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Preparation and Evaluation of Decitabine loaded liposomes for Effective Chemotherapy

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Abstract : Decitabine, an epigenetic drug, is a potent hypomethylating agent. However, its effect is transient due to its instability in acidic environment and enzymatic degradation. Therefore, the present study was designed toincorporate decitabine in liposomal formulation, which offers a dynamic and flexible technology for enhancing drug solubility due to their biphasic characteristic and variety in design, composition and assembly. DEC liposomes were prepared by modified thin film hydration method and characterized by particle size distribution, zeta potential, scanning electron microscopy (SEM), and entrapment efficiency. We tested the efficacy of decitabine liposomes in various cell lines, MCF-7, MDA-MB-231, LN-CaP. Decitabine liposomes significantly enhanced ($p< 0.05$) the antiproliferative effect when compared to the plain drug.
Keywords: Decitabine liposomes, Antiproliferative activity, MCF-7, MDA-MB-231 and LN-CaP.

Introduction

Decitabine is an epigenetic drug that inhibits DNA methylation. Decitabine can reactivate tumour suppressor genes silenced by aberrant DNA methylation, a frequent event in all types of cancer. Since, DNA methylation is reversible; it represents very promising therapeutic targets for breast cancer and prostate cancer treatment. However, the effective delivery of a drug into solid tumors, encounters many potential barriers. The i.v. administration of almost all small-molecule chemotherapeutic agents leads to a large volume of distribution[1,2] resulting in narrow therapeutic index due to a high level oftoxicity in healthy tissues. This volume of distribution can be controlled by encapsulating the drugs in liposome, which also enhances the concentration of drug in the tumour.[3,4]

In the present study, we designed and evaluated the lipid based strategy, liposomes, for the effective delivery of decitabine. Liposomes are used for increasing the effectiveness of medicines by encircling an aqueous solution with a membrane of phospholipids. Encapsulating anadequate amount of the drug is one of the most desirable properties that make liposomes usable. The liposomes can encapsulate hydrophilic drugs within an aqueous component, and the lipophilic drugs within the lipid bilayer.[5]The main advantages are smaller size, biocompatibility, lipophilic nature and great specificity.

Starting with soya-phosphatidylcholine (PC)/cholesterol, the liposomes incorporating decitabine, were prepared. Further, Poly(ethylene glycol)-lipid (PEG-lipid), a widely used conjugate in the field of liposomal drug delivery, was incorporated, to provide a polymer coat that can confer favorable pharmacokinetic characteristics on particles in the circulation.[6]The influence pegylation on incorporation as well as the release of decitabine wasalso investigated.

2. Materials and Methods

2.1 Cell cultures

Two human breast cancer cell lines MCF-7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative), one human prostrate carcinoma cell line LNCaP were obtained from NCCS, Pune. MCF-7 and MDA-MB-231, cells were cultured and maintained in DMEM while LNCaP cells were cultured in RPMI medium, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (100,000 U/l penicillin, 100 mg/l streptomycin), at 37˚C in a humidified atmosphere containing 5% carbon dioxide.

2.2 Materials

Dimethylsulfoxide (DMSO), isopropanol and glacial acetic acid were purchased from Qualigens fine chemicals (Mumbai, India). L-α-Phosphatidylcholine, cholesterol, MTT reagent, dulbecco's modified eagle's medium (DMEM), RPMI medium, fetal bovine serum (FBS), were procured from Sigma Aldrich (St. Louis, MO, USA). Triton-X 100, trypsin,and common laboratory chemicals were purchased from Himedia lab Pvt. Ltd. (Mumbai, India). HPLC grade solvents were procured from SD fine chemicals. All other chemicals used in the study were of analytical grade.

