Chapter 8

In vitro cytotoxicity studies

8.1 INTRODUCTION

While cytotoxicity testing is currently conducted using animals, studies published in recent years have shown a correlation between *in vitro* and *in vivo* acute toxicity. These studies suggest that *in vitro* methods may be helpful in predicting *in vivo* cytotoxicity. The use of cell cultures *in vitro* as alternatives to predict acute lethality *in vivo* has been under study for almost 50 years (Pomerat and Leake, 1954; Eagle and Foley, 1956; Smith *et. al.,* 1963).

Numerous demonstrations of correlations between cytotoxicity *in vitro* and animal lethality *in vivo* exist. (Phillips *et. al.,* 1990; Garle *et. al.,* 1994). Recently, several major international *in vitro* initiatives have been directed toward reducing the use of laboratory animals for acute toxicity testing (Curren *et. al.,* 1998; Ekwall *et. al.,* 2000; Ohno *et. al.,* 1998a, 1998b, 1998c; Seibert *et. al.,* 1996; Spielmann *et. al.,* 1999).

8.2 MEASUREMENT ENDPOINTS FOR CYTOTOXICITY

The measurement of cell viability and growth is a valuable tool in a wide range of research areas. Many measurement endpoints for cytotoxicity are well established and have been used to assess basal cytotoxicity. For inclusion of inhibitory concentration, IC50 values in the registry of cytotoxicity (RC), the following endpoints were accepted as sufficiently characteristic of basal cytotoxicity (Spielmann *et. al.,* 1999):

1) **Inhibition of cell proliferation**

- Cell number
- Cell protein
- DNA content, DNA synthesis
- Colony formation

2) **Cell viability - metabolic markers**

- Metabolic inhibition test (MIT-24)
- Mitochondrial reduction of tetrazolium salts into insoluble dye (MTT test), or, more recently, into soluble dye (MTS test or XTT test).

3) **Decreased cell viability - membrane markers**

- NRU into cell lysosomes
- Trypan Blue exclusion
- Cell attachment, cell detachment

4) **Differentiation markers**

- Functional differentiation within cell islets
- Morphological differentiation within cell islets
- Intracellular morphology

Markers of the release of intracellular components, such as the enzyme lactate dehydrogenase (i.e., LDH release test), or of dye introduced into the cells previous to chemical exposure (e.g., fluorescein leakage [FL] test or Neutral Red Release [NRR] test) were not considered to be characteristic for basal cytotoxicity because they specifically detect damage of the outer cell membrane and generally are associated with short-term chemical exposure. A chemical that specifically damages only cell membranes, however, will be detected correctly in one of the tests for basal cytotoxicity listed above.

8.3 CYTOTOXICITY PROTOCOLS

Since the RC was constructed with data from many different *in vitro* protocols, a number of different *in vitro* cytotoxicity protocols might produce correlations with *in vivo* acute lethality similar to the correlation produced by the RC. It is strongly suggested, however, that any proposed *in vitro* protocol incorporate the following conditions:

(a) Use a cell line (or primary cells) that divides rapidly with doubling times of less than 30 h under standard culture conditions, preferably with normal serum types, e.g., calf serum (CS), newborn calf serum (NBCS), or serum-free medium.

(b) Use only cells in the exponential phase of growth. Never use cells immediately after thawing them from frozen stock. Allow cells to grow 1-2 passages before they are used in the cytotoxicity test.

(c) The chemical exposure period should be at least the duration of one cell cycle, i.e., 24 –72 h (Riddell *et. al.,* 1986).

(d) Initial seeding should be done at a density that allows rapid growth throughout the exposure period.

(e) Use appropriate positive and vehicle control materials for which cytotoxicity, or lack of cytotoxicity, has been well characterized by the performing laboratory.

(f) Use solvents only at levels previously shown not to cause cytotoxicity to the cell system over the entire period of the assay.

(g) Use a measurement endpoint that is well established and that has good interlaboratory reproducibility. Preference is given to endpoints that determine either cell proliferation or cell viability (e.g., NRU, MTT, XTT). Simple endpoints such as total protein content are not recommended, as they may under - predict the toxicity of certain test chemicals by staining dead cells.

