CHAPTER 6:

*p*53 Mediated Chemo-sensitization of Anticancer Drugs

6.1 Introduction

NSCLC is a very aggressive type of lung cancer accounting for significant mortality. Surgery, combination chemotherapy and radiotherapy have been traditionally used in treatment of NSCLC. However, these therapies have shown limited success with cancer recurrence and resistance against chemotherapy which requires use of higher quantity of drug administration leading to side-effects and unwanted cytotoxicity. *p*53 based gene therapy have been reported to be successful in controlling the tumor cell growth by inducing the apoptosis in *p*53 deficient cancer cells, restoring their normal cell function and reducing the non-pump BCL2 mediated antiapoptotic cell resistance towards chemotherapy, hence enhancing the regularization of cell cycle events along with sensitizing the resistant cells to DNA-damaging agents (e.g. chemotherapy and radiotherapy) for enhanced chemotherapeutic action [K.F. Pirollo et al (1997), L. Xua, et al (2001), M. Saad et al (2008)].

Recent advances in non-viral gene delivery vectors have allowed efficient gene transfection into tumor cells *in vitro* and *in vivo* even in presence of chemotherapeutic agents. Delivery of genes i.e. *p*DNA, siRNA and AS ODN have been successfully attempted with nanoparticulate and liposomal vectors encapsulating the chemotherapeutic agent, thus demonstrating co-administration of drug and gene [M. Saad et al (2008); Y. Wang et al (2006); F. Liu et al (2004); L. Xua et al (2001); R.I. Pakunlu et al (2004)]

Co-administration of drug and gene in the same vehicle not only can improve patient compliance due to the reduced number of injections, but can also achieve a synergistic therapeutic effect because both drug and gene can be delivered to the same cancer cells or tissues. These vectors have shown dual advantage of cytotoxic behavior of drug along with expression of gene for producing or blocking the desired protein for improving the cellular entry of chemotherapeutic agent and its anticancer activity though multiple mechanisms of action, thus helping to overcome the resistance.

In this section, we have investigated the liposomal system for delivering anticancer agents as Etoposide (ETP) and Docetaxel (DTX) as cytotoxic agents along with *p*53 for lung cancer treatment *in vitro*. We have attempted to develop a multicomponent gene delivery system containing 1) Blank cationic liposomes without drug, 2) Etoposide or Docetaxel encapsulated in above liposomes (same composition), 3) *p*53 lipoplex and 4) *p*53 complexed with ETP and DTX encapsulating liposomes to form ETP-*p*53 and DTX-*p*53 lipoplex. These developed systems

were investigated for studying the effect of pretreatment and co-administration of *p*53 on cytotoxicity of liposomally encapsulated ETP and DTX in *p*53 null H 1299 and *p*53 (wt) A-549 lung adenocarcinoma cell lines. These studied were based on the hypothesis that, *p*53 restoration in *p*53 deficient / mutated cancer cells would ameliorate the altered apoptotic pathway and also reduce the BCL-2 mediated antiapoptotic non pump resistance thus sensitizing the cells towards chemotherapeutic agent [Yu-ling Wu et al (2001)]. The effect of pre-sensitization and co-administration on comparative cytotoxicity in two different cell lines with varying *p*53 character was determined to demonstrate effect of time of *p*53 delivery and added advantage offered by multicomponent gene delivery system. The studies were also aimed to develop DPI formulations of these lipoplexes for studying the lung deposition pattern for direct lung delivery and targeting, thus enhancing the therapeutic efficiency, reducing unwanted side effects and lowering the dose of the drug.

6.2 Development, Optimization and Characterization of Etoposide and Docetaxel Liposomes

6.2.1 Materials: Etoposide (ETP) and Docetaxel trihydrate (DTX) were obtained as generous gift from Intas Pharmaceuticals Ltd., Ahmedabad, India. The lipids 1,2-dioleoyl-3-trimethylammonium– propane (DOTAP), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were obtained as gift sample from Genzyme Pharmaceuticals, Switzerland and Lipoid GmbH, Germany. Cholesterol was purchased from Sigma Aldrich, Mumbai, India. 5 ([4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid] (HEPES free acid), was purchased from HiMedia, Mumbai, India. All other chemicals used were of analytical reagent grade and were confirmed for purity before use.

6.2.2 Methods: Development of Liposomes by Thin Film Hydration (TFH):

ETP and DTX containing liposomes were prepared by TFH method. The drug and lipids as DOTAP, DOPE, DPPC and cholesterol were mixed in the various molar ratios and dissolved in chloroform : methanol (varying ratios) in round bottom flask. The solvent mixture was allowed to evaporate under vacuum with rotation to form dry thin film and was kept overnight in desiccator to remove residual solvent if any. The prepared dry thin film was then hydrated using aqueous hydration medium (Water, Phosphate buffer saline pH 6.8, HEPES buffer pH 8.0). The drug containing liposomes were optimized by varying process parameters as vacuum applied, rotation of flask, hydration temperature and formulation parameters as drug : lipid molar ratio,

lipid composition, lipid : cholesterol molar ratio, selection of hydration medium and volume of hydration medium used. The liposomes were size reduced by extrusion through 0.45 and 0.22 micron polycarbonate filter papers and lyophilized [Heto Dry Winner] using suitable cryoprotectants (sucrose, lactose, trehalose) with varying lipid : sugar ratio for stabilizing the formulation. Higher entrapment and more significantly, higher drug loading was achieved by varying lipid composition, drug : lipid ratio, lipid : cholesterol ratio and volume of hydration medium.

6.2.3 Characterization of Liposomes

6.2.3.1 Particle Size

The particle size (Z average) and poly-dispersity index (PDI) of the diluted extruded liposomes was analyzed by photon correlation spectroscopy technique (PCS) using a Malvern Zetasizer Nano ZS equipment (Malvern Instruments; UK).

6.2.3.2 Zeta potential analysis

The zeta potential of various undiluted liposomal suspensions was measured by micro electrophoresis using Malvern Zetasizer Nano ZS (Malvern Instruments, UK). The instrument works on the principal of Brownian motion and measured the light by Phase Analysis Light Scattering (PALS). Zeta potential of the undiluted liposomes was measured to achieve highest sensitivity, accuracy and resolution of zeta potential.

6.2.3.3 CryoTEM studies

Morphology, lamellarity and size of the liposomes were studied using Cryo-TEM (TECNAI G2 Spirit BioTWIN, FEI – Netherlands) operating at 200 kV with resolution of 0.27 nm and magnifications of the order of 750,000X.

6.2.3.4 Entrapment Efficiency (%) and Drug Loading (%)

Percentage drug entrapped in the liposomes was determined by UV-Visible spectrophotometer [UV- 1701 – Shimadzu]. Liposomal suspension (100 µl) containing ETP or DTX was dissolved in methanol by bath sonicating and warming. Drug content was analyzed and calculated by UV-Visible Spectrophotometer against methanol with plain liposomes as a blank at 289 nm for ETP and 229 nm for DTX. The entrapment efficiency and % drug loading of ETP and DTX in liposomes was calculated using the formula:

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Quantity of drug encapsulated}}{\text{Total quantity of drug added}} \times 100$$

$$\text{Drug Loading (\%)} = \frac{\text{Quantity of drug encapsulated}}{\text{Total quantity of lipids added}} \times 100$$

6.2.3.5 *In Vitro* Drug Release

The lyophilized drug loaded liposomes were dispersed in 1ml of phosphate buffer saline [PBS buffer] (pH 7.4) with 1 % tween 80 at drug concentration of about 1 mg/mL, and were placed in a dialysis membrane tube with molecular weight cut of 12 kda. The tube was then immersed in a beaker containing 50 mL of phosphate buffer saline (PBS) buffer (pH 7.4) containing 1 % tween 80 as a release medium for ETP and DTX liposomes, and was shaken at a speed of 50 rpm, and incubated at 37^0C. At specific time intervals, 1 ml of solution was withdrawn from the release medium and replaced with fresh release medium. The drug content in the samples was analyzed by diluting the samples with methanol and analyzing the drug using spectrophotometer. The release studies were also performed at pH 5 to simulate acidic tumor and intracellular lysosomal environment. The *in vitro* release experiment was conducted in 3 parallel batches for all the formulations and the variation between batches was represented with the error bar.

6.2.4 Results and Discussion

Liposomes encapsulating ETP and DTX were developed by TFH method. The developed liposomes were optimized on the basis of physical observation, size and zeta potential, drug encapsulation, drug loading and drug holding and leakage tendency on standing. In both drugs, the DOTAP, DOPE, DPPC and cholesterol were used as formulation components. DPPC was used as a principle lipid because of its prevalence in lung lipid composition and its compatibility during transfection. DOTAP and DOPE were used for its transfection ability of pDNA. However, because of their lower Tg, DOTAP and DOPE alone were unable to hold the drug inside liposomes. Cholesterol was used to impart stability to the liposomes and also improving the DNA transfection because of its fusogenic properties during cell line studies. [X. Gao et al (1991)]. In general, good multi-lamellar liposomes with little lipid precipitation was observed at higher rotation of flask (120 – 150 RPM) during solvent evaporation leading to thinner film followed by slower rotation (50-75 RPM) during hydration leading to enhanced contact between lipids and hydration medium. Further, use of methanol in combination with chloroform (in the ratio 1:1) during lipid film formation lead to better and thinner film as compared with film formed from chloroform alone. The slower evaporation of methanol under vacuum enhanced the uniformity and smoothness of the lipid film, which resulted in better liposome formation during

hydration. Further, HEPES buffer pH 8.0 formed comparatively better liposomes than with plain distilled water (pH 5-6) and ionic PBS pH 6.8. The better hydration with HEPES buffer pH 8.0 may be attributed to its non ionic nature theirby avoiding the interaction with charged lipids as DOTAP and DPPC and the alkaline pH favoring better hydration of DOPE [N. J. Zuidam et al (1997)]. Further, the liposomes without lipid precipitation were observed only after removal of all traces of organic solvents from the lipid layer. This required use of absolute vacuum for complete removal of solvents. Further, complete hydration of lipids was observed at 45 ^{0} C, which is above the glass transition temperature of all lipids used in the system. Liposomal suspension formed after hydration with HEPES buffer; pH 8.0 was downsized by extrusion through 0.45 and 0.22 μm polycarbonate membranes (3 cycles each) and was used for measurement of entrapment efficiency and % drug loading.

The effect of formulation parameters was studied on ETP and DTX liposome formulation with objective of highest drug loading and entrapment efficiency. In both the drugs, with increased drug : lipid ratio, higher entrapment was observed. Comparatively, higher entrapment was observed with HEPES buffer pH 8.0 than with water and PBS because of lipid precipitation and instability of liposomes in ionic buffer. Further, volume of HEPES buffer also affected the liposome formation and % entrapment. ETP and DTX liposomes showed maximum entrapment at hydration volume of 1 ml per 20 mg of lipid for ETP and 30 mg of lipid for DTX. Lower hydration volume resulted in lipid precipitation, whereas higher hydration volume resulted in drug leakage during hydration and standing of liposomes. The lipid : cholesterol ratio and lipid composition were also optimized for highest drug entrapment. Increased DOTAP and DOPE content resulted in drug leakage from the liposomes during hydration as well as on standing. Increased cholesterol helped to impart stability to liposomes but increasing the lipid : cholesterol ratio beyond 8:2 lead to lipid precipitation with reduced entrapment during hydration. The optimized liposomes were formed at lipid composition of DOTAP, DOPE, DPPC and cholesterol in the ratio of 0.5:0.5:7:2 and drug : lipid ratio of 1:10 for ETP and 1:20 for DTX. Further, the size and entrapment efficiency of liposomes was varied with extrusion cycles. With increase in no. of extrusion cycles through 0.45 and 0.22 μm polycarbonate filter papers, size of the liposomes was reduced with increase in size uniformity and lower polydispersity index. However, the entrapment of ETP and DTX was reduced after every extrusion cycle. Finally 3

extrusion cycles through 0.45 and 0.22 µm polycarbonate filter paper were optimized to yield ETP and DTX liposomes with particle size 212.7 ± 4.2 nm and 227.9 ± 3.1 nm respectively.

Table 6.1 Optimization Studies for Etoposide Liposomes

Batch No	Drug : Lipid Ratio	Lipid Composition (DOTAP : DOPE : DPPC : Cholesterol)	Hydration Medium and Volume	% Entrapment (Mean ± SEM) (n=3)	Remarks
E1	1:5	0.5:0.5:7:2	0. 5 ml water / 20 mg lipid	15.23 ± 6.45	Lipid Precipitation
E2	1:5	0.5:0.5:7:2	1 ml water / 20 mg lipid	36.47 ± 4.12	Partial Lipid Precipitation
E3	1: 10	0.5:0.5:7:2	1.5 ml water / 20 mg lipid	38.23 ± 3.23	Partial Lipid Precipitation (No improvement)
E4	1:10	0.5:0.5:7:2	1.5 ml PBS pH 6.8 / 20 mg lipid	42.56 ± 2.98	Liposome instability and precipitation on standing
E5	1:10	0.5:0.5:7:2	1 ml HEPES buffer pH 8.0 / 20 mg lipid	72.36 ± 1.34	Very stable liposomes with low drug leakage
E6	1: 10	0.5:0.5:8:1	1 ml HEPES buffer pH 8.0 / 20 mg lipid	78.12 ± 4.34	Higher entrapment than E 5 but drug leakage on standing observed
E7	1: 10	0.75:0.75:7.5:1	1 ml HEPES buffer pH 8.0 / 20 mg lipid	63.62 ± 3.40	Higher drug leakage during hydration because of lower Tg of lipid system with enhanced DOTAP and DOPE ratio.
E8	1: 10	0.5:0.5:6:3	1 ml HEPES buffer pH 8.0 / 20 mg lipid	59.26 ± 3.13	Partial lipid precipitation with increase in cholesterol content.
E9	1:10	0.5:0.5:7:2	1.5 ml HEPES buffer pH 8.0 / 20 mg lipid	67.36 ± 1.34	Higher drug leakage with increased hydration volume.