2.2 Preparation of Decitabine loaded liposomes

Decitabine liposomes composed of L-α-Phosphatidylcholine(PC)/cholesterol (CH) (molar ratio 5:1), and pegylated decitabine liposomes composed of PC/CH/DSPE-MPEG 2000 (molar ratio 5:1:0.5) were prepared using modified thin film hydration method[7,8]. The lipophilic excipients, PC and CH were dissolved in chloroform/ methanol (9:1) solution in a 250 mL round bottom flask (RBF). The organic solvent was evaporated using rotary evaporator (Buchi Rotavapor, R-215, Switzerland) to obtain a thin lipid film. Thereafter, the solvent traces were removed from the film by keeping the RBF under vacuum overnight. The drug was dissolved in KN buffer and the film was hydrated by rotating the RBF until completely dissolved. The suspension was probe sonicated for 10 min with pulse and passed through 0.22 µm pore size syringe filters (millex, Millipore, MA, USA).Untrapped decitabine was removed by centrifuging at 22000 × *g* for 60 min. The liposomes were lyophilized and stored at 4°C in air tight containers for further experiments.[9]

2.3 Characterization of Decitabine liposomes

2.3.1 Particle size and zeta potential

The mean particle size, polydispersity index (PDI) and zeta potential (ZP) of liposomes were determined before and after freeze-drying by dynamic light scattering using photon correlation spectroscopy (PCS) technique at 25°C, using Malvern NanoZS (Malvern Instruments Ltd., Worcestershire, UK).

2.3.2 Transmission Electron Microscopy (TEM)

The surface morphology of liposomes was determined by transmission electron microscopy (Philips Tecnai 20, Hillsboro, OR, USA). Decitabine liposomes were dispersed in distilled water; one drop was incubated on carbon coated copper grid and negatively stained with 1% uranyl acetate. The copper grid was fixed onto the sample holder, placed in the vacuum chamber of the transmission electron microscope and observed under low vacuum.

2.3.6 Drug entrapment efficiency (EE)

The EE was determined by separation of liposomes from the aqueous phase that contained the unentrapped drug after ultra-centrifugation.[10,11,12] The supernatant and pellet were analysed by HPLC. Chromatographic separation was achieved on Phenomenex Luna C-18 column, 250 x 4.6 mm diameter, 5 µ pore size. The mobile phase, 0.01 M ammonium acetate buffer and methanol (98:2), pH 6.4 at flow rate of 1.5 mL per min was optimized and retention time 8.4± 0.1 min was obtained. The column temperature was 25ºC

and UV detection done at 230 nm. Standard plot of decitabine was prepared within the concentration ranges of 1-50 µg mL^{-1}. The supernatant and pellet were diluted with deionized water, 1 x 10^{-2} times before analysis.The entrapment efficiency was calculated as: % EE = {Pellet/ (supernatant+pellet)} *100

2.5 In-vitro release studies

The dialysis bags,molecular weight cut off 12 kda, were washed in tap water over night and rinsed with distilled water before use to remove preservatives. Liposomal decitabine was transferred to the dialysis sac, and mounted in beaker filled with 100 mL of PBS 7.4over a magnetic stirrer. The temperature was set at 37 ± 1 °C, the rotation speed at 250 rpm and for 120 h. At different time intervals, 1 mL aliquots of the release medium were withdrawn and replaced by same amount of fresh medium. The release rate was calculated by plotting absorbance versus time graph.[13,14]

2.6 Stability studies

The lyophilized decitabine liposomes were subjected to stability studies for three months. The stability of the decitabine liposomes was evaluated upon storage at -20°C, +4°C and 25°C for three months. Upon reconstitution, the samples were analyzed for particle size, PDI, entrapment efficiency and release profile.[15]

2.7. Cytotoxicity study by MTT assay

The cytotoxicity of decitabine and decitabine liposomes was determined by the MTT assay[17,18]. Briefly, 5×10^3 cells/well were plated in 96-well tissue-culture plates. DEC and freeze-dried decitabine liposomes were diluted in culture media and 100 µL of different concentrations added to wells. DEC solution was replaced after every 24 h for 3 days while DEC-NPs were added once. After 72 h, the supernatant was flicked off, 50 µl of MTT (0.5 mg/mL) added to each well and incubated for 4 h. The unreduced MTT and medium were then discarded. Each well was washed with 200 µl of PBS. 200 µl of DMSO was added to each well to dissolve the MTT formazan crystals. Plates were shaken for 20 min and absorbance was measured at 540 nm using the microplate reader (Bio-Tek, ELX-800 MS).[19] The IC_{50} values were graphically calculated from concentration-effect curves, considering the optical density of the control well as 100%. The experiments were repeated five times.