(h) The protocol should be compatible with 96-well plates and apparatus such as spectrophotometers that allow a quick and precise measurement of the endpoint.

(i) Complete a detailed concentration-response experiment using a progression factor that yields graded effects between no effect and total cytotoxicity. Any desired toxicity measure can be derived from a well-designed concentration-response experiment. Experiments that seek to detect only a marker concentration, such as the highest tolerated dose or the lowest cytotoxic dose, are characterized by a lack of information and a low level of accuracy.

The *in vitro* cytotoxicity of the anticancer drugs and their liposome formulations can be evaluated by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. MTT cell proliferation assay offers a quantitative, convenient method for evaluating a cell population's response to external factors, whether it is an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis.

8.4 PRINCIPLE OF MTT ASSAY

The antitumor activity of the drugs can be evaluated by MTT assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is a water soluble chemical. Active mitochondrial dehydrogenases of living cells convert the yellowish MTT to an insoluble purple formazan crystal (Mosmann, 1983). This conversion does not take place in dead cells. Tetrazolium salts are cleaved to formazan by the "succinate-tetrazolium reductase" system, which belongs to the respiratory chain of the mitochondria, and is only active in metabolically intact cells. This water insoluble formazan can be solubilized using dimethyl sulphoxide (DMSO) and the dissolved material can be measured spectrophotometrically.

The reduction of tetrazolium salts is now recognized as a safe, accurate alternative to radiometric testing. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation. Among the applications for the method are drug sensitivity, cytotoxicity, response to growth factors, and cell activation.

8.4.1 Advantages of MTT method

1. **Proven technology:** The utility of the MTT method has been documented in the literature for many different applications.
2. **Accurate measurements:** The spectrophotometric procedure can detect slight changes in cell metabolism, making it much more sensitive than trypan blue staining.
3. **Easy to use:** The procedure is relatively simple and uses equipment already available in most laboratories.
4. **Rapid processing:** Assays are run in a 96-well plate and read with a microtitre plate reader, allowing high-throughput handling of samples.
5. **Convenient storage:** The kit is stable for 18 months when stored under refrigeration in the dark.

8.5 EXPERIMENTAL

8.5.1 CELL LINES

The cerebral glioma cell line (BMG-1, wild type p53) was established in Institute of Nuclear Medicine and Allied Sciences, New Delhi. The cells were maintained as monolayer cultures in plastic flasks (Tarsons, India), and passaged twice every week by seeding 0.2 x 10^6 cells in two T-25 flasks. BMG-1 cell line was maintained on Dulbecco's modified eagles medium (DMEM) supplemented with 10% fetal bovine calf serum and antibiotics.

8.5.2 SOLUTIONS

8.5.2.1 MTT solution

5mg/ml MTT (Promega) in phosphate buffer saline pH 7.4 (PBS). This solution was filtered through a 0.2μm filter and stored at 2-8°C.

8.5.2.2 Solution of Methotrexate (MTX) and its liposomal formulations

Stock solution of MTX (1mM) was prepared by dissolving 4.54mg of methotrexate in 10ml of sterile PBS. The liposomal formulations containing MTX were also diluted to give a stock solution of final concentration of 1mM solution.

Suitable aliquots of the stock solution of MTX and its liposomal formulations were added into the wells containing DMEM medium to give final concentrations of 100, 500, 1000, 2000, 5000 and 10000nM of MTX.

8.5.2.3 Solution of Tamoxifen citrate and its liposomal formulations

Stock solution of TMX (1mM) was prepared by dissolving 5.63mg of tamoxifen citrate in 10ml of sterile PBS containing 0.5% cremophor EL-35 as surfactant for solubilizing TMX. The liposomal formulations containing TMX were also diluted to give a stock solution of final concentration of 1mM solution.

Suitable aliquots of the stock solution of TMX and its liposomal formulations were added into the wells containing DMEM medium to give final concentrations of 100, 500, 1000, 2000, 5000 and 10000nM of TMX.