Table 6.2 Optimization Studies for Docetaxel Liposomes: (n=3)

Batch No	Drug : Lipid Ratio	Lipid Composition (DOTAP : DOPE : DPPC : Cholesterol)	Hydration Medium and Volume	% Entrapment (Mean ± SEM) (n=3)	Remarks
D1	1:10	0.5:0.5:7:2	1 ml HEPES buffer pH 8.0 / 20 mg lipid	65.23 ± 6.45	Lipid precipitation during hydration
D2	1:15	0.5:0.5:7:2	1 ml HEPES buffer pH 8.0 / 20 mg lipid	75.23 ± 6.45	Drug leakage and lipid precipitation on standing
D3	1:15	0.5:0.5:7:2	1 ml HEPES buffer pH 8.0 / 30 mg lipid	69.54 ± 2.87 %	Stable liposomes with lower drug leakage
D4	1: 15	0.75:0.75:7:1.5	1 ml HEPES buffer pH 8.0 / 30 mg lipid	62.12 ± 1.53	Lower entrapment with more drug leakage on increasing DOTAP and DOPE content.
D5	1:20	0.5:0.5:7:2	1 ml HEPES buffer pH 8.0 / 30 mg lipid	72.54 ± 1.99	Stable liposomes with lower drug leakage but lower drug loading than D3
D6	1:15	0.5:0.5:7:2	1.5 ml HEPES buffer pH 8.0 / 30 mg lipid	67.36 ± 1.34	Higher drug leakage with increased hydration volume.

These size reduced liposomes showed entrapment of 72.36 ± 1.34 % for ETP and 69.54 ± 2.87 % for DTX and drug loading of 6.073± 0.125 % for ETP and 4.34 ± 0.19 % for DTX. The summary of the optimization study for ETP and DTX liposomes has been tabulated in **Table 6.1 and 6.2.**

The developed liposomes showed positive zeta potential because of presence of cationic lipid DOTAP. The optimized liposomal ETP and DTX formulations showed zeta potential of 24.6 ± 1.3 and 23 ± 2.1mV respectively. The particle size of drug containing liposomes was comparatively higher and the zeta potential was comparatively lower than the blank liposomes

(with similar lipid composition, without drug). The order was observed as DTX liposomes > ETP liposomes > Blank liposomes for particle size and the opposite order was observed for zeta potential. The formulated liposomes when observed for cryoTEM showed unilamellar liposomes in the range of 150-200 nm (**Figure 6.1**) supporting the data provided by Malvern particle size analyzer. The bilayer structure of liposomes was clearly visible in case of both the drug formulations.

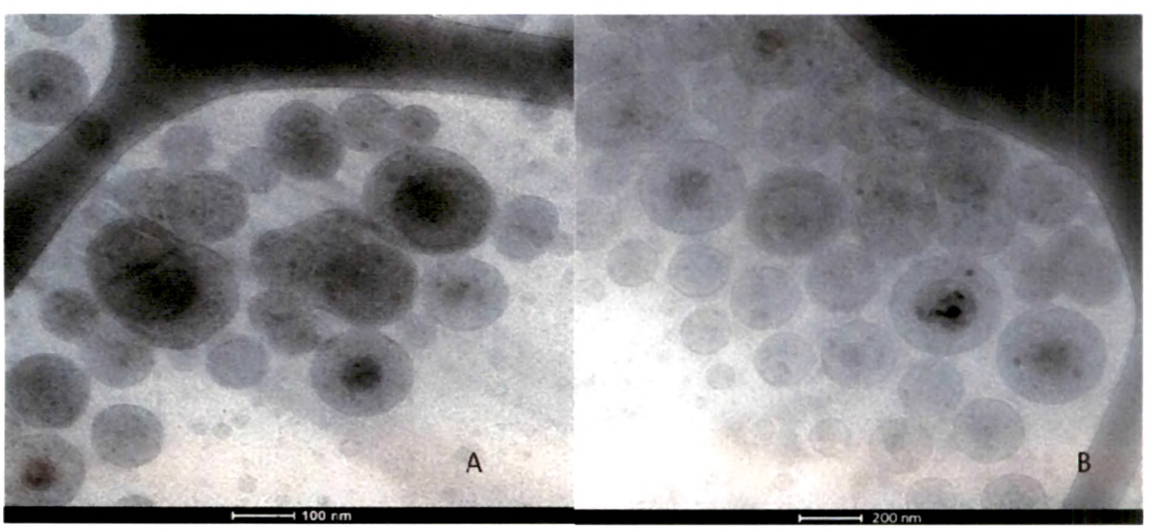

Figure 6.1 CryoTEM studies of Etoposide and Docetaxel Liposomes showing unilamellar liposomes. (A – Etoposide, B – Docetaxel)

The optimized liposomes were lyophilized for enhanced stability of the formulations and DPI development for direct lung deposition. The lyophillization was optimized to achieve same physical properties of the liposomes when they come in contact with aqueous medium to release nanosized liposomes. Of the three cryoprotectants, trehalose showed best compatibility with the lipids and showed comparatively non-significant change in particle size and entrapment efficiency of drugs when compared with sucrose and lactose as other cryoprotectants. The particle size and entrapment efficiency of ETP and DTX liposomes before and after lyophillization has been tabulated in Table 6.3 and 6.4.

Table 6.3 Lyophillization of ETP liposomes by cryoprotectants: (n=3) (Mean± SEM)

Liposomes + Cryoprotectant used	Lipid : Cryoprotectant Ratio (w/w)	Particle Size after resuspension in HEPES buffer pH 8.0	% Drug Retention after resuspension in HEPES buffer pH 8.0
ETP Liposomes + Lactose Monohydrate	1:1	839.4 ± 31.3 nm	57.4 ± 3.9
	1:3	418.7 ± 36.1 nm	69.9 ± 4.1
	1:5	334.5 ± 19.3 nm	88.9 ± 3.1
ETP Liposomes + Sucrose	1:1	591.2 ± 29.3 nm	74 ± 2.1
	1:3	438.2 ± 30.9 nm	81.1 ± 4.7
	1:5	301.5 ± 11.3 nm	91.9 ± 2.6
ETP Liposomes + Trehalose	1:1	516.2 ± 19.1 nm	88.1 ± 3.1
	1:3	238.2 ± 11.9 nm	97.6 ± 2.7
	1:5	233.2 ± 8.9 nm	98.9 ± 1.6

Table 6.4 Lyophillization of DTX liposomes by cryoprotectants: (n=3) (Mean± SEM)

Liposomes + Cryoprotectant used	Lipid : Cryoprotectant Ratio (w/w)	Particle Size after resuspension in HEPES buffer pH 8.0	% Drug Retention after resuspension in HEPES buffer pH 8.0
DTX Liposomes + Lactose Monohydrate	1:1	1222.4 ± 128.3 nm	39.4 ± 7.1
	1:3	916.7 ± 66.1 nm	52.9 ± 3.8
	1:5	538.5 ± 21.8 nm	68.1 ± 3.2
DTX Liposomes + Sucrose	1:1	613.2 ± 67.2 nm	61.9 ± 2.3
	1:3	498.1 ± 41.9 nm	83.1 ± 5.1
	1:5	291.5 ± 14.8 nm	93.9 ± 1.6
DTX Liposomes + Trehalose	1:1	606.2 ± 21.1 nm	71.1 ± 2.9
	1:3	241.2 ± 14.1 nm	99.2 ± 2.1
	1:5	245.2 ± 10.9 nm	98.9 ± 1.1

After lyophillization at different lipid : cryoprotectant ratios, particle size of the liposomes in the cake was determined after resuspension in 2 ml of HEPES buffer followed by vortexing and bath sonicating for 10 seconds to break the lumps. Further, % drug retained in the cake after resuspension was determined after extruding the suspended cake through 0. 45 and 0.22 µm polycarbonate filter paper (1 cycle) followed by dissolving the suspension in methanol and determining the % drug retained in liposomes considering entrapment before lyophillization as 100 %. At lipid : trehalose 1: 3 ratio, non-significant change in particle size and near 100 % drug

retention was observed in case of both ETP and DTX liposomes. No further improvement was observed on increasing the ratio of trehalose hence, lipid : trehalose ratio of 1:3 was optimized and used for all batches.

In vitro drug release study of ETP and DTX liposomes was performed in pH 7.4 PBS buffer and pH 5.4 acetate buffer and the results are tabulated in table 6.5. The drug loaded liposomes showed sustained release of drugs up to 24 hrs in pH 7.4 release media. ETP loaded liposomes showed 97.23 ± 1.67% drug release after 24 hrs and DTX loaded liposomes showed 99.32 ± 1.32% drug release after 24 hrs. Comparatively, faster release of DTX from the liposomes may be attributed to its higher molecular weight which might have caused the faster drug leakage and release from the vesicle membrane. Previously, liposomes demonstrating drug release up to 72

Table 6.5 Release pattern of ETP and DTX Liposomes in PBS pH 7.4 and Acetate Buffer pH 5.4 release media:

Time in Hrs	% Drug Release from Liposomes [mean ± SEM (n=3)]			
	ETP Liposomes pH 7.4	ETP Liposomes pH 5.4	DTX Liposomes pH 7.4	DTX Liposomes pH 5.4
1	20.18 ± 4.19	21.42 ± 3.11	26.19 ± 3.7	29.9 ± 3.56
2	28.18 ± 3.02	35.16 ± 2.96	37.1 ± 2.91	41.38 ± 3.11
4	37.12 ± 3.97	51.44 ± 4.09	51.06 ± 3.67	62.68 ± 4.02
8	53.62 ± 2.66	70.53 ± 3.42	62.09 ± 3.13	81.95 ± 3.18
12	70.47 ± 3.18	85.18 ± 3.65	74.12 ± 3.54	99.69 ± 3.04
16	84.39 ± 2.05	99.28 ± 2.98	86.89 ± 2.89	99.62 ± 2.24
20	96.71 ± 1.78		99.63 ± 1.07	
24	97.23 ± 1.67		99.32 ± 1.32	

hrs have been reported. But, the liposomes in the current investigation tend to show faster and complete drug release probably because of presence of DOTAP and DOPE having lower glass transition temperature causing faster rupture of the vesicles. However, when release is carried out in acidic conditions stimulating tumor environment, release was found to be comparatively faster with complete drug release in 16 hrs. ETP loaded liposomes showed 99.28 ± 2.98 % drug release after 16 hrs and DTX loaded liposomes showed 99.62 ± 2.24 % drug release after 16 hrs. The faster release in acidic pH may be attributed to membrane disruption and lipid fusion

property of DOPE at acidic pH which has also been previously observed [M.S. Hong et al (2002)].

6.3 Development, Optimization and Characterization of Etoposide and Docetaxel Containing Lipoplexes:

Optimized ETP and DTX liposomes were further used for development of lipoplexes with *β galactosidase* and *p53* *p*DNA by the method as described previously. The lipoplexes were prepared in HEPES buffer 8.0 as well as DMEM medium without serum. For transfection and other cell line studies, freshly prepared lipoplexes in DMEM were used, where as for release studies and DPI development, lipoplexes in HEPES buffer were used.

The lipoplexes were prepared at different N / P ratios (From 1/1 to 40/1) calculating molar quantity of DOTAP and *p*DNA as charged moieties. For preparing the lipoplexes, unless mentioned, quantity of *p*DNA was kept constant at 300 nm and the quantity of liposomes was varied accordingly. The developed lipoplexes were studied for size and size distribution by Malvern particle sizer Nano ZS, zeta potential, % drug released, agarose gel retardation on 1 % ethidium bromide pre-stained gel and DNAse I protection assay by the protocols described previously. It is expected that, all the lipoplexes with *p53* and *β* galactosidase would behave similarly at same N/P ratios with similar size, zeta potential, drug release, agarose gel retardation assay, DNAse I treatment assay, cellular uptake and transfection efficiency.

6.3.1 Size, Zeta Potential and % Drug Release of ETP and DTX Lipoplexes:

All the developed lipoplexes showed higher size and reduced zeta potential as compared with blank liposomes because of complexation of anionic DNA and cationic liposomes. The size and zeta potential of various liposomes and lipoplexes at various N/ P ratios are recorded in **table 6.6** and is diagrammatically shown in **Figure 6.2.**

The size of drug loaded lipoplexes was higher than the respective blank lipoplexes and the zeta potential was reduced when compared with blank lipoplexes. In all cases, at higher N/P ratio the lipoplex size was comparatively lower as compared with N/P ratio near neutrality i.e. N / P ratio of 1/1 with comparatively higher zeta potential. All the lipoplexes showed size range near to 200-500 nm range and a cationic zeta potential of 10-35 mV. Such smaller size and cationic charge of the lipoplexes are desirable properties for easy cellular uptake for efficient gene / drug delivery.

