P-052	**Doxorubicin encapsulated solid lipid nanoparticles for the mangement of brain cancer**

Kharya P. and Jain S.K. [1 *]
[1] Departement of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar, India
* Supervisor # kharya_parul@yahoo.co.in

INTRODUCTION

In spite of huge advances in brain research, brain and central nervous system (CNS) disorders remain the world's leading cause of disability, and account for more hospitalizations and prolonged care than almost all other diseases combined. The major problem in drug delivery to brain is the presence of the blood brain barrier (BBB). Drugs that are effective against diseases in the CNS and reach the brain via blood compartment must pass the BBB (Misra et al., 2003). Active targeting is a non-invasive approach, which consists in transporting drugs to target organs using site-specific ligands. Endogenous and chimeric ligands binding to carriers or receptors of the BBB have been directly or indirectly conjugated to nanocarriers.

MATERIALS AND METHODS

Materials Doxorubicin HCl (Doxo HCl) was a gift from Khandelwal laboratory (Mumbai, India). Hydrogenated soya phosphatidyl choline (HSPC) distearylphospatidylethanolamine (DSPE) and DSPE-mPEG were gifted from Lipoid, Ludwigshafen, Germany. Triton X-100, Phenylalanine (PA), Sephadex G-50, Dextran sulphate (DS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma Chemicals (St Louis, MO, USA).

Method of Preparation HSPC: tristearin (100:100), DSPE: DSPE-mPEG (1:1) were added to ethanol in a definite ratio and the solution was warmed in water bath to about 40°C to dissolve the lipid mixture in the solvent. Aqueous phase was prepared containing 0.1% v/v of tween 80 and doxo HCl: Dextran sulphate (2:1) in deionised water. Now, organic phase i.e. ethanol solution containing lipids was added to prewarmed aqueous solution at the 40°C temperature with the help of syringe under continuous mechanical agitation at constant speed for a definite time interval. This leads to the formation of lipid suspension which was then further sonicated using probe sonicator to form solid lipid SLNs. Free drug was removed by dialysis. Dextran sulphate is used as counter ion for the drug. Stevens et al.,(2004). Conjugation of SLNs with PA was done as the method reported by Gupta et al., 2007 with slight modification.
Preparation of FITC-BSA encapsulated SLNs was also done. Characterization of SLNs done on the basis of Size, size distribution, zeta potential, entrapment efficiency and in vitro drug release from both the uncoupled and PA coupled SLNS (PA-SLNs).

In vitro cytotoxicity studies MTT assay C6 glioma cells were grown as a suspension culture in Dulbecco's Modified Eagle Medium (DMEM) seeded supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic and incubated at 37±1°C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were seeded in 24-well Nunclon plates at a cellular density of 2×10^4 cells/well for 24 h, in 0.2 ml DMEM. After culture, the growth medium was removed and growth medium containing all the three formulation free drug solution, drug encapsulated SLNs, drug encapsulated PA-SLNs respectively, to each well representing the same concentration of Doxo HCl and plates were further incubated for 72 h. Then 10µL of fresh medium containing 5 mg/ml 3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was added to each well and the cells were incubated for four hours in dark at 37±0.2°C. After incubation for 3 hour, C6 glioma cells were detached by pipetting and collected by centrifugation at 1000 RPM for 10 min. Suspension was discarded then washed again with PBS (pH 7.4). 1 ml of 0.04M HCl-isopropanol solution was added and allowed to stand for 10 min. Suspension was collected, centrifuged at 1000 RPM for 10 min, supernatant was taken out and absorbance was measured at 650 nm.

The absorbance was read on an Elisa plate reader at the dual wavelengths 570 and 650 nm. Finally, the cytotoxicity (IC50) of different formulations were calculated which is based on its effect on the color yield of treated cells following MTT exposure. The IC_{50} value was defined as the drug concentration required inhibiting growth by 50% relative to controls.

Cell uptake studies The uptake study was performed at 1, 3 and 5 hours time interval using fluorescence activated cell sorters instrument. Cell lines used in the study were cultured in Falcon 12-well flat bottom plate at a density of 2×105 cells/well for in 0.5 ml of Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 1% antibiotic and incubated at 37 ±0.2°C in a humidified atmosphere containing 5% CO_2 for 24 h to attach.
Cells were incubated with the FITC encapsulated SLNs formulation (in a concentration of 50, 100 and 200 µg per ml) in a growth medium for different time. Cells were washed twice with PBS (pH 7.4) then 40 µl was added to trypsin PBS solution (2.5mg/mL), incubated for 5 min, and cells were harvested by adding 1mL PBS followed by treatment with probe-type ultrasonicator for 5 times to obtain the cell lysate. Finally cell lysate was centrifuged at 10,000 RPM for 10 min and the supernatant was supplied to florescence assay by using FACS.

RESULTS AND DISCUSSION

Cytotoxicity of blank SLNs No cytotoxicity was observed in case of plain uncoupled SLNs and plain PA-SLNs. Cytotoxicity test showed that cell viability was not found to be decreased even when SLNS are used at higher concentration, this show that SLNs are non toxic at higher concentration as well. The SLNs principally is made up of Lecithin which is physiologically acceptable lipid.