8.5.2.4 Solution of Cytarabine and its liposomal formulations

Stock solution of CYT (1mM) was prepared by dissolving 2.43mg of cytarabine in 10ml of sterile PBS. The liposomal formulations containing CYT were also diluted to give a stock solution of final concentration of 1mM solution.

Suitable aliquots of the stock solution of CYT and its liposomal formulations were added into the wells containing DMEM medium to give final concentrations of 100, 500, 1000, 2000, 5000 and 10000nM of CYT.

8.5.3 MTT ASSAY

Human cerebral glioma cancer cell line (BMG-1) cells were harvested using 0.05% trypsin and about 5000cells/ well were seeded into 96 well flat-bottomed plate in 200 µl of DMEM containing 10% fetal bovine serum for 24h to allow the cells to attach and reach the exponential growth phase. After the addition of 200µl DMEM containing various concentrations of drugs and their liposome formulations, the plates were incubated for 24 and 48h at 37°C in a humidified incubator (5% CO_2 and 95% air). 20µl of MTT solution was added to each well and incubated at 37°C in a humidified incubator for 2h. The medium was then removed and 150µl of DMSO was added to each well. The plate was mechanically shaken for a few minutes to dissolve the purple colored formazan crystals. The optical density was measured at 570nm by keeping the reference at 630nm using an Elisa Plate Reader.

Figure 8.1 Time dependent *in vitro* cytotoxicity study of methotrexate

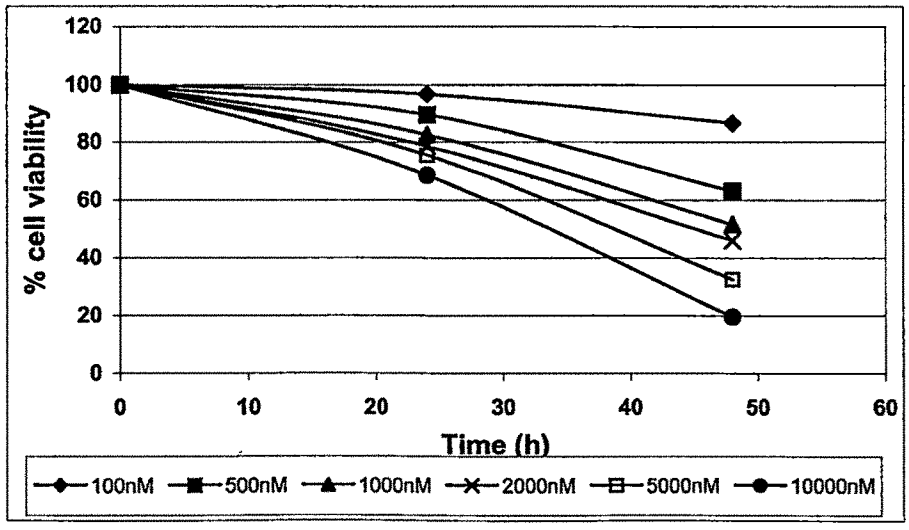
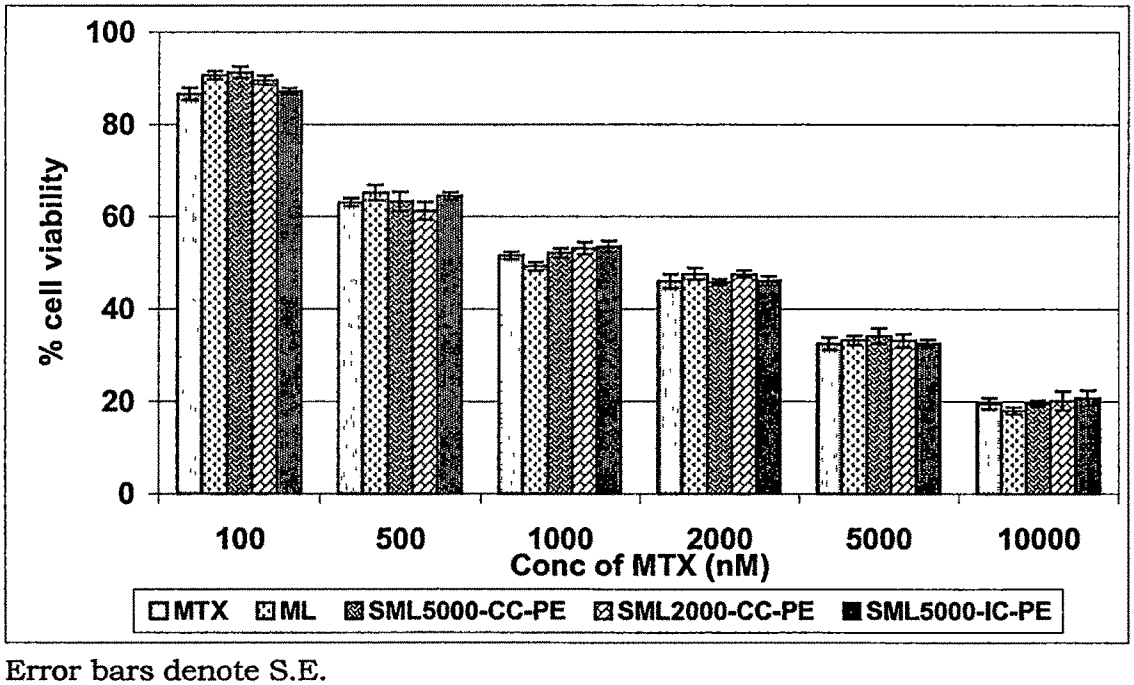