Table 6.6 - Size and Zeta Potential of Blank, ETP and DTX loaded lipoplexes (Mean ± SEM , n=3)

N:P ratio	Blank Lipoplex		ETP Lipoplex		DTX Lipoplex	
	Size	Zeta	Size	Zeta	Size	Zeta
1 : 0	167.3 ± 4.9	34.9 ± 2.0	212.7 ± 4.2	24.6 ± 1.3	227.9 ± 3.1	23 ± 2.1
1: 1	325.6 ±16.6	19.2 ± 3.9	425.8 ±23.3	14 ± 4.1	468.1 ±21.9	15.2 ± 3.1
2: 1	282.1 ± 5.7	23.4 ± 2.1	389.5 ±16.4	22.3 ± 3.2	481.6 ±20.8	21.4 ± 2.9
3: 1	279.3 ± 7.1	26.8 ± 3.1	394.6 ±14.9	26.4 ± 2.9	422.2 ±17.1	24 ± 3.1
4: 1	258.4 ±10.3	25.9 ± 2.2	375.7 ±16.0	25.1 ± 3.1	407.2 ±22.0	23.9 ± 2.9
5: 1	261.3 ± 9.1	27 ± 1.9	349.1 ±16.8	24.8 ± 1.1	390.2 ±17.4	25.6 ± 3.0
10: 1	229.1 ± 5.1	29.8 ± 1.1	298.9 ±13.6	29 ± 3.5	320.8 ±12.9	29.1 ± 4.5
15: 1	228 ± 10.4	28.9 ± 3.3	271.2 ±14.8	28 ± 1.9	309.1 ±20.6	31 ± 3.1
20: 1	238.9 ±11.0	30.1 ± 3.2	284.7 ±11.9	31.1 ± 3.4	315.8 ±17.0	28.1 ± 2.9
25: 1	223.6 ± 8.9	28.1 ± 2.8	274.5 ±10.3	28.9 ± 3.2	329.4 ±14.7	31.3 ± 2.1
30: 1	214 ± 7.6	29.6 ± 2.8	275 ± 13.1	29.9 ± 2.9	310.4 ±18.0	28.9 ± 3.1
35: 1	220.5 ±10.1	30.2 ± 3.9	281.3 ±12.9	30 ± 1.6	300.3 ±11.9	30.5 ±1.9
40: 1	219.6 ± 5.2	29.7 ± 2.1	270.4 ± 9.9	32.1 ± 3.1	310.5 ±13.0	32 ± 3.2

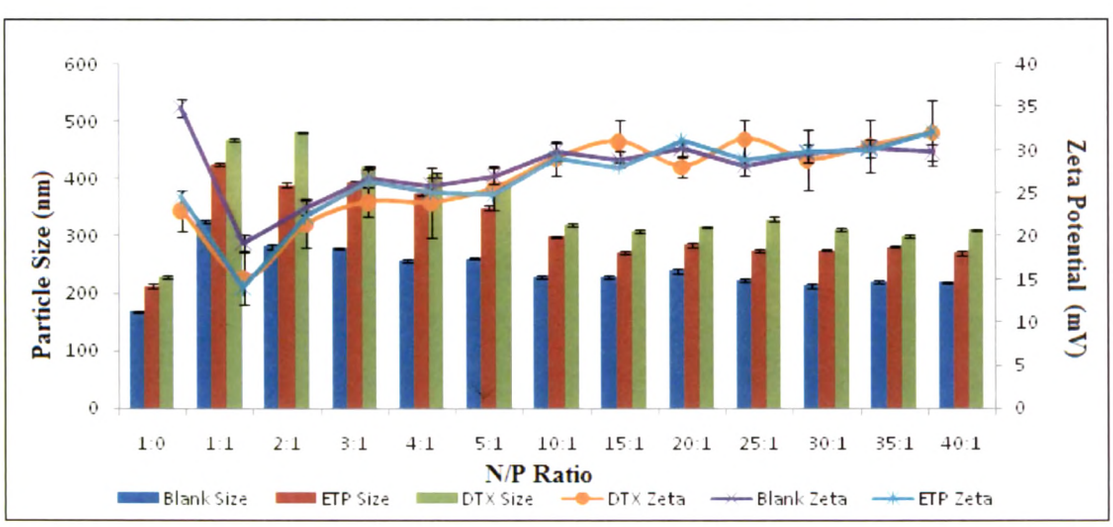

Figure 6.2 - Size and zeta potential of Blank, ETP and DTX loaded lipoplexes (n=3)

In vitro drug release study of ETP and DTX lipoplexes in pH 7.4 PBS buffer and pH 5.4 acetate buffer was carried out and comparative results between liposome and lipoplex release are shown in **Table 6.7** and **Figure 6.3**.

Table 6.7 Release pattern of ETP and DTX Lipoplexes in PBS pH 7.4 and Acetate Buffer pH 5.4 release media (Mean ± SEM , n=3):

Time in Hrs	ETP Lipoplexes – pH 7.4	ETP Lipoplexes – pH 5.4	DTX Lipoplex – pH 7.4	DTX Lipoplex – pH 5.4
1	8.78 ± 2.11	18.56 ± 2.06	18.31 ± 2.18	24.57 ± 3.74
2	19.39 ± 2.9	31.72 ± 1.96	31.41 ± 2.79	39.48 ± 2.89
4	30.51 ± 1.89	47.33 ± 3.67	43.5 ± 1.96	57.91 ± 2.57
8	45.9 ± 2.19	67.21 ± 3.81	53.93 ± 2.85	76.78 ± 3.01
12	64.87 ± 3.54	84.42 ± 3.32	66.78 ± 1.49	92.44 ± 2.62
16	73.12 ± 2.96	98.69 ± 2.78	79.1 ± 2.18	99.4 ± 2.51
20	81.05 ± 3.14		91.13 ± 2.04	
24	89.35 ± 3.12		96.98 ± 2.17	

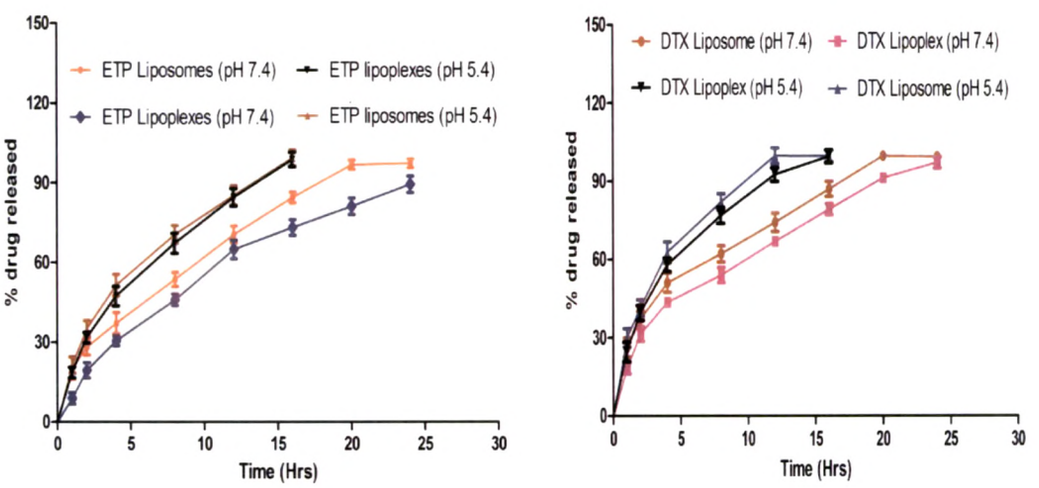

Figure 6.3: Comparative % drug release of A) ETP liposomes and lipoplexes and B) DTX liposomes and lipoplexes in pH 7.4 PBS buffer and pH 5.4 Acetate buffer (n=3)

The drug loaded lipoplexes showed comparatively more sustained release of drugs than respective liposomes. Presence of tightly condensed plasmid *p*DNA in the lipoplexes slowed the drug release by 5-10 % at all-time points when compared with corresponding liposomes,

probably because of tightly condensed system. ETP loaded lipoplexes showed 89.35 ± 3.12 % drug release after 24 hrs and DTX loaded lipoplexes showed 96.98 ± 2.17 % drug release after 24 hrs. In acidic lysosomal and tumor simulating conditions of acetate buffer, drug release was found to be comparatively faster with complete drug release in 16 hrs. ETP loaded lipoplexes showed 98.69 ± 2.78 % drug release after 16 hrs and DTX loaded lipoplexes showed 99.40 ± 2.51 % drug release after 16 hrs.

6.3.2 Agarose gel retardation assay and DNAseI protection study

6.3.2.1 Method

The developed lipoplex formulations (blank lipoplexes without drug) with varying N: P ratios ranging from 1: 2 to 10:1 were used for agarose gel retardation assay. After mixing with gel loading dye, the DNA alone and the complexes were run on 1 % agarose gel containing ethidium bromide and were visualized under UV trans-illuminator for gel retardation of the DNA. Further, the integrity of these lipoplexes and its ability to protect complexed pDNA against DNAse I enzyme was studied by treatment of naked DNA and complexed pDNA with DNAse I enzyme by the protocol described previously.

6.3.2.2 Results and Discussion

The gel retardation assay as demonstrated in **Figure 6.4** showed capability of developed lipoplexes to effectively condensate and protect *p*DNA. The liposomes were able to bind with the DNA efficiently because of opposite charge and electrostatic attraction resulting in retardation of DNA on agarose gel. With increasing the liposomal concentration, more retardation of the DNA was observed. Complete gel retardation of the *p*DNA mobility as observed in Lane 5 **(Figure 6.4 A)** indicated complete complexation of *p*DNA and liposomes at the N/P ratio of 2:1. At all further combinations with increasing N/P ratio, complete *p*DNA retardation was observed. Further, complete protection of the DNA against extracellular DNAse offered by the complexation with liposomes was confirmed by treatment of DNAse-I **(Figure 6.4B).** The naked DNA on treatment with DNAse I showed complete degradation (Lane 2). However, the DNA after complexation with liposomes showed no degradation as observed in lane 4 **(Figure 6.4 B).** The results displayed development of a stable lipoplex system which has a capability of intracellularly delivering the stable non-degraded *p*DNA against extracellular DNAse in cellular environment. The ETP and DTX loaded lipoplexes showed exactly the same pattern of DNA condensation and DNAseI protection.

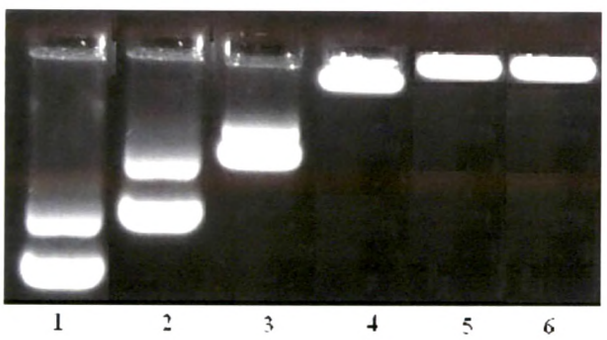

Figure 6.4 A

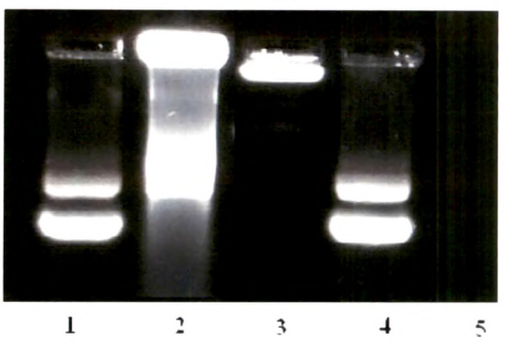

Figure 6.4 B

Figure 6.4: Agarose gel retardation of Plasmid alone and after complexation with liposomes at different N / P ratios [Figure 6.4 A - Lane 1: Plasmid alone, Lane 2 – Lipoplex (N / P – 1/2), Lane 3 - Lipoplex (N / P – 1/1), Lane 4 - Lipoplex (N / P - 1.5 / 1) , Lane 5 : Lipoplex (N / P – 2 / 1), Lane 6 : Lipoplex (N / P – 3 / 1)] and stability of DNA after formation of lipoplex against DNAse-I [Fig 6.4 B – Lane 1 : Plasmid Alone, Lane 2: Degraded Plasmid after treatment with DNAse I, Lane 3: Lipoplex at N / P ratio 2/1 , Lane 4 : Stable DNA after treatment of lipoplex with DNAse I followed by DNA purification]

6.4 Cell Uptake Studies

6.4.1 Materials

*p*53 and *β-galactosidase* *p*DNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and used. A 549 cell line was obtained from cell repository, National Centre for Cell Sciences, Pune, India and *p*53 null Human lung adenocarcinoma H 1299 cell line was obtained from Dr. Samit Chattopadhyay, Scientist, National Centre for Cell Sciences, Pune, India. Both A 549 and H 1299 cells were grown in high glucose DMEM medium (HiMedia, Mumbai, India) supplemented with 10 % fetal bovine serum (HiMedia, Mumbai, India) and 2mM L-glutamine, $100Uml^{-1}$ Penicillin, 100 μgml^{-1} Streptomycin and 50 μgml^{-1} of Amphotericin B. The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. All experiments were performed on cells in the exponential growth phase in between the passage no. 91-101 for H 1299 and passage no. 25-35 for A 549 cells. All other chemicals were used as discussed earlier and were confirmed for purity before use.

6.4.2 Development of 6 – Coumarin Liposomes and Lipoplexes

The fluorescent liposomes for estimating cell uptake was formulated by TFH technique for encapsulating 6-coumarin instead of ETP or DTX. The 6-Coumarin liposomes were formulated by replacing ETP with 6 coumarin and keeping the lipid composition, hydration medium, volume and extrusion cycles the same. The liposomes showed particle size of 230.6 nm and zeta potential of 26 mv. Further, the 6-coumarin loaded lipoplexes were prepared by complexing *β-gal p*DNA with the liposomes at N/P ratios of 2:1 which showed complete DNA complexation, agarose gel retardation and DNAse I protection. The lipoplex showed size of 409.9 nm and zeta potential of 19 mv. These liposomes and lipoplexes were used for cell uptake studies in A 549 and H 1299 cell line.

6.4.3 Qualitative and Quantitative Cell Uptake of 6 – Coumarin Liposomes and Lipoplexes:

Cell uptake of liposomes and lipoplexes were studied qualitatively and quantitatively and in H-1299 and A-549 cell lines using 6 Coumarin encapsulated liposomes with similar lipid composition and DNA content (N/P ratio of 2:1). Quantitative cell uptake was studied with a flow cytometer (BD FACS Caliber). Cells were first seeded on 6 well plates at a seeding density of 1 X 10^6 cells /well and grown overnight in DMEM growth medium supplemented with 10 % FBS and 1% Penicillin–Streptomycin- Amphotericin B solution. On next day, cells were treated with free 6-Coumarin suspension, 6- Coumarin liposomes and 6-Coumarin lipoplexes prepared in complete medium at a concentration of 0.5 mg / ml. After 3 hrs of incubation, cells were washed with sterile PBS (pH 7.4) for 5 times followed by trypsinization and centrifugation at 1400 RPM, 4°C, 5 min to harvest them as pellet. The cell pellet after resuspending in 250 µl PBS was analyzed for fluoroscence positive cells to estimate lipoplex uptake by cells by FACS Calibur (BD Biosciences). The fluorescent intensity measurement was referenced to that given by the control cells without any coumarin and liposome treatment to eliminate the possibility of auto-fluorescence effect given by the cells. The measurements were done with excitation and emission wavelengths of 430 nm and 485 nm respectively [K.Y. Win et al (2005)].

As a qualitative comparison, an independent set of experiments was performed by fluoroscence imaging of the cells treated under the same conditions as above. Briefly, the cells were seeded in 6 well plate on a sterile cover slip at a seeding density of 0.5 X 10^6 cells per well and grown overnight. After 24 hrs, the cells were incubated with free coumarin suspension, coumarin loaded liposomes and coumarin loaded lipoplexes at a concentration of 0.25 mg / ml in complete

medium. After 3 h of incubation, the cells were washed thoroughly with PBS, permeabilized with 0.01 % Triton X 100 in PBS pH 7.4, stained with nucleus specific 4, 6-diamidoino-2-phenylindole (DAPI) dye and imaged under a fluorescent microscope (Nikon) with excitation and emission wavelengths of 350 and 470 nm respectively for DAPI. Coumarin uptake was analyzed at excitation and emission wavelengths of 430 nm and 485 nm respectively. The fusion image by mixing the 6 Coumarin and DAPI fluoroscence was created with the help of cell quest software and was compared for qualitative uptake.

