Research Article CODEN: IJPRNK IMPACT FACTOR: 1.862 ISSN: 2277-8713
Patel H, IJPRBS, 2014; Volume 3(3): 581-590 IJPRBS

INTERNATIONAL JOURNAL OF
PHARMACEUTICAL RESEARCH AND BIO-SCIENCE

IN-VITRO CYTOTOXICITY STUDIES FOR CALCIUM COATED LIPOSOME

PATEL H, PATEL H, CHUDASMA A, PATEL N
Shri J. J. T. University, Jhunjhunu, Rajasthan.

Accepted Date: 07/06/2014; Published Date: 27/06/2014

Abstract: Cytotoxicity testing is currently conducted using animals, studies published have shown a correlation between in vivo and in vitro acute toxicity. The use of cell culture in vitro as an alternative approach to predict acute lethality in vivo has been under study for almost 50 years. Numerous demonstration of correlations between cytotoxicity in vitro and animal lethality in vivo exist. In-vitro cytotoxicity studies were performed for Vinblastine sulfate loaded liposomes. For H1299cells, after 48 h incubation, calcium phosphate coated liposome (IC_{50}=1.5µM) exhibit superior cytotoxic activities to liposome (IC_{50}=4.25µM) and free VBS (IC_{50}=6.75µM). Liposomal formulation show higher cytotoxicity compared to free VBS. However, calcium phosphate coated liposome exhibit higher cytotoxicity than liposome and free VBS.

Keywords: Cytotoxicity, Liposome, In-Vitro.

Corresponding Author: MR. HARESH PATEL

Access Online On:

www.ijprbs.com

How to Cite This Article:

Patel H, Patel H, Chudasma A, Patel N; IJPRBS, 2014; Volume 3(3): 581-590

PAPER-QR CODE

INTRODUCTION

While cytotoxicity testing is currently conducted using animals, studies published have shown a correlation between in vivo and in vitro acute toxicity. These studies suggest that in vitro methods may be helpful in predicting in vivo cytotoxicity (Eagale and Forey, 1956). The use of cell culture in vitro as an alternative to predict acute lethality in vivo has been under study for almost 50 years. Numerous demonstration of correlations between cytotoxicity in vitro and animal lethality in vivo exist (Phillips et al. 1990).

PROTOCOL

The current literature indicates the in vitro cytotoxicity in murine and human cell lines with in vivo acute lethality in animals. However, all the in vitro cytotoxicity protocols incorporate the following condition (Freshney R. I. et al.)-

Use a cell line that divides rapidly with doubling times of less than 30 hours under standard culture conditions , preferably with normal serum type e.g. calf serum , new born calf serum or serum free medium.

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

(b) The chemical exposure period should be at least the duration of one cell cycle .i.e. 24 to 72 h.

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

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

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

(f) Use a measurement end point that is well established that has inter-laboratory reproducibility. Preference is given to end points that determine either cell proliferation or cell viability (e.g., NRU, MTT, XTT).

(g) The protocol should be compatible with 96 well plates and apparatus such as spectrophotometer and microplate ELISA reader that allows a quick and precise measurement of the end point.

(h) Complete a detailed concentration response experiment using a progression factor that yields graded effect between no effect and total cytotoxicity. Any desired toxicity study

measure can be derived from a well designed concentration response experiments. Experiments that seek to detect only a marker concentration such as the highest tolerated dose or a lowest cytotoxicity dose are characterized by lack of information and a low level of accuracy.

MTT ASSAY

Cell cytotoxicity assay was carried out to measure the ability of the cells to survive and to continue to proliferate. The *IN-VITRO* cytotoxicity of the anticancer drugs and their formulations can be evaluated by using MTT Assay. The existing well described MTT assays to evaluate the cellular sensitivity to chemotherapeutics drugs was employed because of ease of use, optimal condition, measurement requirement, etc. MTT assay offers advantages like, rapid, versatile, quantitative and highly reproducible, Adaptable to large-scale screening, relevant for most cells.

This assay is a sensitive, quantitative and reliable colorimetric assay that measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product which is insoluble in water.