2.8 Statistical Analysis

Data is represented as mean±SD of four readings.

3. Results and Discussion

3.1 Characterization of decitabine liposomes

The particle size distribution of decitabine liposomes was found to be in the range of 90-120 nm. The selected conventional liposomal formulation of decitabine has mean particle size 115.3 ± 6.4 nm while the long circulating pegylated form of decitabine liposomes were 143.4 ± 5.2 nm. The PDI of all the formulations was around 0.1 to 0.3, which indicated a homogeneous particle size distribution; results are shown in **table 1**.

Table 1. Composition and characterization of selected decitabine liposomes

S No	Composition	MPS (nm)	PDI	ZP (mV)	EE (%)
DL1	PC:CH = 3:1	129.3±12.2	0.210±0.04	-52.4±2.7	31.7±4.7
DL2	**PC:CH = 5:1**	**115.3±6.4**	**0.133±0.01**	**-56.3±1.7**	**54.2±4.6**
DL3	PC:CH = 7:1	159.7±10.6	0.361±0.1	-55.4±4.2	58.3±4.8
DL4	PC:CH = 9:1	210.4±4.5	0.305±0.04	-57.7±2.5	57.5±5.1
DL5	PC:CH:PEG_{2000}=5:1:0.15	152.3±7.5	0.210±0.22	-60.3±3.1	47.9±6.3
DL6	**PC:CH:PEG_{2000}=5:1:0.25**	**143.4±5.2**	**0.241±0.37**	**-60.3±2.7**	**57.73 ± 2.1**
DL7	PC:CH:PEG_{2000}=5:1:0.50	194.4±13.2	0.352±0.08	-63.3±1.1	55.7±4.3

PC, Phosphatidylcholine; CH, Cholesterol; MPS, mean particle size; ZP, zeta potential; EE, entrapment efficiency. The ratio of liposome composition is molar ratio (%). Each value represents the mean ± S.D., (*n*=4).

The pegylated liposomes had the highest mean particle size and PDI. Zeta potential for all prepared formulation was below -30 mv which indicted their stability. The desirable particle size is more than +30 mv and less than -30 mv.[6]The surface Morphology by was observed by TEM analysis. Decitabine liposomes were discrete and round structured **(Fig. 1)**with a mean particle size range from 100 to 200 nm. HPLC method was used to analyse the entrapped decitabine in liposomes. Drug entrapment efficiency of optimised liposomes was found to be more than 50%. Results are shown in **table 1**.

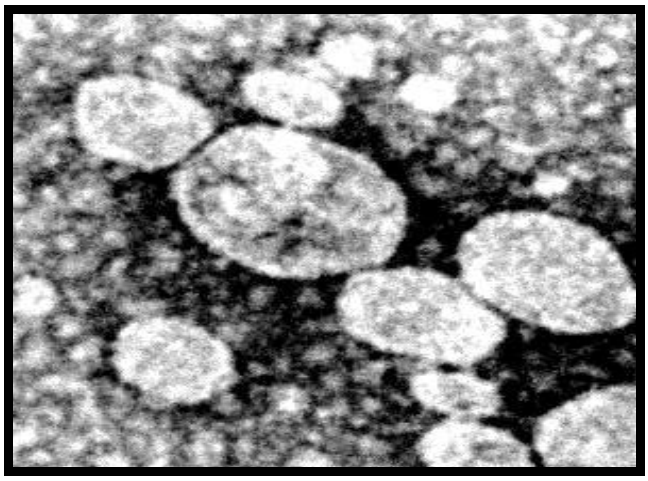

Fig. 1TEM image of decitabine liposomes. The surface morphology of the prepared liposomes was studied by TEM analysis. The liposomes were round and discrete. The particle size was correlating with the size analyzed by particle size analyzer.

3.2 In vitro release of decitabine liposomes

The drug release rate of all the optimised formulations was often comparable. The free drug was released in the medium in approximately 4 h and from the conventional liposomes in 72 h. Furthermore, the rate of release of decitabine from pegylated liposomes was slower than that of conventional liposomes**(Fig.2)**.