Cytotoxicity of Doxorubicin HCl encapsulated SLNs The cytotoxicity of SLNs formulations and free drug were evaluated using C6 glioma cell lines using an MTT assay. The Doxo HCl concentration in all the formulations was adjusted to be the same as that of free drug. It was observed that doxo encapsulated PA-SLNs were found to be showed significant cytotoxicity than other formulations in C6 glioma cell lines in 72 hrs period of incubation. The amount of doxo HCl required to achieve 50% of growth inhibition (IC_{50}) was much lower in PA-SLNs than in solution. This may be due to the higher expression of PA occurs on C6 glioma cells so a significant amount of drug will be able to intercalate with the DNA of cell result in cell death.

Table 1: MTT cytotoxicity assay of formulation.

Concenration in µg/mL	Absorbance		
	Doxo HCl solution	Doxo encapsulated SLNS	Doxo encapsulated PA-SLNs
2	0.443	0.408	0.332
4	0.396	0.356	0.300
6	0.358	0.321	0.248

Absorbance of Control i.e. cells only is 0.593

Concentration in µg/mL	% cell viability		
	Doxo HCl solution	Doxo HCl encapsulated SLNS	Doxo encapsulated PA-SLNs
2	74.70	68.80	55.98
4	66.78	60.03	50.59
6	60.37	54.13	41.82

IC50 value was found to be 4.07µg/mL for doxo encapsulated PA-SLNs

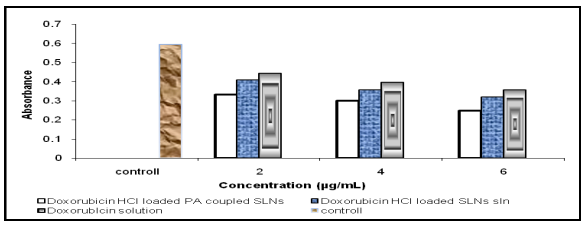

Figure 1: MTT cytotoxicity assay of doxo solution, doxo encapsulated SLNs, and PA- SLNs.

Cell uptake studies Cell uptake studies were performed on C6 glioma cells using FITC as a fluorescent marker in 1, 3 and 5h time interval. Cell uptake study shows that amount of the FITC-BSA was found to be entered into the C6 glioma cells from the formulations were 71.57% by FITC-BSA encapsulated PA-SLNs, 27.68% by FITC-BSA encapsulated SLNs and only 12.20% by FITC-BSA solution between the concentration range 2-6 µm/mL. Remarkable variations in the cytotoxicity response and cell uptake studies of the formulation in comparison to that of drug were observed. A reason can be the ligand receptor affinity (PA-SLNs). C6 glioma cells over express LAT1 receptor and phenylalanine having higher affinity to this receptor site so PA-SLNs shows higher cytotoxicity as well cell uptake. Also SLNs inhibit P-glycoprotein induced efflux pump which could also lead to decreased doxo efflux from the cell. Doxo being cationic is substrate of PGP efflux pumps. PA-SLNs provided better cytotoxicity response and increase cell uptake in C6 glioma cell line.

Table 2: cell uptake study of formulations.

Time in hrs	% Cell uptake		
	Plain FITC-BSA solution	FITC-BSA encapsulated SLNS	FITC-BSA encapsulated PA-SLNs
1	0.00	13.47	22.94
3	1.54	22.41	44.62
5	12.20	27.68	71.57

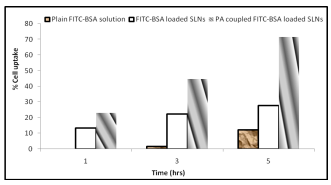

Figure 2: Cell uptake study of FITC-BSA solution, FITC-BSA encapsulated plain SLNs and PA coupled FITC-BSA encapsulated SLNs

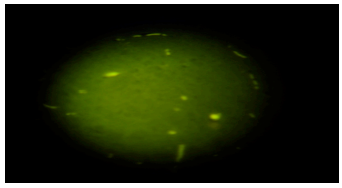

Figure 3 Photomicrograph of brain tissues using fluorescent microscope

CONCLUSIONS

Neither of the unencapsulated SLN formulations, obtained with different lipid matrices, are toxic to either of the cell-lines studied. (2) The cytotoxicity of PA-SLNs is consistently higher than that of the drug solutions as well as uncoupled SLNs on C6 glioma cell lines.

The higher sensitivity of the cells to the Doxo encapsulated PA-SLNs than to free Doxo in solution as well as uncoupled Doxo encapsulated SLNs may be related to the marked uptake and accumulation of Doxo encapsulated PA-SLNs in the cells, where the encapsulated SLNs should release the drugs, so enhancing their action. PA-SLNs may be proposed as alternative colloidal drug carriers for the brain delivery of chemotherapeutic agents.

REFERENCES

• Misra, A. et al. (2003) Drug delivery to the central nervous system: a review. J Pharm Pharmaceut Sci. 6(2):252-273.

• *Lin, R. et al. (2005)* In vitro study of anticancer drug doxorunicin in PLGA-based microparticles. *Biomaterials 26:4476–4485.*

• *Muller. et al. (1999),* Solid lipid Nanoparticles: Phagocytic uptake, in vitro cytotoxicity and in vitro biodegradation. *Pharm. Ind. 61, 564–569.*