6.4.4. Results and Discussion

6-Coumarin, a hydrophobic fluorescent marker molecule has similar physical properties as Etoposide and Docetaxel and was internalized inside the liposomes by thin film hydration method with similar lipid composition and process parameters. This allows for the investigation of coumarin internalization inside the cells using fluoroscence microscopy and flow cytometry when treated as suspension and as encapsulated in liposomes. **Figure 6.5 (a) and (b)** shows quantitative analysis of cellular uptake of coumarin performed in H 1299 and A 549 cell lines using a flow cytometer. As shown in figure, compared to control, there is a shift in histogram with increased intensity indicating fluoroscence emission from the cells. The histogram indicated about 25- 30 % increment in number of cells internalizing the coumarin after it is encapsulated in liposomes. The order of number of cells emitting the coumarin signal was 6-coumarin liposomes > 6-coumarin lipoplexes > 6-coumarin suspension indicating that, cells internalized more the quantity of coumarin in encapsulated form when delivered as liposomes compared to coumarin suspension. Higher cationic charge and lower size of liposomes attributes slightly higher uptake of liposomes as compared to lipoplexes.

Both H 1299 and A 549 cell lines showed similar order of uptake as evidenced in **table 6.8,** however, in all formulations, H 1299 cell line showed comparatively higher uptake.

Further, qualitative studies using fluorescent microscopy in H 1299 and A 549 cell lines treated with coumarin suspension, coumarin loaded liposomes and lipoplexes also confirmed the quantitative findings. Intense fluorescent signals were observed in the cells treated with coumarin loaded liposomes as compared to those treated with coumarin loaded lipoplexes and free coumarin indicating that the cells took up more coumarin molecules when they were delivered by liposomes as shown in **Figure 6.6**. Comparatively, higher uptake of liposomes in H 1299 than in A 549 cells was observed. Higher cell uptake is attributed to higher zeta potential /

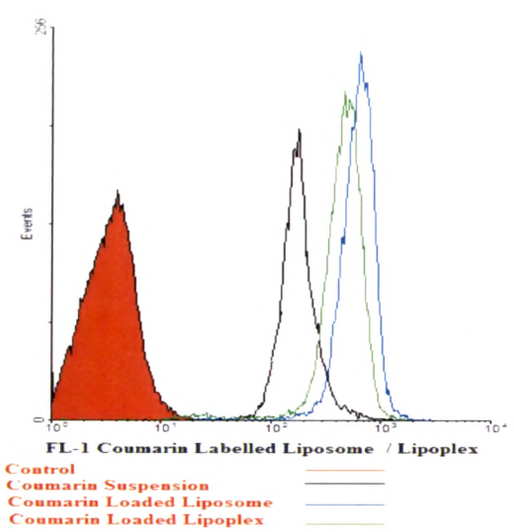

Figure 6.5 a

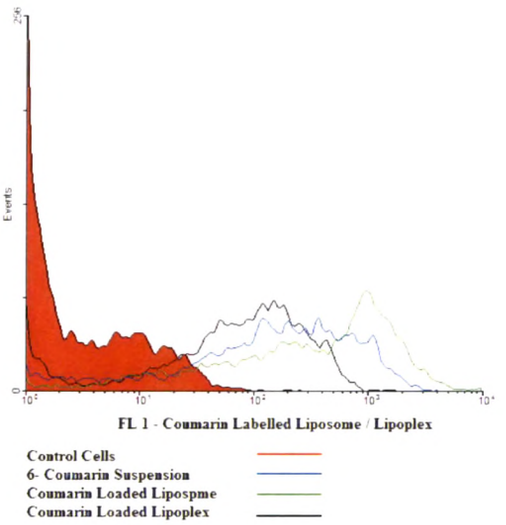

Figure 6. 5 b

Figure 6.5 : Quantitative analysis of cellular uptake of 6-coumarin performed in H 1299 (6.5 a) and A 549 (6.5b) cell lines by flow cytometer .

Table 6.8 : Quantitative uptake of 6-Coumarin formulations by H 1299 and A 549 cell line

Treatment Substrate	% Cells emitting the fluoroscence ((**Mean ± SEM , n=3**)	
	H 1299 Cell line	A 549 Cell line
6-Coumarin Suspension	62.7 ± 2.12	58.1 ± 1.88
6-Coumarin loaded liposome	89.9 ± 1.1	86.3 ± 2.1
6-Coumarin loaded lipoplex	81.8 ± 2.8	79.7 ± 1.3

charge on liposomes and their smaller sizes compared to their lipoplex counterpart. In addition, coumarin was seen to be dispersed throughout the cell at higher intensity in liposomes and lipoplex treated cells because of possible disruption of liposomes at lower pH of endosome indicating efficient release of coumarin inside cells which can be efficiently diffused inside the nucleus. The nucleus of the cells was identified by the nuclear DNA specific DAPI dye which intercalates with nucleus to give blue fluoroscence.

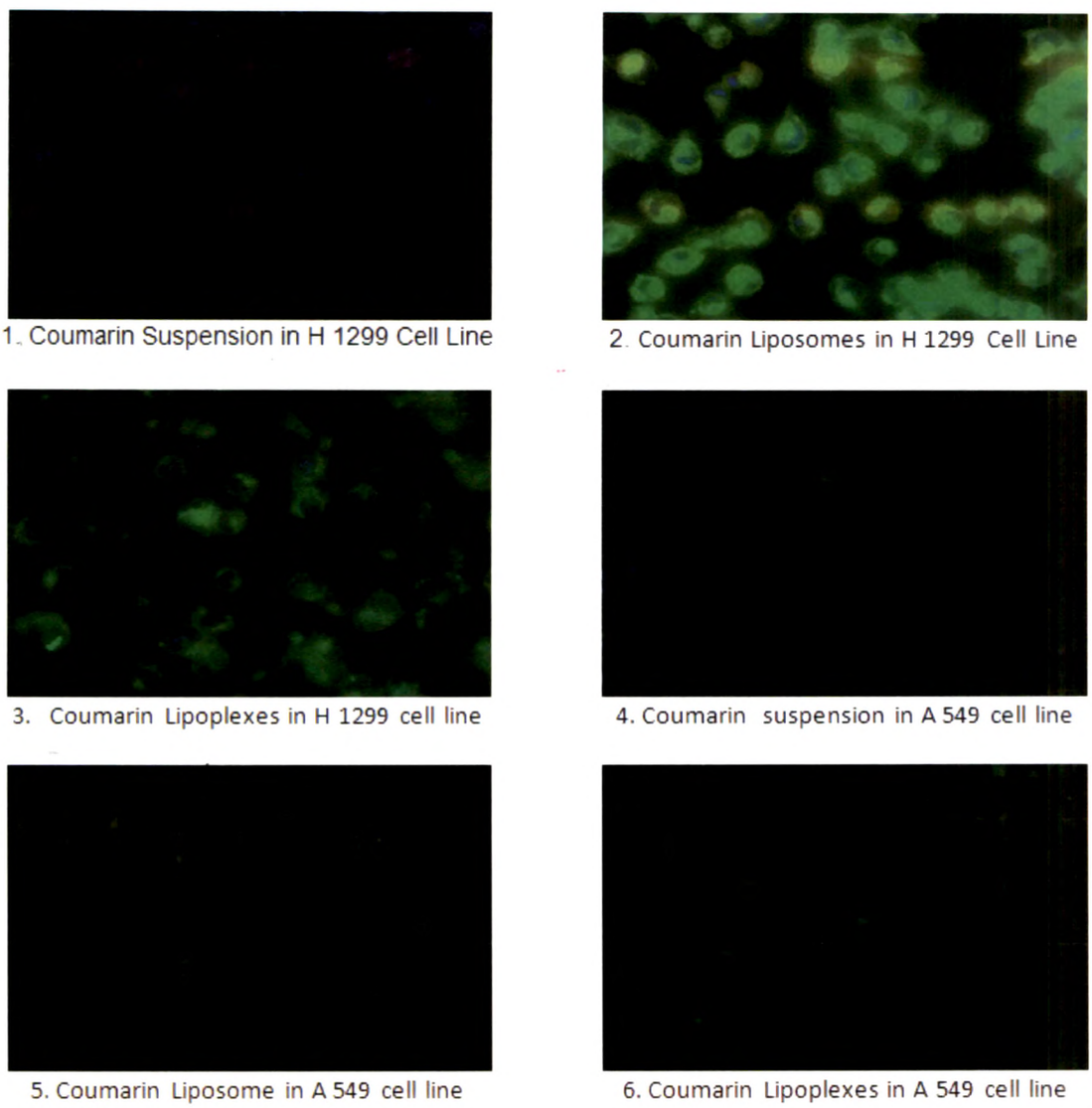

Figure 6.6: Intracellular uptake of 6-coumarin in H 1299 and A 549 Cell line

6.5. Transfection Studies

6.5.1 Materials: *p*53 and *β galactosidase* *p*DNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and used. *p*53 wt A 549 and *p*53 null H 1299 cell line were used to study transfection. The cells were grown in high glucose DMEM medium as described previously. All other chemicals were used as discussed earlier and were confirmed for purity before use.

6.5.2 Transfection of Drug Containing Lipoplexes in A 549 and H 1299 cell line:

Efficiency of the developed cationic liposomes to induce gene expression in H 1299 and A 549 cells was determined using *β gal* reporter *p*DNA. The expression is studied at various N/P ratios at which complete *p*DNA condensation was achieved. The cells were seeded in 96 well plate at a density of 5000 cells / well in 100 µl of DMEM growth medium supplemented with 10 % FBS and 1% Penicillin–Streptomycin- Amphotericin B solution. After 24 Hrs, the culture medium was replaced with fresh complete medium containing DNA/Liposome complexes prepared at different N/P ratios keeping the quantity of *p*DNA constant (300 ng). After 4 hrs of incubation of the lipoplexes with cells, the culture media was removed; the cells were washed with PBS pH 7.4 and were replaced with complete media. After 48 hrs, the culture media was removed, cells were washed with PBS pH 7.4 and were lysed using 50 µl lysis buffer (0.5 % Nonidet P 40 in Tris buffer pH 8.0) and were treated with 50 µl of 2 X ONPG solution, a substrate for β galactosidase protein. After 20 min of incubation at 37°C, the intensity of yellow color was measured in ELISA micro well plate reader (Biorad, Model 680 XR, Mumbai, India) at 405 nm as a function of quantity of β galactosidase protein expressed in the cells which converts the ONPG into O- Nitro phenol yielding the yellow color. In all the experiments, naked *p*DNA transfected cells were used as negative control and the Lipofectamine (Invitrogen) transfected cells were used as a positive control.

The effect of chemotherapeutic agents as ETP and DTX on transfection of β gal pDNA was also studied on the cell lines. Drug containing lipoplexes with N/P ratio 20 to 40 carrying significant and increasing level of encapsulated drug with suitable gene expression level were studied for relative change in transfection in presence of drug. The present of cytotoxic drug is expected to lower the *p*DNA transfection. Hence it is important to determine the maximum significant drug concentration still allowing significant *p*DNA transfection.

6.5.3 Results and Discussion

Lipid based non-viral gene delivery system has attracted more attentions in recent years due to their non-immungenic properties as opposed to viral ones, but their relatively low transfection efficiency usually limits their application. In order to evaluate the transfection capability of the developed lipoplexes, *in vitro* transfection experiment was performed on both H 1299 and A 549 cell line. Transfection efficiency of liposomes complexed with marker *β-gal p*DNA was measured by comparative determination of yellow color developed by ONPG hydrolysis to

Ortho Nitro Phenol, which is directly proportional to the quantity of β galactosidase protein formed after *p*DNA transfection. The transfection in terms of β gal activity has been tabulated in **table 6.9.** Transfection with naked uncomplexed *β-gal p*DNA was kept as negative control which showed very negligible transfection and transfection with Lipofectamine was considered to be positive control, which showed maximum transfection. Transfection achieved at different N/P ratio in both the cell lines is shown in **figure 6.7.** The results showed higher transfection of H 1299 cells than A 549 cells throughout the experiment. The transfection was observed to also varied by lipoplex N/P ratio, size and zeta potential. In H 1299 cell line, maximum transfection was achieved at N/P ratio of 2/1 which showed zeta potential near zero thus having minimum effect with serum anionic proteins, followed by lipoplex at N: P ratio in excess of 20/1 which showed lower particle size resulting in higher cell uptake. A 549 cell line showed similar transfection tendency suggesting significant role of particle size and zeta potential.

Encapsulation of the chemotherapeutic agent inside liposomes lead to lowered transfection efficiency as compared to plain lipoplexes. The effect of ETP and DTX on transfection efficiency has been diagrammatically shown in **figure 6.8.** With increasing concentration of ETP / DTX, lowering of the transfection efficiency was observed in both H 1299 and A 549 cell lines demonstrating significant role of drug concentration, which may cause cell cytotoxicity after achieving significant therapeutic concentration thereby impeding the expression of the gene in host cell [30]. Further, comparatively lower transfection efficiency in DTX loaded lipoplexes than ETP loaded lipoplexes was observed, which may had to do with lower particle size of ETP lipoplexes at all N/P ratios, theirby enhancing the cell uptake and transfection. At N/P ratio 20/1 insignificant change in transfection was observed indicating therapeutically ineffective drug concentration. As drug concentration increases with increase in N/P ratio, decrease in transfection efficiency was observed with lowest *β-gal* transfection at N/P ratio 40/1. Significant transfection with comparatively lower change after drug loading was observed as N/P ratio 25/1 when compared with transfection at higher N/P ratios. This N/P ratio of liposome to *p*53 *p*DNA was used for further cytotoxicity and flow cytometry studies, which will allow or maximum p53 expression with significant cytotoxic effect of ETP and DTX.

