CHAPTER 9

INVITROCYTOTOXICITY STUDY OF LEUPROLIDE ACETATE LIPOSOMES

9.1 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).

9.2 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 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.

9.3 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 and antiproliferative 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.

9.4 PRINCIPLE OF THE MTT ASSAY

The antiproliferative activity of the drugs can be evaluated by MTT assay. Cell proliferation and viability assays are of particular importance for routine applications in cell biology. Tetrazolium salts (*e.g.*, MTT, XTT, WST-1) are particularly useful for this type of analysis. 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. The figure below shows an example of this type of reaction.

MTT is used for the quantitative determination of cellular proliferation and activation (*e.g.*, in response to growth factors and cytokines such as IL-2 and IL-6). It is also used for the quantitation of antiproliferative or cytotoxic effects (*e.g.*, mediated by tumor necrosis factor-alpha or -beta) and for the measurement of interferon action. In cancer research, the MTT assay is used for the quantitation of *in vitro* tumor cell chemosensitivity, for the assessment of photoradiation therapy, and for the screening of anticancer compounds. MTT dissolved in agarose overlays is also used to improve the visualization of virus-infected cells in plaque assays.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is a water soluble chemical. The assay is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells (Mosmann, 1983). The MTT formazan crystals are insoluble in aqueous solution, but may be solubilized by adding dimethyl sulphoxide (DMSO) solution, then incubating the plates overnight in a humidified atmosphere (*e.g.*, 37°C, 6.5% CO_2). The solubilized formazan product can be spectrophotometrically quantitated using an ELISA reader. An increase in the number of living cells results in an increase of total metabolic activity, which leads to a stronger color formation.

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.

9.4.1 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 labs.

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.

9.5 EXPERIMENTAL

9.5.1 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.

9.5.2 SOLUTIONS

9.5.2.1 MTT solution

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

9.5.2.2 Solution of leuprolide acetate and its liposomal formulations

Stock solution of leuprolide acetate (1mM) was prepared by dissolving 12.7mg of leuprolide acetate in 10ml of sterile PBS. The liposomal formulations containing leuprolide acetate were also diluted to give a stock solution of final concentration of 1mM solution.

Suitable aliquots of the stock solution of leuprolide acetate and its liposomal formulations were added into the wells containing DMEM medium to give a final concentration 0.001μM, 0.01μM, 0.1μM, 1μM, 10μM and 100μM of leuprolide acetate.