Table 8.1 ***In vitro* cytotoxicity study of methotrexate and its liposomes**

Conc of MTX (nM)	% Cell Viability (± S.E.)*				
	MTX	ML	SML5000-CC-PE	SML2000-CC-PE	SML5000-IC-PE
100	86.59±1.32	90.56±0.89	91.22±1.22	89.54±0.97	87.21±0.64
500	63.12±0.88	65.23±1.65	63.33±2.06	61.25±1.99	64.52±0.79
1000	51.61±0.75	49.24±0.98	52.13±1.06	53.14±1.33	53.46±1.21
2000	45.97±1.52	47.56±1.26	45.75±0.68	47.54±0.74	46.12±0.99
5000	32.48±1.33	33.26±1.02	34.15±1.66	33.16±1.44	32.54±0.88
10000	19.54±1.22	18.02±0.78	19.52±0.68	20.16±2.05	20.77±1.65

*n=3

Figure 8.2 ***In vitro* cytotoxicity study of methotrexate and its liposomes**



Error bars denote S.E.

Figure 8.3 Time dependent *in vitro* cytotoxicity study of tamoxifen citrate

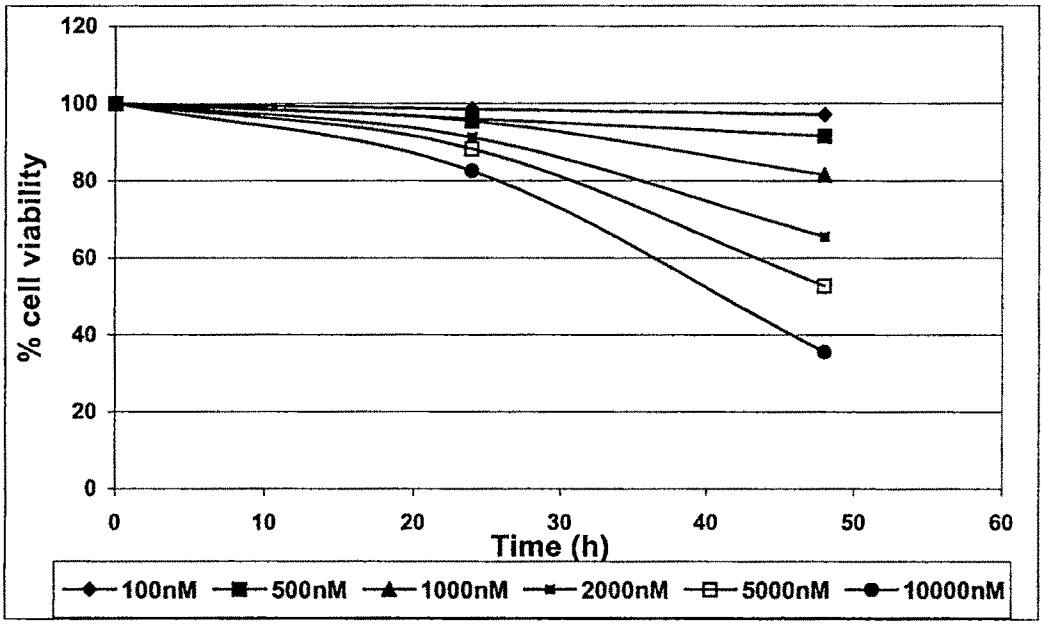
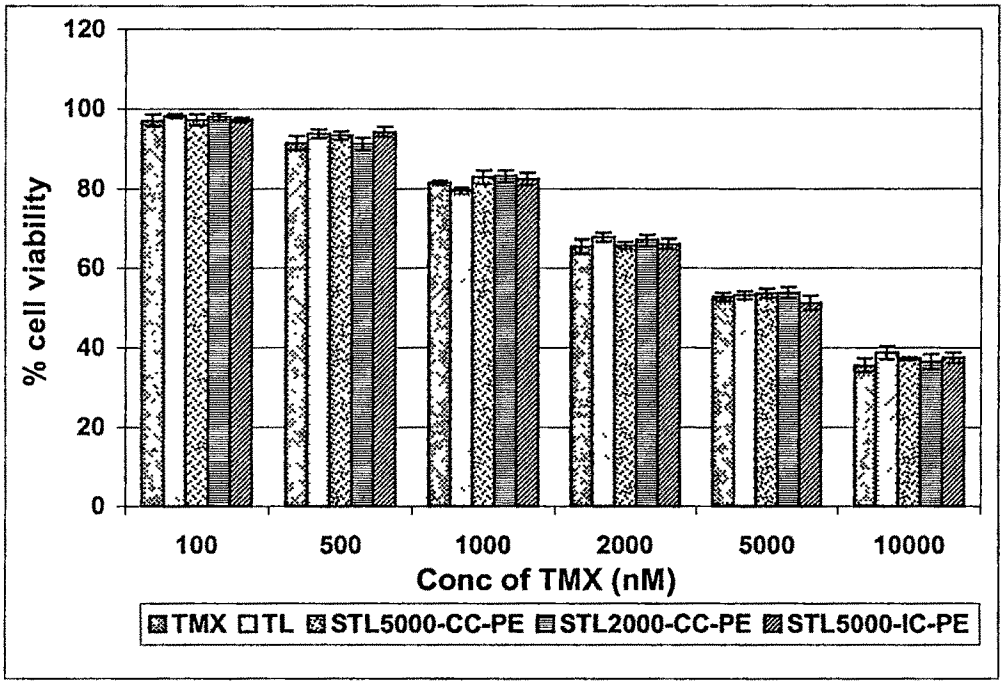


Table 8.2 *In vitro* cytotoxicity study of tamoxifen citrate and its liposomes

Conc of TMX (nM)	% Cell Viability (± S.E.)*				
	TMX	TL	STL5000-CC-PE	STL2000-CC-PE	STL5000-IC-PE
100	97.12±1.47	98.26±0.54	97.22±1.42	98.11±0.77	97.28±0.55
500	91.52±1.75	93.83±1.11	93.39±1.05	91.25±1.45	94.22±1.29
1000	81.52±0.48	79.45±0.78	82.86±1.75	83.14±1.45	82.46±1.54
2000	65.47±1.79	67.74±1.16	65.65±0.87	67.04±1.34	66.02±1.29
5000	52.74±1.08	53.22±1.02	53.65±1.21	53.76±1.44	51.26±1.78
10000	35.55±1.74	38.72±1.68	37.22±0.44	36.54±1.87	37.47±1.31

*n=3

Figure 8.4 ***In vitro*** **cytotoxicity study of tamoxifen citrate and its liposomes**



Error bars denote S.E.