Table 6.9 Transfection of β gal activity at different N/P ratios of blank lipoplexes in H 1299 and A 549 cell line. (Mean ± SEM , n=3)

Transfection Reagent	β gal activity (H 1299 cell line (mU))	β gal activity (A 549 cell line (mU))
β gal cDNA	0.06 ±0.04	0.02±0.02
Lipofectamine	1.89±0.21	1.42±0.11
1:1	0.59±0.12	0.99±0.16
2:1	1.45±0.21	0.81±0.21
3:1	0.92±0.17	0.45±0.13
4:1	0.79±0.11	0.43±0.11
5:1	0.91±0.08	0.41±0.18
10:1	1.03±0.07	0.73±0.09
20:1	1.21±0.12	0.78±0.14
25:1	1.36±0.13	0.8±0.11
30:1	1.08±0.17	0.78±0.16
35:1	0.99±0.10	0.84±0.11
40:1	1.01±0.09	0.78±0.08

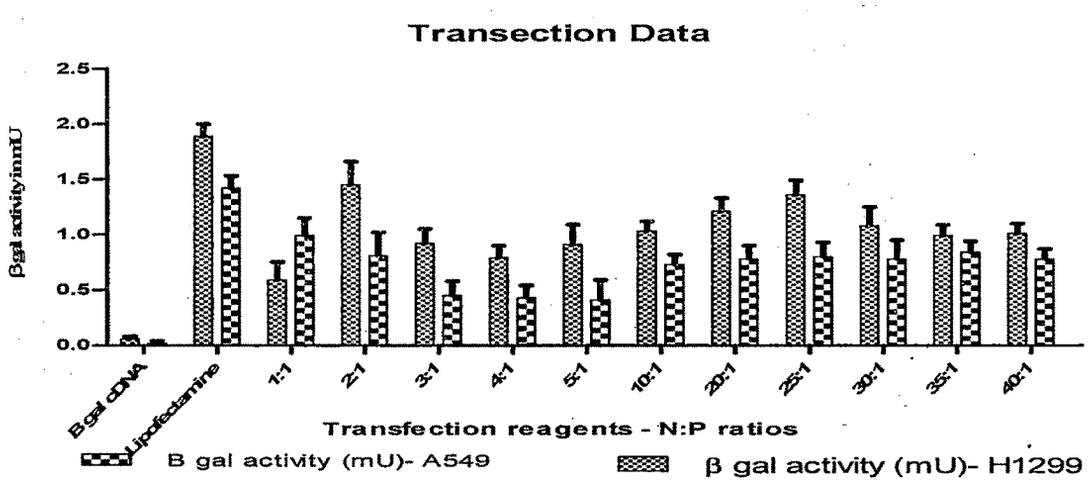

Figure 6.7: Transfection of *β-gal* *p*DNA after complexing with cationic liposomes at different N/P ratios in H 1299 and A 549 cell line (n=3)

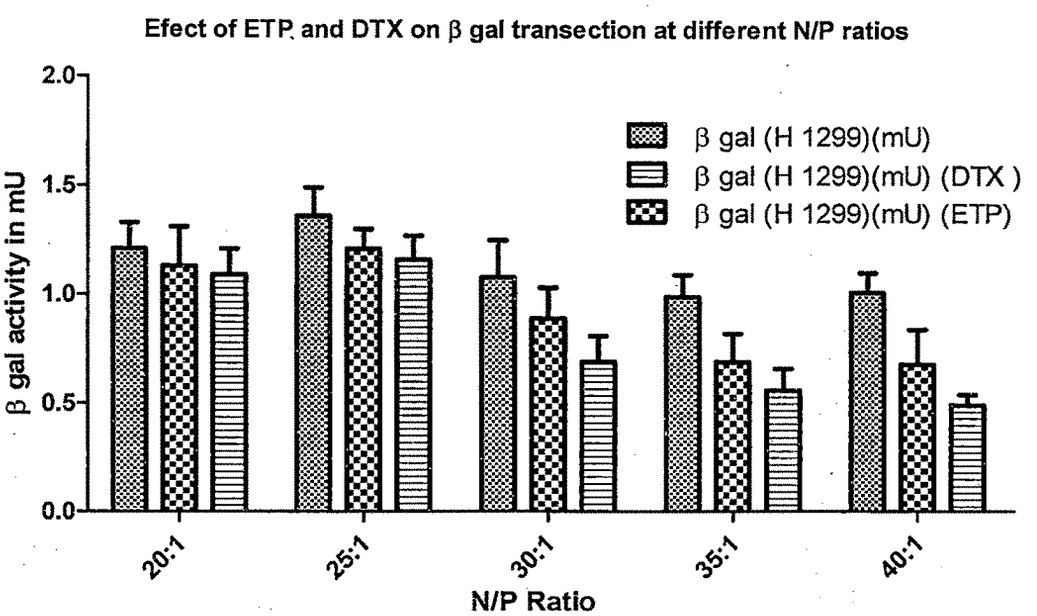

Figure 6.8 A

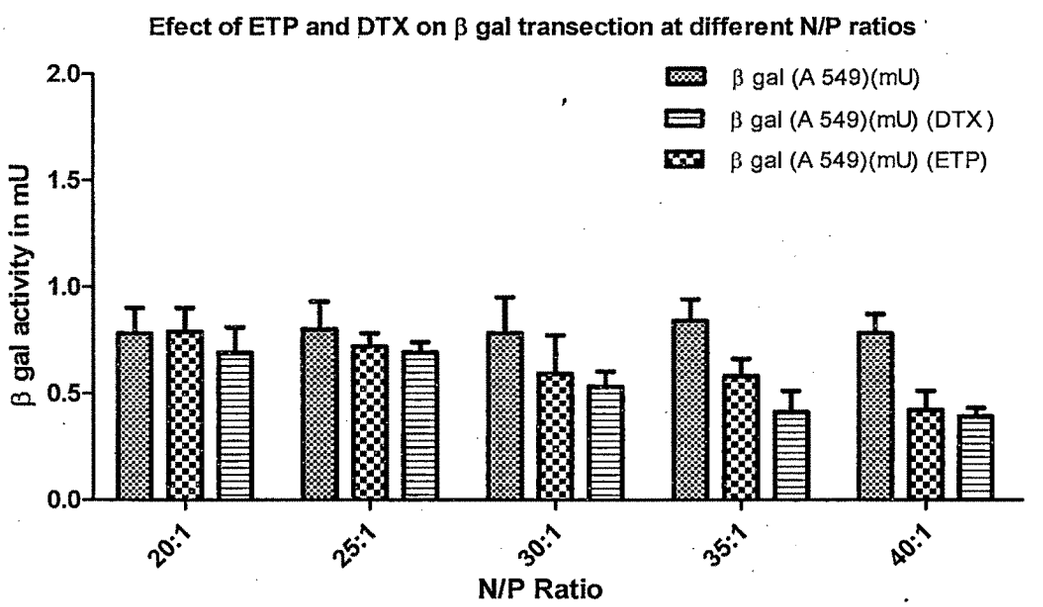

Figure 6.8 B Figure 6.8: Effect of cytotoxic chemotherapeutic agents on transfection of *β-gal p*DNA in H 1299 (8 A) and A 549 (8 B) cell lines.

Table 6.10: Effect of cytotoxic chemotherapeutic agents on β gal cDNA cell transfection in H 1299 and A 549 cell lines (Mean ± SEM , n=3)

Transfection Reagent	Average β galactosidase activity in H 1299 cell line (mU)	Average β galactosidase activity in A 549 cell line (mU)	Average β galactosidase activity in H 1299 cell line (mU) (In presence of ETP)	Average β galactosidase activity in A 549 cell line (mU) (In presence of ETP)	Average β galactosidase activity in H 1299 cell line (mU) (In presence of DTX)	Average β galactosidase activity in A 549 cell line (mU) (In presence of DTX)
Lipoplex (N:P) 20:1	1.21 ± 0.12	0.78 ± 0.12	1.13 ± 0.18	0.79 ± 0.11	1.09 ± 0.12	0.69 ± 0.12
Lipoplex (N:P) 25:1	1.36 ± 0.13	0.80 ± 0.13	1.21 ± 0.09	0.72 ± 0.06	1.16 ± 0.11	0.69 ± 0.05
Lipoplex (N:P) 30:1	1.08 ± 0.17	0.78 ± 0.17	0.89 ± 0.14	0.59 ± 0.18	0.69 ± 0.12	0.53 ± 0.07
Lipoplex (N:P) 35:1	0.99 ± 0.10	0.84 ± 0.10	0.69 ± 0.13	0.58 ± 0.08	0.56 ± 0.10	0.41 ± 0.10
Lipoplex (N:P) 40:1	1.01 ± 0.09	0.78 ± 0.09	0.68 ± 0.16	0.42 ± 0.09	0.49 ± 0.05	0.39 ± 0.04

6.6 Cytotoxicity Studies

6.6.1 Materials: *p*53 and *β galactosidase* *p*DNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and used. *p*53 wt A 549 and *p*53 null H 1299 cell line were used to study transfection. The cells were grown in high glucose DMEM medium as described previously. All other chemicals were used as discussed earlier and were confirmed for purity before use.

6.6.2 Cytotoxicity Studies of ETP / DTX formulations along with *p*53 pretreatment and co-administration:

*p*53, a cell growth regulator, is actively involved in inducing apoptosis and exerting cytotoxic effect on cancer cell lines. *p*53 has also shown enhancement in the cytotoxicity of anticancer agents when given along with them. In this investigation, we have studied the synergy in the anti-tumor activities between *p*53 and ETP/DTX in terms of decreased of cell viability by pretreatment of *p*53 with the cancer cells to sensitize them towards chemotherapy as a pretreatment approach and *p*53 and ETP/DTX co-delivery for synergistic anticancer activity.

The concentration of ETP and DTX to be used in the cytotoxicity study was determined on the basis of results obtained in terms of highest *β gal* expression in presence of ETP and DTX. The *p*53 concentration was kept same as that of *β gal* (300 ng) and was constant through out the study. The lipoplexes with *p*53 were prepared in a similar manner at the same conditions having N/P ratio which resulted in maximum *β gal p*DNA expression as studied earlier and the cytotoxicity assay was performed. Various formulations by combination of *p*53, ETP / DTX, cationic liposomes prepared for studying the effect of *p*53 in cell transfection and associated cytotoxicity are as mentioned below:

1. Control group treated only with medium
2. Blank cationic liposomes
3. *p*53 solution as Negative control
4. *p*53 lipoplexes (N/P ratio 25:1)
5. Cationic Lipid- Non therapeutic *β gal p*DNA at N/P ratio 25: 1
6. ETP / DTX solution
7. ETP / DTX liposome
8. *p*53 solution + ETP / DTX solution (Co-administration)
9. *p*53 lipoplex + ETP / DTX solution (Co- administration)
10. *p*53 lipoplex + ETP / DTX liposome (Co- administration)
11. *p*53-ETP lipoplex / *P*53 – DTX lipoplex(Co- administration)
12. Liposomal *p*53 pre-delivery followed by ETP / DTX solution after 24 Hrs (Pre-treatment)
13. Liposomal *p*53 pre-delivery followed by ETP / DTX liposome after 24 Hrs (Pre-treatment)

Briefly, cytotoxic effect of all these formulations was estimated by modified MTT cell viability assay. The cells H 1299 and A 549 were seeded in 96 well plates at cell density of 5000 cells/ well and grown overnight. On the next day the cells were incubated with all the formulations (Formulation 1-11 for both drugs) for 4 Hrs, rinsed well with PBS and the cells were allowed to grow for 48 hrs. Simultaneously, in another set of experiments, the cells were first pretreated with *p*53 lipoplexes for 4 hrs and allowed to grow for 20 hrs. These *p*53 pretreated cells were incubated with ETP and DTX solution / liposome for 4 hrs, rinsed with PBS and then allowed to grow for another 48 hrs. After 48 hrs, the cells were rinsed with PBS and then treated with 100

µl of MTT solution in DMEM (without serum) at concentration of 1mg/ml and incubated for 4 hrs. After the incubation, the medium was replaced with 200µl DMSO to dissolve the formazan crystals formed after internalization of MTT by live cells. The samples were assayed with Microplate reader (Biorad) at 570 nm keeping reference absorbance at 630 nm as blank. The absorbance of cells without any chemical treatment is used as positive control and the absorbance of cells treated by *p*53 solution is used as negative control. The percentage cell viability (%) is determined as

$$\text{Cell Viability (\%)} = \frac{\text{Sample absorbance at 570 nm}}{\text{Control absorbance at 570 nm}} \times 100$$

The cytotoxicity tests were repeated three times, and the data is expressed as mean and standard deviation of 3 replicates. The results were compared statistically with single factor ANOVA.

6.6.3 Results and Discussion

The cytotoxicity assay of *p*53 along with ETP and DTX was performed to evaluate the effect of *p*53 pretreatment and co-administration along with drugs by modified MTT test. The cytotoxicity results in terms of % cell viability after the treatment have been tabulated in **table 6.11** and are diagrammatically shown in **figure 6.9** as below. Cationic liposomes and *p*53 solution showed non-significant toxicity and were considered as controls. Cytotoxicity studies were performed in two cell lines as H 1299 (p53 null) and A 549 (p53 wt), which mimics the deleted / mutated / normal but inactive *p*53 character in majority of lung cancers *in vivo* [D. Sidransky et al (1996); L. Xua et al (2001).

During the studies, both ETP and DTX as liposomal formulations showed higher cytotoxicity than their drug solutions, because of higher cell uptake of nanosized drug loaded cationic liposomes than their solutions. The higher cell uptake was also observed during cell uptake studies by FACS and fluoroscence microscopy studies. Both the drugs showed cytotoxicity of 20-25 % when compared with control cells, however, the cytotoxicity was higher in H 1299 cell line as compared to A 549 cell line. The higher cytotoxicity in *p*53 devoid H 1299 cell line may have been because of initial absence of *p*53, which when expressed after transfection controls the entire growth and promotes apoptosis in the cell line. When liposomally encapsulated drugs were treated, cytotoxicity was enhanced by 10-20 %. DTX showed more potent action on cell lines

with higher cytotoxicity than ETP in all formulations, suggesting need of lower dose of DTX than ETP when used as liposomal formulation.