Figure 9.1 Time dependant *in vitro* cytotoxicity study of leuprolide acetate

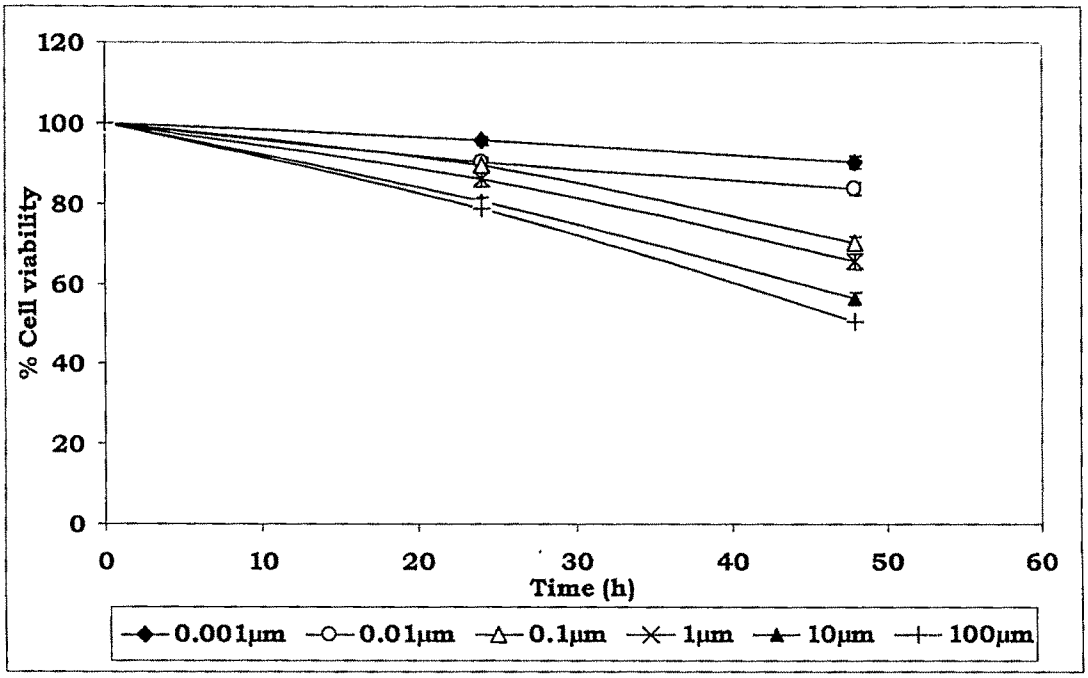
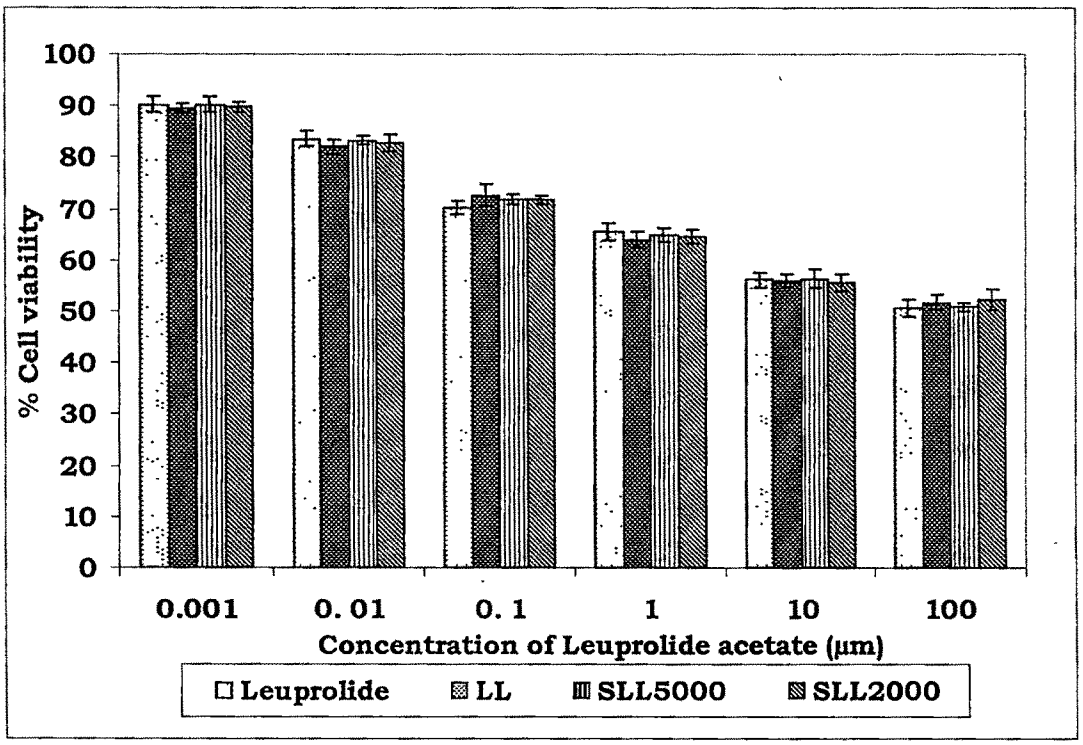


Table 9.1 *In vitro* cytotoxicity study of leuprolide acetate and its liposomes

Concentration of Leuprolide acetate (µM)	% Cell Viability (± S.E.)*			
	Leuprolide acetate	LL	SLL5000	SLL2000
0.001	90.2±1.62	89.52±0.75	90.22±1.63	89.67±0.92
0. 01	83.6±1.53	81.98±1.56	83.26±0.69	82.69±1.63
0. 1	70.2±1.45	72.65±2.02	71.92±0.99	71.69±0.69
1	65.6±1.78	63.98±1.69	64.9±1.23	64.56±1.26
10	56.2±1.56	55.98±1.36	56.39±1.89	55.62±1.69
100	50.7±1.69	51.69±1.48	50.89±0.89	52.36±1.92

Figure 9.2 ***In vitro*** **cytotoxicity study of leuprolide acetate and its liposomes**



9.6 RESULTS AND DISCUSSION

The *in vitro* cytotoxicity study was carried out as a preliminary study before carryout the *in vivo* biodistribution study of the liposomes encapsulated antiproliferative drug (leuprolide acetate). This was carried out to evaluate the cytotoxicity potential of the drug when encapsulated in conventional and sterically stabilized liposomes.