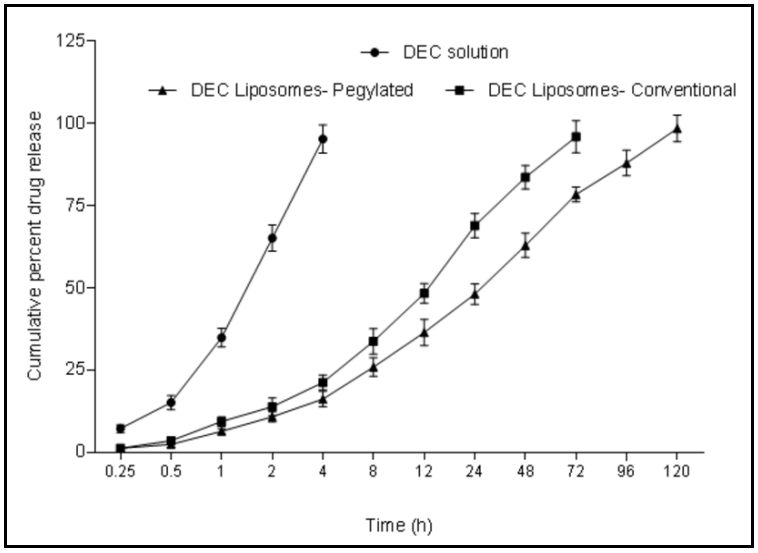

Fig.2In vitro release study. The drug solution (DEC solution) was completely released into the receptor compartment within 4 h. The conventional liposomes released the drug completely into the buffer in approximately 72 h, whereas, the release of decitabine from pegylated liposomes was delayed by 48 h.

3.3 Stability of decitabine liposomes

The stability of optimized decitabine liposomes DL2 and DL6 was evaluated at three different temperatures, 37°C, 20°C and 4°C.There was no significant change in the particle size and drug EE observed for formulations stored at 20°C and 4°C. However, there was a slight increase in the particle size and EE of liposomes stored at room temperature after three months.

3.4 Cytotoxicity studies of decitabine liposomes

The liposomes were tested on breast cancer and prostate cancer cells. The cytotoxicity of decitabine liposomes was significantly ($p< 0.05$) higher than the plain drug in breast cancer and prostate cancer cells. The breast cancer cells MCF-7 and MDA-MB-231 were effectively suppressed.The liposomes showed higher cytotoxicity in the prostate cancer cells LN-CaP.The results clearly showed that the liposomal decitabine had greater penetration leading to higher cytotoxic potential in cell cultures.

Table. 2 IC_{50} of decitabine and decitabine liposomes in 72 h

Cell Line	Source	DEC (µM)	DEC Liposomes (µM)
MCF-7	Human breast adenocarcinoma	0.28	0.08
MDA-MB-231	Human breast adenocarcinoma	0.51	0.09
LN-CaP	Human prostrate carcinoma	0.67	0.29

DEC, decitabine; All values are mean ± SEM of three individual experiments

4. Conclusion

The liposomal drug delivery system for decitabine was established. Decitabine loaded liposomes were competent in delivery of decitabine to the cells which significantly increased the cytotoxic potential. The lipid content of the liposome bilayer was shown to influence decitabine incorporation with maximum incorporation efficiency achieved at the molar ratio of 5:1. Beyond this ratio, increase in the quantity of PC had no significant effect on the entrapment efficiency of decitabine. Addition of DSP-MPEG had no significant effect on the decitabine drug loading, however, there was a slight increase in the mean particle size and zeta potential. The drug release pattern was found to be slower than the conventional liposomes. TEM was used to study the surface morphology of prepared decitabine liposomes. The final conventional formulation with molar ratio 5:1 was selected and investigated on breast cancer and prostate cancer cell cultures. There was a significant increase ($p < 0.05$) in the cytotoxic potential of the liposomes over the free drug, which could be owed to the protection from degradation of the drug by the lipid bilayers.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Speth, P., Q. Van Hoesel and C. Haanen, Clinical pharmacokinetics of doxorubicin, Clin Pharmacokinet, 1988, 15(1), 15-31.