Table 6.11: % Cell viability of H 1299 and A 549 cells after p53 and ETP / DTX formulations treatment: (n=3, mean ± SEM)

Formulation	% Cell Viability (H 1299 Cell line)	% Cell Viability (A 549 Cell line)	% Cell Viability (H 1299 Cell line)	% Cell Viability (A 549 Cell line)
	ETP	ETP	DTX	DTX
Control	100	100	100	100
Blank Cationic Liposomes	97.7 ± 0.53	98.1 ± 1.09	97.7 ± 0.53	98.1 ± 1.09
Drug solution	78.49 ± 1.67	75.92 ± 2.32	72.44 ± 2.13	76.56 ± 2.90
Drug liposome	69.90 ± 1.26	72.11 ± 2.15	63.19 ± 2.71	69.56 ± 2.63
P53 solution	95.6 ± 0.67	97.1 ± 0.35	95.6 ± 0.67	97.1 ± 0.35
P53 Lipoplexes (N: P Ratio – 25:1)	79.17 ± 1.54	91.29 ± 4.12	79.17 ± 1.54	91.29 ± 4.12
B gal Lipoplexes (N:P Ratio – 25:1)	98.27 ± 0.13	98.31 ± 0.89	98.27 ± 0.13	98.31 ± 0.89
P53 solution + Drug solution (Co-delivery)	76.92 ± 0.94	73.12 ± 2.10	70.98 ± 2.62	75.98 ± 1.43
P53 lipoplex + Drug solution (Co-delivery)	69.34 ± 0.78	70.87± 1.22	61.09 ± 2.17	68.79 ± 1.34
P53 lipoplex + Drug liposome (Co-delivery)	59.21 ± 1.21	63.19 ± 2.01	48.75 ± 1.12	60.12 ± 1.49
P53- Drug lipoplex (Co-delivery)	50.67 ± 2.11	58.63 ± 1.82	38.83 ± 3.06	49.12 ± 2.89
Liposomal P53 pre-delivery followed by drug solution after 24 Hrs	62.23 ± 1.69	66.02 ± 2.18	58.36 ± 1.80	63.50 ± 2.09
Liposomal P53 pre-delivery followed by drug liposome after 24 Hrs	31.91 ± 0.55	42.25 ± 2.45	19.78 ± 1.95	33.65± 1.58

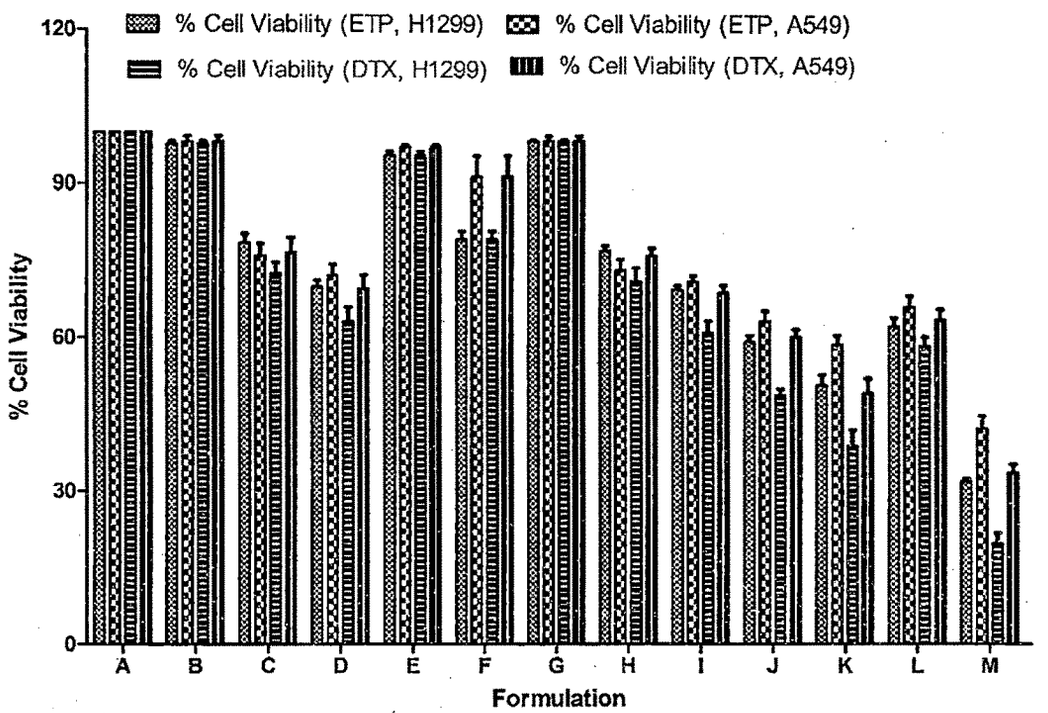

Figure 6.9: Cytotoxicity of *p*53 and ETP / DTX formulations in H 1299 and A-549 cell lines after pre-treatment and co-administration approach [**A:** Control group treated with medium, **B:** Blank cationic liposomes, **C:** *p*53 solution as negative control, **D:** *p*53 lipoplexes (N/P ratio 25/1), **E:** Cationic Lipid- *β-gal- p*DNA (N/P ratio 25/1), **F:** ETP/DTX solution, **G:** ETP/DTX liposome, **H:** *p*53 solution + ETP/DTX solution (Co-administration), **I:** *p*53 lipoplex + ETP/DTX solution (Co-administration), **J:** *p*53 lipoplex + ETP/DTX liposome (Co-administration), **K:** *p*53-ETP lipoplex / *p*53 – DTX lipoplex (Co-administration), **L:** *p*53 lipoplex pre-treatment followed by ETP/DTX solution after 24 Hrs, **M:** *p*53 lipoplex pretreatment followed by ETP/DTX liposome after 24 Hrs]

*p*53 lipoplex when formulated similar to *β-gal* lipoplexes showed good transfection and cytotoxicity of 20 % as therapeutic action at N/P ratio 25/1. At N/P ratio 2/1, the cytotoxicity produced by *p*53 lipoplex was 2 folds higher than the cytotoxicity at N/P ratio 25/1 in H 1299 and A 549 cell lines indicating efficient cytotoxic action of *p*53 in lung cancer as well as transfection ability of the carrier. However, this N/P ratio was not used for further studies because of very poor drug loading in liposomes at this ratio. Comparatively, *p*53 lipoplex at N/P

ratio 25/1 showed significant cytotoxicity in H 1299 cell line than A 549 cell line (Single factor ANOVA, $p < 0.05$), consistent with the transfection studies performed earlier. The higher cytotoxicity was also because of the initiation of *p53* dependant apoptosis and normalization of cell cycle in transfected H 1299 cells. The presence of wild type *p53* in A 549 may also play a role in apoptosis induced by transfected *p53* in A 549 cell line.

The *p53* mediated cell cytotoxicity has been observed because of normalization of apoptosis function, with suppression of BCL-2 protein. The BCL-2 protein is postulated to block the release of cytochrome C after the initiation of apoptosis, which prevents the downstream propagation of the death signal, thereby promoting cell survival [L. Shyh-Dar et al (2006)]. To confirm the cytotoxicity because of *p53*, and not because of the lipoplex treatment, a control *β-gal* lipoplex at N/P ratio 25/1 was treated with cell lines, which showed non-significant cytotoxicity.

Further enhancement of cytotoxicity was observed when *p53* and the chemotherapeutic agent as ETP or DTX were administered together as a co-administration approach. The *p53* solution co-administered with ETP or DTX solution or liposomes showed non-significant enhancement in cytotoxicity, because of lack of *p53* transfection after administration as solution. However, coadministration of *p53* lipoplex along with ETP and DTX solutions and liposomes lead to 10-30 % increase in drug mediated cytotoxicity. The results were more significant particularly in H 1299 cell line and more in case of DTX liposomes than other formulations. The enhancement in cytotoxicity may be attributed to simultaneous transfection of *p53* resulting in regaining normal apoptosis function theirby causing cytotoxicity as well as suppressing the level of BCL-2 anti-apoptotic cellular defense resistance protein, thus sensitizing the cells towards the action of chemotherapeutic agent [Yu-ling Wu et al. (2001)]. Further, the apoptosis inducing property of ETP also plays a significant role in enhancing the apoptosis of H 1299 and A 549 cells [Z.A. Butenko et al (2000)]. It has been observed that, delivery of chemotherapeutic agent induces the cytotoxicity in cancer cells, but it is accompanied by the drug resistance as efflux pump and

antiapoptotic cellular defense resistance [Yu-ling Wu et al. (2001) ; S. Maha et al (2008)]. The chemotherapeutic action is enhanced by the higher cell uptake of the liposomes as compared to drug solution, which also overcomes the efflux pump resistance by shielding the drug inside the nanostructure but also enhances the cell apoptosis and regularizes and simultaneously suppresses the antiapoptotic cellular defense BCL 2 protein resistance thereby leading to effective induction of cell death in cancer cells [L. Shyh-Dar et al (2006)].

Further, when *p*53 complexed drug liposomes (*p*53- drug lipoplexes) were treated with the cells, significant enhancement by 10-15 % of the drug cytotoxicity was observed than simultaneous administration of drug liposomes and *p*53 lipoplexes treated together ($p<0.05$). The enhancement was more potent in case of DTX than in case of ETP and more in case of H 1299 cell line. This enhancement may be attributed to the uptake of drug and the DNA in appropriate concentrations in the same cell theirby enhancing the effect. The results of experimental testing of this multicomponent lipoplex system showed that cationic liposomes were able to efficiently deliver the *p*53 *p*DNA and ETP / DTX inside the cells. Simultaneous delivery of chemotherapeutic agent and the apoptosis inducing *p*DNA which also lowers the antiapoptotic cellular defense resistance led to the effective apoptosis induction and killing of drug-resistant lung cancer cells.

Further, pretreatment of *p*53 enhanced the cell sensitization towards the action of ETP and DTX by 40-60 % as compared to drug solution and liposomes. The cytotoxic action was 25-40 % higher than the *p*53- ETP / DTX co-administration with 20 to 35 % of cell viability after drug treatment. The cells showed viability of 31.91 and 19.78 % after *p*53 followed by ETP / DTX treatment in H 1299 cells and 42.25 and 33.65 % in A 549 cell line. The cells especially H 1299 showed high chemosensitization than in A 549 which may be attributed to higher *p*53 transfection in H 1299 than A 549. DTX showed comparatively higher cytotoxicity than ETP formulation thereby showing higher chemosensitization and potency of the molecule. Further, when *p*53 is treated alone followed by drug treatment as in pretreatment approach, the *p*DNA has shown higher transfection than when treated along with the drug solution or liposome. The *p*53 transfection in turn makes the cells more responsive towards chemotherapy by restoring the normal cell apoptotic function and reducing BCL 2 mediated cellular antiapoptotic defense. [L. Shyh-Dar et al (2006)]

Further, the bystandard action of *p*53 protein after expression in lung cancer cell lines has also reported to play a significant role in enhancing the action of *p*53 throughout the complete cells even after possibly getting transected in fewer cells thereby enhancing the cytotoxic action of the drug [D. K. Frank et al (1998)]. This bystandard effect nullifies the disadvantage of possible *p*53 and drug liposome uptake in different cells in different proportion which may reduce the synergism. However, chemosensitization by *p*53 was observed to be sequence dependant. Addition of drug solution / liposome followed by *p*53 lipoplex did not showed any significant improvement of drug cytotoxicity making the action of *p*53 ineffective [L. Shyh-Dar et al (2006)].

6.7 *p*53 detection by flow cytometry

6.7.1 Materials

*p*53 pDNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and used. *p*53 null H 1299 cell line were used to study expression of p53 in the cells. The cells were grown in high glucose DMEM medium as described previously. p53 selective (p53 [DO-1] mouse monoclonal IgG2a antibody, raised against amino acids 11-25 of p53 of human origin) primary and secondary (Alexafluorr 488, goat antobody antimouse primary antibody) antibodies and all other chemicals were used as discussed earlier and were confirmed for purity before use.

6.7.2 Lipoplex mediated p53 expression in H 1299 cells

Expression and restoration of native *p*53 in *p*53 null H 1299 cell line was determined using flow cytometry by the help of *p*53 specific antibodies. The studies were not performed in A 549 cell line because of presence of *p*53 (wt) in the A 549 cell line. Briefly, the cells were seeded in the 6 well plates at the cell density of 0.25 X 10^6 cells per well. The cells were allowed to grow overnight and were transfected next day with various transfection reagents at a proportionately higher dose of 2 µg of *p*53 and corresponding liposomal concentration (blank / drug containing) per well. Transfection experiments with *p*53 pre-treatment followed by ETP / DTX delivery and p53 co-administration with ETP / DTX were carried out. After 48 hrs, the cells were rinsed with PBS, trypsinized, fixed with 4 % paraformaldehyde for 15 min at 4° C, permeabilized with 0.1 % Triton X 100 in PBS and were treated with human *p*53 specific mouse anti *p*53 primary antibody followed by goat Alexafluor 488 secondary antibody against the primary antibody. After washing the cells 5 times, the cells were resuspended in 250 µl PBS and the fluoroscence was measured using FACS Calibur (BD Biosciences). Experiments with control cells without any

treatment were also carried out to eliminate cell autofluoroscence and were considered as a negative control. The results were statistically compared with single factor ANOVA test.

6.7.3 Results and Discussion

The expression of p53 after liposomal delivery in H 1299 cell line was determined in terms of % cells emitting the fluoroscence signal because of binding of p53 specific antibodies to the translated protein developed after transfection. The results have been depicted in the **Figure 6.10 and 6.11** below.

The control group of *p*53 null, H 1299 cells showed no fluoroscence. Moreover, p53 solution and plain ETP and DTX liposomes treatment also displayed non-significant p53 expression. However, treatment with p53 lipoplex showed significant fluoroscence shift of cells in the histogram and demonstrated significant p53 expression ($p < 0.05$). At ratio N/P (2/1), 63.94 % cells showed p53 expression. However, at N/P ratio of 25/1, the cells showed lower transfection with 51.5 % of cells showing fluoroscence. The lowering of transfection may have to do with higher cationic charge of the liposomes, which may interact with anionic serum proteins to form aggregates theirby reducing their cell uptake when compared with lipoplexes with N/ P ratio 2/1. Further, treatment with *p*53 - drug lipoplex at N/P ratio 25/1 showed lower transfection possible because of cytotoxic effect of the drug. The cytotoxic effect was more pronounced in case of DTX. The *p*53-DTX and *p*53-ETP lipoplex showed *p*53 expression of 17.49 % and 28.26 % respectively in H 1299 cells. This lowered transfection was consistent with the transfection and cytotoxicity data provided earlier. However, the pretreatment approach showed significantly higher *p*53 expression irrespective of the drug treated later. *p*53 followed by ETP and DTX liposomes, both showed *p*53 transfection in 44-45 % cells. This higher transfection followed by significant drug action was responsible for higher cytotoxicity of the cells.