9.6.1 LEUPROLIDE ACETATE AND ITS LIPOSOMES

Various concentrations (0.001µM, 0.01µM, 0.1µM, 1µM, 10µM and 100µM) of leuprolide acetate and its liposomes (LL, SLL5000 and SLL2000) were added to the 96 wells flat bottomed plate containing BMG-1 cells grown in DMEM medium. The figure 9.1 explains about the relationship between the cytotoxicity of leuprolide acetate with respect to the time. The cytotoxicity was evaluated at three time points (0, 24 and 48h). The cytotoxicity effect of leuprolide acetate was found (78 to 95% 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 leuprolide acetate was carried out after 48h.

The cytotoxicity effect produced by leuprolide acetate and its liposomes after 48h of incubation at 37°C in a humidified incubator (5% CO_2 and 95% air) is shown in table 9.1 and Figure 9.2. 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 leuprolide acetate. The cytotoxicity produced by the leuprolide acetate 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 50% of cell viability at a concentration of 100μM of leuprolide acetate. The reason for the similar cytotoxicity effect may be due to the drug release from the liposomes when incubated at 37°C. The addition of the polymers (mPEG5000-CC-PE, and mPEG2000-CC-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 leuprolide acetate is due to the released drug and not due to any components of the liposome bilayer.

REFERENCES

Curren, R., Bruner, L., Goldberg, A., Walum, E. (1998). 13th meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Validation and acute toxicity testing. *Environ. Health Persp.*, 106, (Suppl. 2), 419-425.

Eagle, H., Foley, G.E. (1956). The cytotoxic action of carcinolytic agents in tissue culture. *Am. J. Med.*, 21, 739-745.

Ekwall, B., Ekwall, B., Sjostrom, M. (2000). MEIC evaluation of acute systemic toxicity: Part VIII. Multivariate partial least squares evaluation, including the selection of a battery cell line tests with a good prediction of human acute lethal peak blood concentrations for 50 chemicals. *ATLA* 28 (Suppl. 1), 201-234.

Garle, M., Fentem, J.H., Fry, J.R. (1994). In vitro cytotoxicity tests for the prediction of acute toxicity in vivo. *Toxicol. In Vitro.*, 8, 1303-1312.

Mosmann, T. (1983). Rapid Colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods.*, 65, 55-63.

Ohno, T., Asakura, M., Awogi, T. (1998a). Validation study on five cytotoxicity assays by JSAAE. I. Overview of the study and analyses of variations of ED50 values. *AATEX.*, 5,1-38.

Ohno, T., Futamura, Y., Harihara, A. (1998b). Validation study on five cytotoxicity assays by JSAAE. VI. Details of the LDH release assay. *AATEX.*, 5, 99-118.

Ohno, T., Futamura, Y., Harihara, A. (1998c). Validation study on five cytotoxicity assays by JSAAE. VIII. Details of the neutral red uptake assay. *AATEX* ., 5, 131-145.

Phillips, J.C., Gibson, W.B., Yam, J., Alden, C.L., Hard, G.C. (1990). Survey of the QSAR and in vitro approaches for developing non-animal methods to supersede the in vivo LD50 test. *Food Chem. Toxicol.* 28, 375-394.

Pomerat, C.M., Leake, C.D. (1954). Short term cultures for drug assays: general considerations. *Ann. New York Acad. Sci.*, 58, 1110-1128.

Riddell, R.J., Panacer, D.S., Wilde, S.M., Clothier, R.H., Balls, M. (1986). The importance of exposure period and cell type in in vitro cytotoxicity tests. *ATLA*. 14, 86-92.

Seibert, H., Balls, M., Fentem, J.H., Bianchi, V., Clothier, R.H., Dierickx, P,J., Ekwall B., Garle, M.J., Gómez-Lechón, M.J., Gribaldo, L., Gülden, M., Liebsch, M., Rasmussen, E., Roguet, R., Shrivastava, R., Walum, E. (1996). Acute toxicity testing in vitro and the classification and labeling of chemicals: The report and recommendations of ECVAM Workshop 16. *ATLA*, 24, 499-510.

Smith, C.G., Grady, J.E., Northam, J.I. (1963). Relationship between cytotoxicity in vitro and whole animal toxicity. *Cancer Chemoth. Rep.*, 30, 9-12.

Spielmann, H., Balls, M., Dupuis, J., Pape, W.J.W., Pechovitch, G., De Silva, O., Holzhütter, H.G., Clothier, R., Desolle, P., Gerberick, F., Liebsch, M., Lovell, W.W., Maurer, T., Pfannenbecker, U., Potthast, J.M., Csato, M., Sladowski, D., Steiling, W., Brantom, P. (1998a). EU/COLIPA "In vitro phototoxicity" validation study, results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. *Toxicol. In Vitro.*, 12, 305-327.