Figure 8.5 **Time dependent** ***in vitro*** **cytotoxicity study of cytarabine**

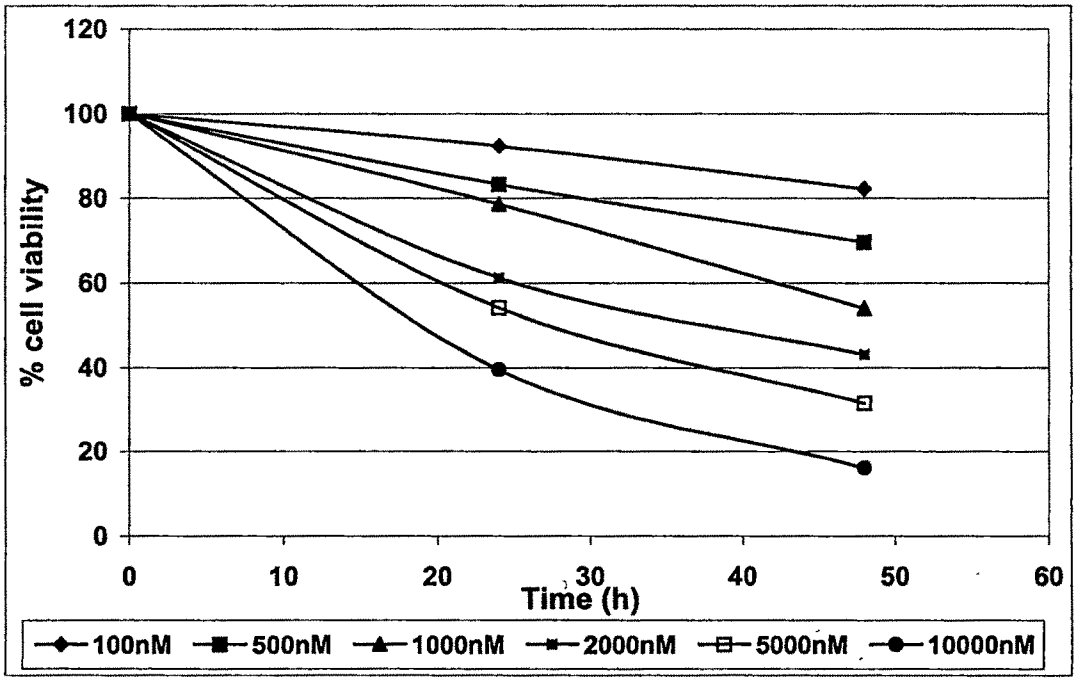
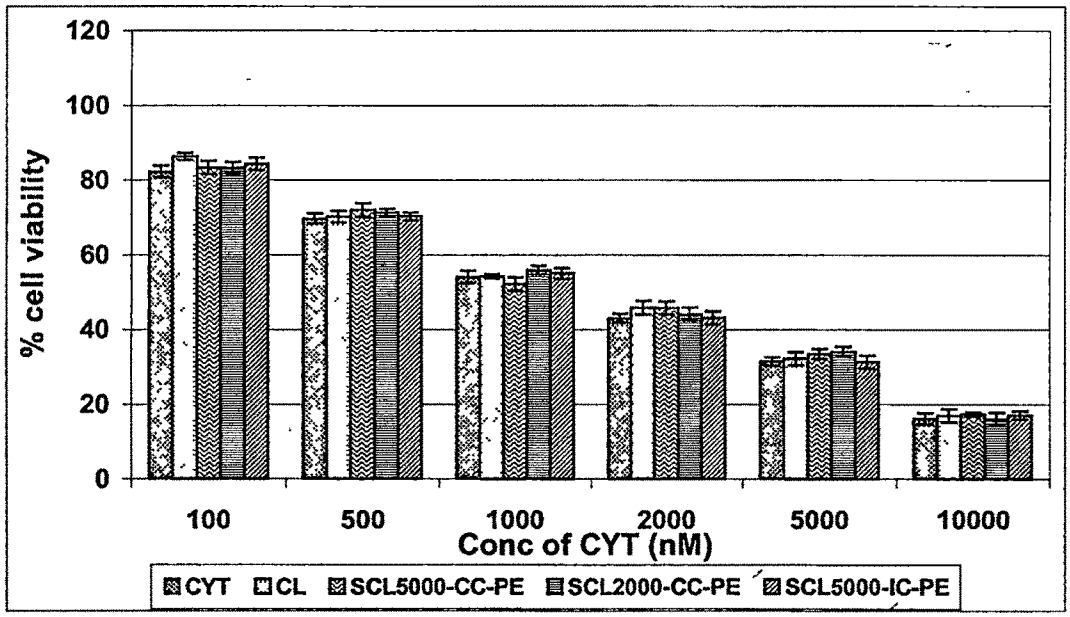