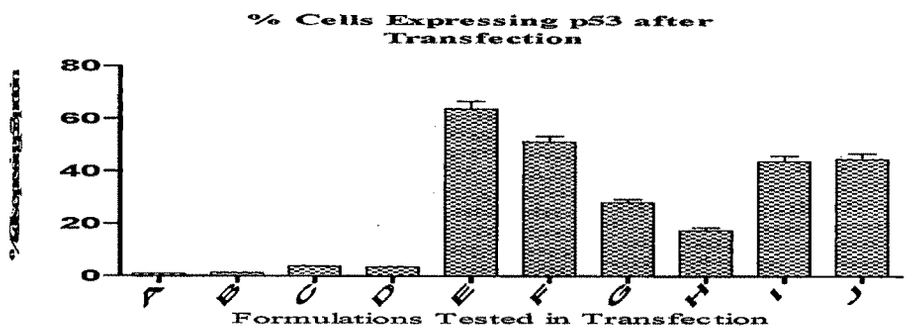

Figure 6.10 : Percentage cells expressing *p*53 after transfection in H 1299 cell line. (A: Control Cells, B: P53 solution, C: ETP liposome, D: DTX liposome, E : P53 lipoplex (N:P – 2:1), F: P53 lipoplex (N:P – 25:1), G: P53 – ETP lipoplex, H: P53-DTX lipoplex, I: P53 followed by ETP liposome, J: P53 followed by DTX liposome)

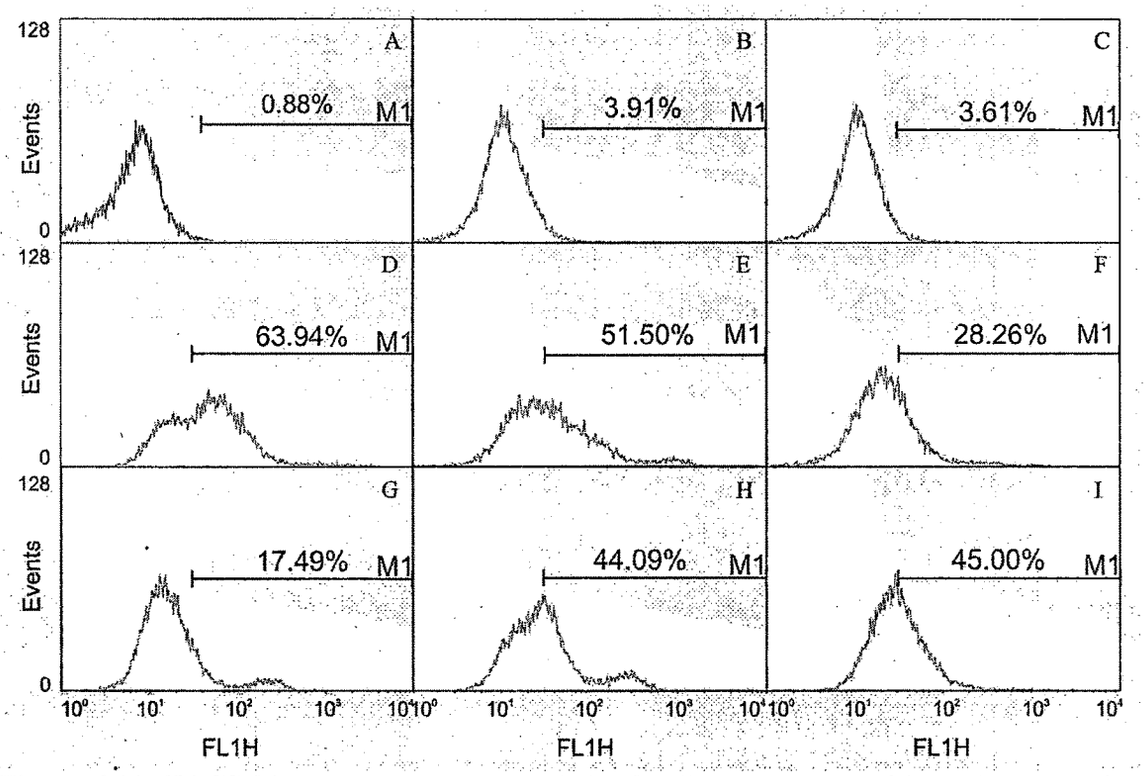

Figure 6.11: FACS histograms showing shift in the fluoroscence positive p53 expressing cells after transfection in H 1299 cells (A: Control Cells, B: ETP liposome, C: DTX liposome, D: P53 lipoplex (N:P – 2:1), E: P53 lipoplex (N:P – 25:1), F: P53 – ETP lipoplex, G: P53-DTX lipoplex, H: P53 followed by ETP liposome, I: P53 followed by DTX liposome)

6.8 Annexin V FITC Assay

6.8.1 Materials:

*p*53 pDNA were isolated and purified as discussed earlier (260 / 280 ratio of 1.9) and used. *p*53 null H 1299 and *p*53 wt A-549 cell line were used to study cell apoptosis. The cells were grown in high glucose DMEM medium as described previously. Annexin V FITC kit was procured from Sigma Aldrich and all other chemicals were used as discussed earlier and were confirmed for purity before use.

6.8.2 Methods:

Sensitization of cell cytotoxicity by enhancement in apoptosis after *p*53 pretreatment / co-administration and cell necrosis because of ETP/ DTX treatment was investigated using Annexin V – FITC assay based on binding of Annexin V with phosphatidyl serine of early apoptotic cells. The necrotic cells were identified by binding of potassium iodide with the nucleus of the dead cells. Briefly, the cells were seeded at a cell density of 0.125 X 10^6 cells per well and allowed to grow overnight. After 24 hrs, the cells were transfected with transfection agents using same N/P ratio and keeping *p*53 dose of 2 µg per well. Transfection experiments with *p*53 pre-treatment and co-administration with ETP and DTX were carried out. After 48 hrs, the cells were rinsed with PBS, trypsinized, and treated with Annexin V- FITC conjugate solution and propidium iodide solution (in dark) and incubated for 10 min. The fluorescence of the cells was immediately measured by flow cytometer (FACS Caliber, BD Biosciences) to determine early apoptotic cells (stained with Annexin V – FITC only), live cells (No staining), and necrotic cells (staining by Annexin V FITC as well as propidium iodide). The results were statistically compared using Single factor ANOVA.

6.8.3 Result and Discussion

Lack of functional *p*53 observed predominantly in NSCLC results in inability of tumor cells to initiate apoptosis when treated with chemotherapy. Restoration of functional *p*53 has shown to restore the apoptotic response against chemotherapy by enhancing *p*53 level and theirby reducing BCL 2 mediated antiapoptotic defense. The effect of p53 transfection as p53 pre-treatment followed by ETP/DTX delivery and *p*53 co-administration along with ETP and DTX liposomes was studied by Annexin V FITC assay for determining cell undergoing apoptosis and necrosis. The results of the study are tabulated in table 6.12 and 6.13. The dot plots of the FACS analysis are plotted in **figure 6.12 (a) and (b).** Fluoroscence absorbance of Annexin V (X axis) against

Table 6.12: % stained H 1299 cells after Annexin V-FITC and PI staining demonstrating apoptotic and necrotic cells. (n=1)

Sr No.	Formulation	PI + cells	PI & Ann V + Cells	Unstained cells	Ann V + Cells
1	Control	0.01	0.07	99.91	0.02
2	P53 lipoplex (2:1)	0.55	18.83	58.07	22.57
3	P53 lipoplex (25:1)	2.95	9.53	74.90	12.63
4	ETP liposome	2.98	16.21	61.60	19.22
5	DTX liposome	1.13	20.73	53.20	24.95
6	P53 + ETP lipoplex codelivery	2.40	24.28	41.45	32.18
7	P53 + DTX lipoplex codelivery	10.22	40.53	25.66	23.59
8	P53 pretreatment followed by ETP liposome delivery	11.14	37.31	21.47	30.09
9	P53 pretreatment followed by DTX liposome delivery	4.04	28.69	27.46	39.82

Table 6.13: % stained A 549 cells after Annexin V-FITC and PI staining demonstrating apoptotic and necrotic cells. (n=1)

Sr No.	Formulation	PI + cells	PI & Ann V + Cells	Unstained cells	Ann V + Cells
1	Control	0.01	0.05	99.91	0.04
2	P53 lipoplex (2:1)	0.25	3.29	80.53	15.94
3	P53 lipoplex (25:1)	0.78	2.88	92.02	4.32
4	ETP liposome	21.08	16.35	38.65	23.93
5	DTX liposome	19.17	14.02	47.30	19.52
6	p53 + ETP lipoplex codelivery	6.27	18.97	48.91	25.36
7	p53 + DTX lipoplex codelivery	3.82	20.16	54.52	21.51
8	p53 pretreat. followed by ETP lip. delivery	4.04	28.69	27.46	39.82
9	P53 pretreatment followed by DTX liposome delivery	4.09	17.22	48.74	29.96

propidium iodide (Y axis) was used to distinguish viable cells (negative for both PI and Annexin V – Lower Left quadrant), early apoptotic cells (Annexin V positive but PI negative – Lower right quadrant), late apoptotic and necrotic cells (double positive for Annexin V and PI – Upper right) and damaged necrotic cells (positive for PI only – Upper left quadrant).

The results showed significant increase in Annexin V positive cells and Annexin V and PI positive cells after pretreatment and co-administration of *p*53 and ETP / DTX when compared with plain ETP and DTX liposomes ($p < 0.001$). The enhancement was significant in H 1299 cell line and in presence of DTX ($p<0.001$). After *p*53 pretreatment, followed by ETP and DTX delivery, higher Annexin V positive – early apoptotic cells were observed along with necrotic cells demonstrating chemo-sensitization of the cells. When *p*53- ETP / DTX lipoplex were co-administration approach was used, synergistic action of *p*53 and chemotherapeutic agent was observed. However, % early apoptotic cells were lower than the pretreatment approach. Higher Etoposide apoptosis was observed in A 549 cells (p53 wt), as compared to H 1299 probably because of property of ETP to induce apoptosis inside cells in presence of *p*53 as reported previously [H. Zhang et al (2002), C. Stefanelli1 et al (1998)].

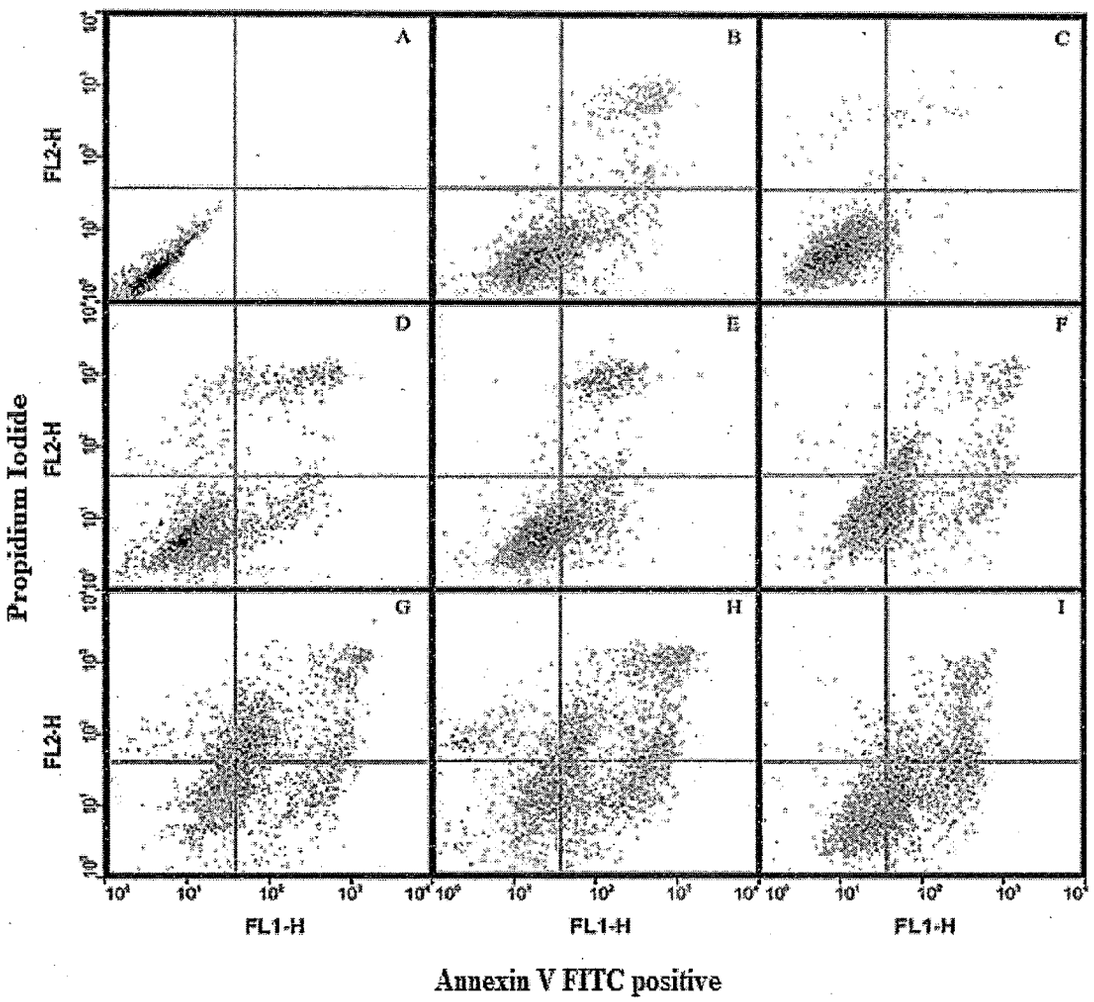

Fig 6.12 A : Annexin V FITC dot plots for determining cell Apoptosis and Necrosis in H 1299 cell line. (A: Control, B: P53 lipoplex (2:1), C: P53 lipoplex (25:1), D: ETP liposome, E: DTX liposome, F: P53 + ETP lipoplex codelivery, G: P53 + DTX lipoplex codelivery, H: P53 pretreatment followed by ETP liposome delivery, I: P53 pretreatment followed by DTX liposome delivery)

