.04µg hr²/ml	
MRT	1.5875 hrs	9.678 hrs	
Elimination rate constant	0.09 hr ⁻¹	1 0.88 hr ⁻¹	
Elimination $t_{1/2}$	0.558 hrs	18.236 hrs	
Absorption Rate Constant	0.59 1 hr ⁻¹	1 0.0585 hr ⁻¹	
Absorption t _{1/2}	1.172 hrs	11.84 hrs	

Table 2: Effect of Gemcitabine Stealth Liposome's treatment on the survival Time and Average Body Weight changes of Tumor Bearing Mice

Treatment	MST (d) in body wt (g) days	Life Span (%)	Average increase	
Tumor control	20 + 1.10	-	18.75 + 1.30	
(Saline 2 ml/kg)				
Pure sample (30 mg/kg)	$35 + 1.15^{a}$	75	5.85 +0.17 ^a	
Stealth Liposome's	$38 + 1.44^{a}$	90	$90\ 4.02\ +\ 0.23\ ^{a}$	
Formulation (30 mg/kg)				

Table 3: Effect of Gemcitabine pure sample and Stealth Liposome's on Solid Tumor volume

Treatment	Dose	Solid tumor volume (ml)			
	(mg/kg)	15 th day	20 th day	25 th day	30 th day
Tumor control	-	3.88 +0.12	4.18 + 0.15	5.65 +0.16	6.15 + 0.45
Pure samples	30 mg/kg	2.86 + 0.13a	3.55 + 0.17	4.02 + 0.10a	4.72 +0.12a
Stealth					
Liposome's	30 mg/kg	2.86 + 0.13a	3.55 + 0.17	4.02 + 0.10a	4.72 +0.12a
Formulation					

Table 4: Tissue distribution studies of Gemcitabine formulation:

Sample	Parameter	Stealth Liposome's
		Formulation
Serum	$c_{max}(\mu g/mL)$	9.73±1.21
	\mathbf{t}_{max} (h)	0.25
	$AUC_{(0-6 h)} (\mu g h/mL)$	11.08 ± 0.57
	t _{1/2} (h)	2.74±.29

	MRT	1.86±.38
Brain	$c_{max}(\mu g/mL)$	1.9 ± 0.06
	t_{max} (h)	0.25
	$AUC_{(0-6 h)} (\mu g h/mL)$	2.85 ± 0.57
	t _{1/2} (h)	1.52 ± 0.21
	MRT	2.24±0.14
Liver	$c_{max}(\mu g/mL)$	3.26±0.42
	t_{max} (h)	0.25
	$AUC_{(0-6 h)} (\mu g h/mL)$	5.52±0.59
	t _{1/2} (h)	2.09 ± 0.75
	MRT	2.44±0.85
Lung	$c_{max}(\mu g/mL)$	4.47±0.35
	t_{max} (h)	0.25
	$AUC_{(0-6 h)} (\mu g h/mL)$	5.49±1.66
	$t_{1/2}$ (h)	2.84 ± 1.99
	MRT	3.89±1.47
Kidney	$c_{max}(\mu g/mL)$	5.11±0.3562
	t_{max} (h)	0.25
	$AUC_{(0-6 h)} (\mu g h/mL)$	8.15 ± 0.48
	$t_{1/2}$ (h)	3.56±0.59
	MRT	3.49±0.4
Spleen	c_{max} (µg/mL)	2.87 ± 1.05
	t_{max} (h)	0.25
	$AUC_{(0-6 h)} (\mu g h/mL)$	5.6 ± 2.07
	$t_{1/2}(h)$	4.21 ± 1.80
	MRT	5.24±2.51
Heart	$c_{max}(\mu g/mL)$	1.48 ± 0.59
	t_{max} (h)	0.25
	$AUC_{(0-6 h)} (\mu g h/mL)$	5.57±1.53
	$t_{1/2}(h)$	2.4±0.3
	MRT	2.37±0.35

DISCUSSION

Pharmacokinetic studies

The stealth liposome's altered the pharmacokinetic profile of Gemcitabine. The serum levels of Gemcitabine were significantly higher for stealth liposome's when compared to free Gemcitabine solution. The parameters evaluated are Absorption rate constant (Ka), Elimination rate constant (Ke) Absorption half-life $(t_{1/2}a),$ Elimination half-life (t 1/2e), tmax, cmax, AUC, AUMC and MRT. The valves are shown in (Table 1). Higher values of the elimination half-life of stealth liposomes compared to free Gemcitabine will have prolonged circulation time in blood. The time taken to reach the peak serum concentration was higher for stealth when compared to free Gemcitabine and the peak serum concentration at

that particular time found to be lower for stealth liposomes. This shows that stealth liposome found to be in blood for prolonged time.

Antitumor activity

Effect on Mean Survival Time and Average Body Weight Changes

The antitumor efficacy of Gemcitabine stealth liposome's formulation were compared with plain Gemcitabine solution; For control group mean survival time was 20 days and average increase in body weight 18.75g. For Gemcitabine pure drug mean survival time was 35 day and life span 75% and average increase in body weight 5.85g, Stealth liposome's formulation mean survival time 38 days and life span was 90% and average increase in body weight was 4.02g. From this study, it found that increased mean survival time, life span and decreased body weight for stealth liposome's formulation. The results are showed in **Table 2**.

Effect on Solid Tumor Volume

Solid tumor volume measured at 30th day, for control, pure drug and stealth liposome's; For control group solid tumor volume was increased to 6.15 ± 0.45 ml, For Gemcitabine pure drug solid tumor volume was 4.72 ± 0.12 ml, For stealth liposome's formulation tumor volume was $4.32 \pm$ 0.25ml. From this study, it found that decrease in solid tumor volume for stealth liposomes formulation. It was very well evident that stealth liposome's formulation has an anti tumor effect. The results are showed in **Table 3**.

Tissue distribution studies

Plasma and tissue concentrations of Gemcitabine at different time points following i.v. administration of Gemcitabine Formulation 2 were determined. The drug distribution is less in the heart when compare with other tissues. The distribution in serum is more and also spleen. The results are showed in **Table 4**

SUMMARY AND CONCLUSION

From the preliminary *in vivo* work, it was observed that stealth liposome's formulation was

better than free Gemcitabine. Stealth liposome's formulation decreased the volume of solid tumor as well as ascites volume, decreased average body weight and increased the life span. *In vivo* pharmacokinetic studies were carried out in lymphoma bearing mice and the drug was detected in plasma even after 24 hours. This reveals that the stealth liposome's formulation had improved stability in the biological fluids.

Tissue distribution studies done in the drug loaded long circulating liposome's showed preferential drug targeting to liver followed by spleen, lungs, and kidneys. Higher concentration of drug was targeted to the organs after administering the dose in the form of long circulating liposome's except in the heart. Drug levels in the heart are closely related to the inherent cardiac toxicity of Gemcitabine. Therefore using liposomal Gemcitabine formulation could reduce the cardiac toxicity of Gemcitabine.

In vivo studies stealth liposome's increased the life span of mice bearing Ehrlich ascites carcinoma. Pharmacokinetic studies have shown that stealth liposome's will have increased elimination half life and the area under the curve also higher when compared with pure Gemcitabine and justify their potential in strengthening the efficacy and safety profile of the drug.

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