Table 8.3 ***In vitro*** **cytotoxicity study of cytarabine and its liposomes**

Conc of CYT (nM)	% Cell Viability (± S.E.)*				
	CYT	CL	SCL5000-CC-PE	SCL2000-CC-PE	SCL5000-IC-PE
100	82.22±1.59	86.24±0.97	83.27±1.79	83.22±1.57	84.28±1.76
500	69.64±1.41	70.12±1.52	71.90±1.85	71.25±1.01	70.29±1.02
1000	54.01±1.64	54.19±0.49	52.16±1.79	55.84±1.16	54.97±1.44
2000	43.07±1.19	45.77±1.87	45.75±1.74	44.15±1.77	43.18±1.77
5000	31.47±1.08	32.22±1.79	33.44±1.41	33.98±1.41	51.26±1.72
10000	16.14±1.44	16.92±1.74	17.27±0.67	16.04±1.74	17.09±1.07

*n=3

Figure 8.6 ***In vitro*** **cytotoxicity study of cytarabine and its liposomes**



Error bars denote S.E.

8.6 RESULTS AND DISCUSSION

The *in vitro* cytotoxicity study was carried out as a preliminary study before performing the *in vivo* biodistribution study of the liposomes encapsulated anticancer drugs (MTX, TMX and CYT). This was carried out to evaluate the cytotoxicity potential of the drugs when encapsulated in conventional and sterically stabilized liposomes.

8.6.1 Methotrexate and its liposomes

Various concentrations (100-10000nM) of MTX and its liposomes (ML, SML5000-CC-PE, SML2000-CC-PE and SML5000-IC-PE) were added to the 96 wells flat bottomed plate containing BMG-1 cells grown in DMEM medium. The Figure 8.1 explains about the relationship between the cytotoxicity of MTX with respect to the time. The cytotoxicity was evaluated at three time points (0, 24 and 48h). The cytotoxicity effect of MTX was found (68 to 96% of cell viability) after 24h, but appreciable amount of cytotoxicity was found after 48h of incubation. Hence, evaluation of the cytotoxic potential of the liposome encapsulated MTX was carried out after 48h.

The cytotoxicity effect produced by MTX and its liposomes after 48h of incubation at 37°C in a humidified incubator (5% CO_2 and 95% air) is shown in Table 8.1 and Figure 8.2.

As the concentration of the drug (in free form as well as in liposomal formulations) increases there was a gradual reduction in the % cell viability, which shows the cytotoxicity effect produced by methotrexate. The cytotoxicity produced by the MTX and by its liposomes was almost similar and no significant difference was found between them. After 48h of incubation, there was a reduction of almost 80% of cell viability at a concentration of 10000nM of MTX. The reason for the similar cytotoxicity effect may be due to the phospholipid used. The use of egg phosphatidyl choline, which is having a transition temperature at around 37°C, may be attributed to the drug release from the liposomes when incubated at 37°C. The addition of the polymers (mPEG5000-CC-PE, mPEG2000-CC-PE and mPEG5000-IC-PE) in the bilayer for producing steric stability to the liposomes did not cause any significant change in the cytotoxicity of the

drug. The cytotoxic potential of the empty liposomes was also tested and found to be non-toxic to the cells, which proves that the cytotoxicity caused by the liposomal MTX is due to the released drug and not due to any components of the liposome bilayer.