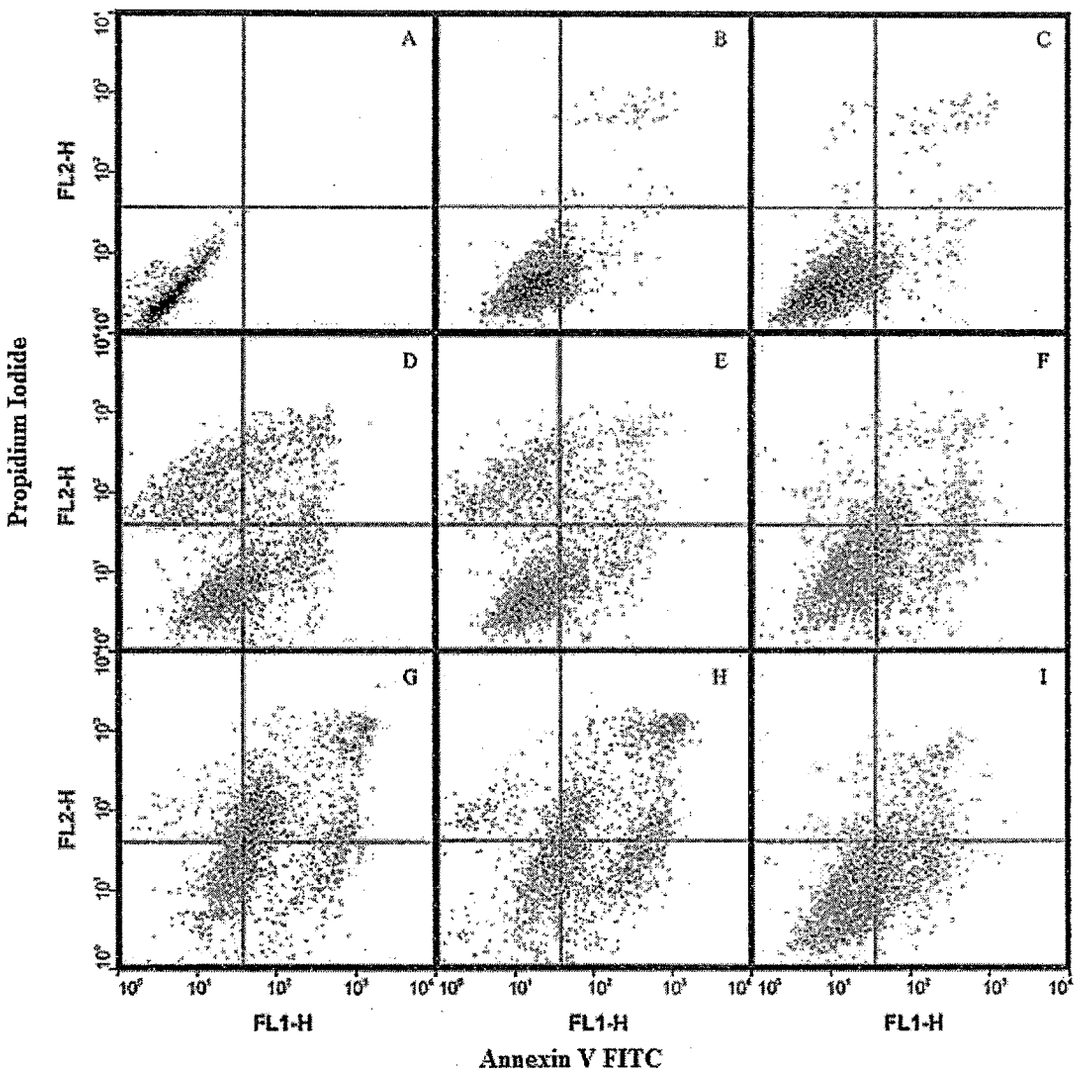

Fig 6.12 B : Annexin V FITC dot plots for determining cell Apoptosis and Necrosis in A 549 cell line. (A: Control, B: P53 lipoplex (2:1), C: P53 lipoplex (25:1), D: ETP liposome, E: DTX liposome, F: P53 + ETP lipoplex coadministration , G: P53 + DTX lipoplex coadministration, H: P53 pretreatment followed by ETP liposome delivery, I: P53 pretreatment followed by DTX liposome delivery)

From the transfection, cytotoxicity and Annexin V FITC assay results, conclusively, p53 pretreatment and p53- ETP/DTX co-administration demonstrated enhanced cytotoxicity with increased cell apoptosis and reduced antiapoptotic defense mechanism. The results overall may be graphically represented as in **Figure 6.13**.

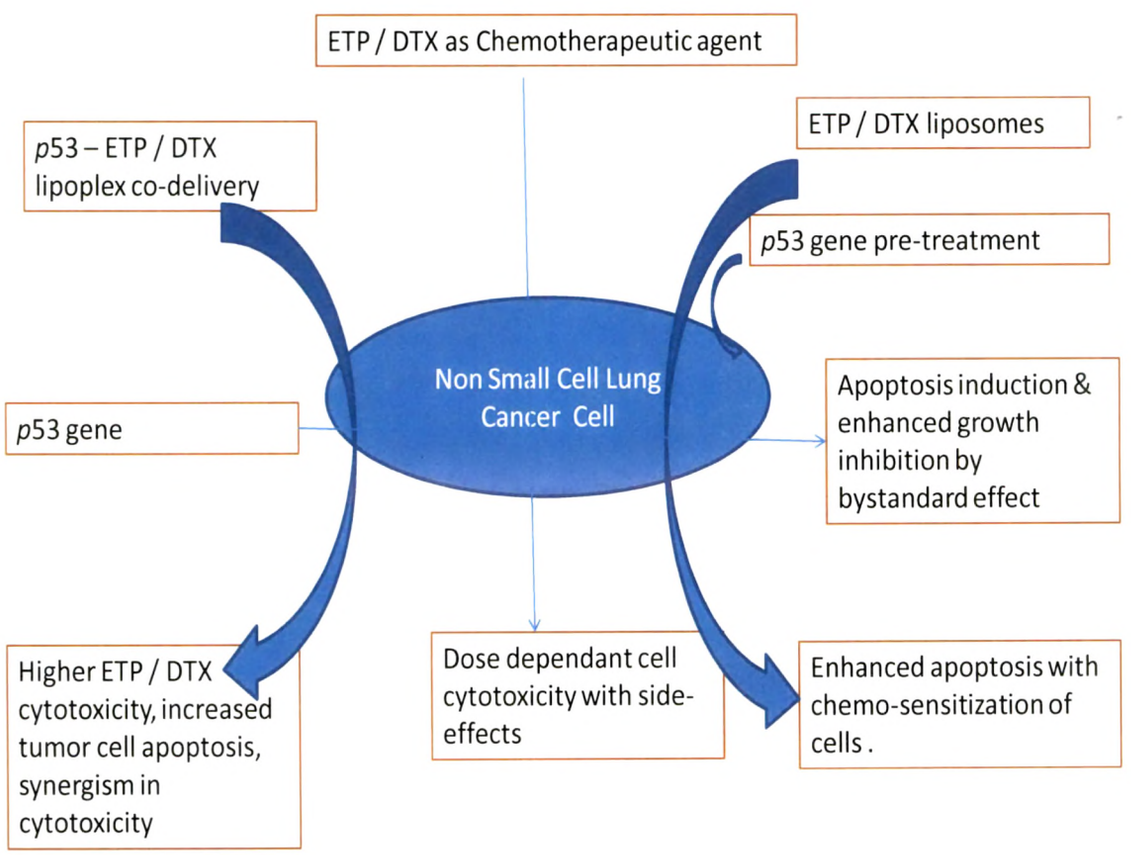

Figure 6.13 : Graphical representation of p53 pretreatment and co-delivery with ETP and DTX for chemo-sensitization and enhanced synergistic cytotoxicity

6.9 Lyophillization of Lipoplexes:

The optimized lipoplexes were lyophilized for enhanced stability of the formulations and DPI development for direct lung deposition. The lyophillization was optimized with trehalose, to achieve same physical properties of the lipoplexes when they come in contact with aqueous medium to release nanosized lipoplexes. Trehalose has shown best compatibility with the lipids in liposome lyophillization and hence was used during lipoplex lyophillization. Previously, sugar molecules as isomaltose and sucrose have shown capacity to protect the liposomes and lipoplexes from aggregation and maintain their transfection ability for 3 months at 2-8^0 C [Y. Maitani et al (2008)]. Our lipoplexes were lyophilized with main objective of maintaining same particle size of lipoplexes which may result in similar transfection efficiency. Similar to liposome lyophillization, trehalose as a weight ratio of 1: 3 of lipid: sugar showed non-significant changes in particle size and zeta potential. The results for physical changes after lipoplex lyophillization after tabulated in table 6.14 below:

Table 6.14: Formulation parameters of liposomes and lipoplexes before after lyophillization (n=3) (Mean± SEM)

Sr No.	Lipoplexes (N/P - 25/1) (Lipid:Sugar Ratio of 1:3)	Parameter	Fresh Formulation	Formulation after lyophillization and resuspension in HEPES buffer pH 8.0
1	p53 lipoplexes	Particle Size (nm)	223.6 ± 8.9	245.2 ± 4.9
		Zeta Potential (mv)	28.1 ± 2.8	26.4 ± 2.5
		% Cell Viability (H 1299 cell line)	79.17 ± 1.54	78.47 ± 3.84
2	ETP-p53 lipoplexes	Particle Size (nm)	274.5 ±10.34	289.6 ± 12.9
		Zeta Potential (mv)	28.9 ± 3.2	24.6 ± 3.1
		% Cell Viability (H 1299 cell line)	50.67 ± 2.11	48.34 ± 1.37
3	DTX-p53 lipoplexes	Particle Size (nm)	329.4 ±14.7	341.8 ± 9.8
		Zeta Potential (mv)	31.3 ± 2.1	30.1± 1.8
		% Cell Viability (H 1299 cell line)	38.83 ± 3.06	35.68 ± 3.61

Further, % drug retained in the cake after resuspension was determined after extruding the suspended cake through 0. 45 and 0.22 µm polycarbonate filter paper (1 cycle) followed by dissolving the suspension in methanol and determining the % drug retained in liposomes considering entrapment before lyophillization as 100 %. At lipid : trehalose 1: 3 ratio, non-

significant change in particle size and near 100 % drug retention was observed in case of both ETP and DTX lipoplexes. No further improvement was observed on increasing the ratio of trehalose hence, lipid : trehalose ratio of 1:3 was optimized and used for all batches.

The developed trehalose based lyophilized formulations on testing for transfection and cytotoxicity showed statistically non-significant changes in results as compared to the freshly prepared formulations theirby offering the more stable formulations for DPI development and *in vivo* drug delivery.

6.10. *In Vitro* lung deposition of liposomes and lipoplexes by Anderson cascade impactor

6.10.1 Materials: Optimized liposome and lipoplex formulations in lyophilized form, Size 2 Capsules, Anderson Cascade Impactor.

6.10.2 Methods: The efficacy of *p*53 complexed liposomes, drug encapsulated liposomes and lipoplexes in the lung environment actually depends on their deep lung deposition and the dose delivered. *In Vitro* lung deposition pattern of the lyophillized drug loaded and *p*53 complexed lipoplexes in the alveolar region was determined by the Anderson cascade impactor. The capsules containing 25 mg of dry lyophilized powders (after passing through 100 #)loaded with therapeutic lipoplexes and trehalose demonstrating maximum apoptosis and necrosis in the cell lines were aerosolized using Rotahaler (Cipla) after applying vacuum at 30 LPM for 10 seconds for determining their lung deposition pattern. 12 Capsules were aerosolized through rotahaler and the powder deposited on each plate was estimated for drug content by spectroscopy and the stability of the *p*DNA in the lipoplex during aerosolization was estimated by phenol-chloroform extraction of the lipoplex followed by agarose gel electrophoresis of isolated and purified *p*DNA. Fine particle fraction and mean mass aerodynamic diameter of the formulation was determined as described previously and correlated with *in vitro* lung deposition of the formulations.

6.10.3. Result and Discussion: Further, the formulations when tested on Anderson Cascade Impactor under the standard conditions of USP, showed significant in vitro lung deposition. The FPF and MMAD of the tested formulations has been tabulated in Table 6.15 below:

All the formulations with their similar nanosized in range of 200-500 nm and same lipid:trehalose ratio showed similar deposition pattern with FPF in range of 33-37 % and MMAD of 3-4 μm. The MMAD of 3-4 μm indicates lung distribution in trachea, upper respiratory area and upper alveoli. When the powder comes in contact with lung fluids, the liposomes get hydrated and after getting detached from sugar moiety. These liposomes then can be internalized

to give intracellular cytotoxic effect as well as pDNA transfection. The powder on impactor plates when collected and purified for DNA extraction showed supercoiled DNA on agarose gel indicating pure and stable cDNA with efficient transfection capacity.

Table 6.15. FPF and MMAD of the Lyophillized Liposomal and Lipoplex formulations (n=3) (Mean± SEM)

Formulation	FPF (%)	MMAD (μm)
ETP Liposomes	36.28 ± 2.31	3.33 ± 0.18
DTX Liposomes	35.12 ± 1.99	3.81 ± 0.21
p53 Lipoplex	33.46 ± 2.92	3.91 ± 0.18
p53-DTX Lipoplex	35.32 ± 1.91	3.56 ± 0.29
p53-ETP Lipoplex	36.11 ± 2.09	3.41 ± 0.16

6.11 Conclusion

The results and discussion of this investigation conclusively demonstrate that pre-treatment and co-administration of p53 tumor suppressor gene lead to increase in cytotoxicity of ETP and DTX by enhanced apoptosis leading to reduced BCl-2 mediated antiapoptotic defense of the cells. Increased apoptosis suggests restored p53 function and reduced antiapoptotic drug resistance. It causes cell sensitization and synergism towards drugs cytotoxicities. This enhancement in cytotoxicity may finally help in reducing drug dose, resistance and side-effects and may also help in remission of the tumor completely by correcting the genetic malfunction. Further, direct lung delivery as dry powder inhaler will help in opening a new strategy to treat lung cancer in patients having drug resistance.

Although DPI formulations favors, the co-delivery approach because of simultaneous entry of cDNA and drug in the same cell and tissue, but the pretreatment approach may also show significant enhancement in cytotoxicity, probably because of the p53 bystandard effect observed in vitro and in vivo, theirby making the direct lung delivery approach a suitable treatment strategy to treat NSCLC especially in cases of drug resistance with need of enhanced target specificity.

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