2. Lazo, J., B. Chabner and D. Longo., Cancer chemotherapy and biotherapy: principles and practice, DL Lippincott-Raven, Philadelphia, 1996, 37.
3. Food and Drug Administration, Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers, Food and Drug Administration, Washington, DC. http://www.fda.gov/cder/guidance, 2005.
4. Neupane, Y. R., M. Srivastava, N. Ahmad *et al.*, Lipid based nanocarrier system for the potential oral delivery of decitabine: Formulation design, characterization, ex vivo, and in vivo assessment, Int J Pharm, 2014,477, 601-612.
5. Gabizon, A., H. Shmeeda and Y. Barenholz., Pharmacokinetics of pegylated liposomal doxorubicin, Clin Pharmacokinet, 2003, 42, 419-436.
6. Jain, P., N. Kumar, V. R. Josyula *et al.*, A study on the role of (+)-catechin in suppression of HepG2 proliferation via caspase dependent pathway and enhancement of itsin vitro and in vivo cytotoxic potential through liposomal formulation, Eur J Pharm Sci, 2013, 50, 353-365
7. NK, S., & Kumar, V. Release Kinetics of Novel Photosensitive Liposome for Triggered Delivery of Entrapped Drug.; International Journal of PharmTech Research, 2015, 8(1), 106-113
8. Meghana, G., Karri, V. N. R., Talluri, S. V., *et al.*, In vitro and In vivo behavior of Liposomal Gel for the Treatment of topical Fungal Diseases.; International Journal of PharmTech Research, 2015, 7(2), 814-820
9. Chonkar, A. D., Rao, J. V., Managuli, R. S.,*et al.*, Development of Fast Dissolving Oral Films Containing Lercanidipine HCl Nanoparticles in Semicrystalline Polymeric Matrix for Enhanced Dissolution and Ex Vivo Permeation. Euro JourPharm Biopharm, 2016, 103, 179-191
10. Nalla, P., Bagam, S., Eedara, B. B.,*et al.*, Formulation and Evaluation of Domperidone Oral Proliposomal Powders.; International Journal of PharmTech Research,2015, 7(1), 108-118.
11. Plimack, E. R., D. J. Stewart and J.-P. J. Issa., Combining epigenetic and cytotoxic therapy in the treatment of solid tumors, J Clin Oncol, 2007, 25, 4519-4521.
12. Joshi, G., A. Kumar and K. Sawant., Enhanced bioavailability and intestinal uptake of Gemcitabine HCl loaded PLGA nanoparticles after oral delivery, Eur J Pharm Sci, 2014, 60, 80-89.
13. Nii, T. and F. Ishii., Encapsulation efficiency of water-soluble and insoluble drugs in liposomes prepared by the microencapsulation vesicle method, Int J Pharm, 2005, 298, 198-205.
14. Karahoca, M. and R. L. Momparler., Pharmacokinetic and pharmacodynamic analysis of 5-aza-2′-deoxycytidine (decitabine) in the design of its dose-schedule for cancer therapy, Clin Epi, 2013, 5, 3.
15. Mohammed, A., N. Weston, A. Coombes, M. *et al.*, Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability, Int J Pharm, 2004, 285, 23-34.
16. Denizot, F. and R. Lang., Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, J Immunol Methods, 1986, 89, 271-277.
17. Osman, M. E., Eid, M. M., Khattab, O. H., *et al.*, In vitro cytotoxicity of biosynthesized Ag/CS NP against MCF7, PC3 and A549 Cancer cell lines.; International Journal of PharmTech Research, 2015, 8 (5), 1011-1017
18. Anggraeni, R., Hadisahputra, S., Silalahi, J., *et al.*, Combinational effects of ethylacetate extract of Zanthoxylum acanthopodium DC. with Doxorubicin on T47D breast cancer cells. International Journal of PharmTech Research, 2015, 7(4), 2032-5
19. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J Immunol Methods, 1983, 65, 55-63.

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