8.6.2 Tamoxifen citrate and its liposomes

Various concentrations (100-10000nM) of TMX and its liposomes (TL, STL5000-CC-PE, STL2000-CC-PE and STL5000-IC-PE) were added to the 96 wells flat bottomed plate containing BMG-1 cells grown in DMEM medium. The Figure 8.3 explains about the relationship between the cytotoxicity of TMX with respect to the time. The cytotoxicity was evaluated at three time points (0, 24 and 48h). The significant cytotoxicity effect of TMX was not found (82 to 98% of cell viability) after 24h, but appreciable amount of cytotoxicity was found after 48h of incubation. The reason may be due to the hydrophobicity of the drug and release of drug from liposomes due to its high lipophilic nature. Hence, evaluation of the cytotoxic potential of the liposome encapsulated TMX was carried out after 48h.

The cytotoxicity effect produced by TMX and its liposomes after 48h of incubation at 37°C in a humidified incubator (5% CO_2 and 95% air) is shown in Table 8.2 and Figure 8.4. As the concentration of the drug (in free form as well as in liposomal formulations) increases there was a gradual reduction in the % cell viability, which shows the cytotoxicity effect produced by tamoxifen citrate. After 48h of incubation, there was a reduction of almost 65% of cell viability at a concentration of 10000nM of TMX. The cytotoxicity produced by the TMX and by its liposomes was almost similar and no significant difference was found between them. The reason for the similar cytotoxicity effect may be due to the phospholipid used. The use of egg phosphatidyl choline, which is having a transition temperature at around 37°C, may be attributed to the drug release from the liposomes when incubated at 37°C. The addition of the polymers (mPEG5000-CC-PE, mPEG2000-CC-PE and mPEG5000-IC-PE) in the bilayer for producing steric stability to the liposomes did not cause any significant change in the cytotoxicity of the drug. The cytotoxic potential of the empty liposomes was also tested and found to be non-toxic to the cells,

which proves that the cytotoxicity caused by the liposomal TMX is due to the released drug and not due to any components of the liposome bilayer.

8.6.3 Cytarabine and its liposomes

Various concentrations (100-10000nM) of CYT and its liposomes (CL, SCL5000-CC-PE, SCL2000-CC-PE and SCL5000-IC-PE) were added to the 96 wells flat bottomed plate containing BMG-1 cells grown in DMEM medium. The Figure 8.5 explains about the relationship between the cytotoxicity of CYT with respect to the time. The cytotoxicity was evaluated at three time points (0, 24 and 48h). The cytotoxicity effect of CYT was found (39 to 92% of cell viability) after 24h, but appreciable amount of cytotoxicity was found after 48h of incubation. Hence, evaluation of the cytotoxic potential of the liposome encapsulated CYT was carried out after 48h.

The cytotoxicity effect produced by CYT and its liposomes after 48h of incubation at 37°C in a humidified incubator (5% CO_2 and 95% air) is shown in Table 8.3 and Figure 8.6. As the concentration of the drug (in free form as well as in liposomal formulations) increases there was a significant reduction in the % cell viability, which shows the cytotoxicity effect produced by cytarabine. The cytotoxicity produced by the CYT and by its liposomes was almost similar and no significant difference was found between them. After 48h of incubation, there was a reduction of around 84% of cell viability at a concentration of 10000nM of CYT. The reason for the similar cytotoxicity effect may be due to the phospholipid used. The use of egg phosphatidyl choline, which is having a transition temperature at around 37°C, may be attributed to the drug release from the liposomes when incubated at 37°C. The addition of the polymers (mPEG5000-CC-PE, mPEG2000-CC-PE and mPEG5000-IC-PE) in the bilayer for producing steric stability to the liposomes did not cause any significant change in the cytotoxicity of the drug. The cytotoxic potential of the empty liposomes was also tested and found to be non-toxic to the cells, which proves that the cytotoxicity caused by the liposomal CYT is due to the released drug and not due to any components of the liposome bilayer. The results obtained were in agreement with the earlier reports (Storm *et. al.,* 1988; Aviva *et. al.,* 1992).

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