The amount of formazan produced is directly proportional to the cell number in range of cell lines. A solubilization solution (usually either dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The absorption maximum is dependent on the solvent employed. The absorbance directly correlates with the cell number. (Twenty man et al., 1987)

EXPERIMENTAL

Materials and Instrument

Table 1 List of Materials and Instrument

Material	Company
H1299 cell line	NCCS, Pune
Dulbecco Modified Eagle medium (DMEM),	HIMEDIA
Trypsin-EDTA solution 1X	HIMEDIA
Antibiotic-antimycotic solution	HIMEDIA

Fetal Bovine serum	HIMEDIA
MTT	HIMEDIA
DMSO	S.D. Fine chemicals, India
Sodium hypochlorite	S.D. Fine chemicals, India
Isopropyl alcohol	S.D. Fine chemicals, India
Polycarbonate 0.2 ,0.4 micron membrane	Wattmann
Calibrated pipettes of 5,10 and 25 ml	Tarson, India
volumetric flasks (10, 25, 50 and 100 ml)	Tarson, India
Beakers (250 ml)	Tarson, India
Flat bottom sterile 96-well plates	Tarson, India
Tissue culture flask (75 ml)	Tarson, India
Serological pipettes (5,10 and 25 ml)	Tarson, India
Phosphate Buffer saline (PBS)	HIMEDIA
Trypan Blue	HIMEDIA
Haemocytometer	
ELISA microplate Reader	Bio-Rad
Laminar air flow system	Swastika, India
IGO 150 incubator	Jouan
CKX41 Inverted Microscope	Olympus

Media preparation

1 liter media was prepared by adding Dulbecco's modified Egale's medium (DMEM) in 1 liter sterile wide mouth bottle. 5 ml Antibiotic solution (containing Penicillin G, Streptomycin and Amphotericin B) was added into it and filtered through 0.2µm filter then 10% FBS and 2mM L-glutamine were added and stored in media bottle and wrapped with aluminum foil. The whole process carried out in sterile hood.

SUBCULTURING PROTOCOL FOR H 1299 cell line

The H1299 cell line was established in NCCS pune. The cells were maintained in above prepared media as monolayer culture in T 75 cell culture flasks (tarsons, india), and Cells were

subcultured every 2–3 days wit h trypsin to retain cell densities below 80% confluence. H1299 cells were cultured in a 95% relative humidity, 5% CO_2 atmosphere at 37°C in a humidified incubator. The medium was changed every other day.

Following procedure was followed for the sub culturing –

1. Remove culture medium from the Tissue culture flask T 75.
2. Add 2.0 ml of Trypsin-EDTA solution to flask shake it and remove it and incubate for 2-3 minutes, tap the flask gently.
3. Observe cells under an inverted microscope for cell detachment.

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37 ^{0}C to facilitate dispersal. Remove the Trypsin-EDTA solution from the flask.

4. Add 10.0 ml of complete growth medium and aspirate cells by gently pipetting. Marking for passage no. on the T 75 culture flask.
5. Incubate cultures at 37 ^{0}C.

CELL COUNTING USING HAEMOCYTOMETER

1. Preparing haemocytometer

1. Ensure the haemocytometer is clean using 70% ethanol.
2. Moisten the shoulders of the haemocytometer and affix the cover slip using gentle pressure and small circular motions. The phenomenon of Newton's rings can be observed when the cover slip is correctly affixed, thus the depth of the chamber is ensured.

2. Preparing cell suspension

1. Make sure the cell suspension to be counted is well mixed by either gentle agitation of the flask containing the cells (or other appropriate container). A serological pipette may be used if required.
2. Before the cells have a chance to settle take out about 1 ml of cell suspension using a serological pipette and place in an Eppendorf tube.
3. Using a 100 µl pipette, mix the cells in this sample again (gently to avoid lysing them).Take out 100 µl and place into a new Eppendorf, add 100 µl trypan blue and mix gently again.

3. Counting

1. Using the micro pipette, draw up some cell suspension containing trypan blue. Carefully fill the haemocytometer by gently resting the end of the Gilson tip at the edge of the chambers. Take care not to overfill the chamber. Allow the sample to be drawn out of the pipette by

capillary action, the fluid should run to the edges of the grooves only. Re-load the pipette and fill the second chamber if required.

2. Focus on the grid lines of the haemocytometer using the 10X objective of the microscope. Focus on one set of 16 corners square as indicated by the circle in the diagram below.

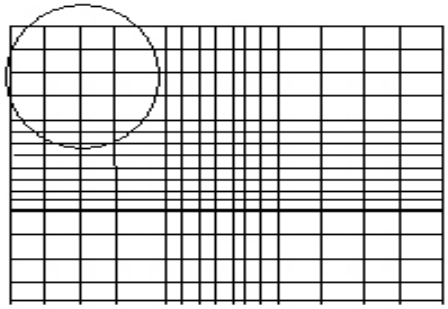

Fig 1 Haemocytometer diagram indicating the 16 corner squares which should be used for counting.

3. Using a hand tally counter, count the number of cells in this area of 16 squares. When counting, always count only live cells that look healthy (unstained by trypan blue). Count cells that are within the square and any positioned on the right hand or bottom boundary line. Dead cells stained blue with trypan blue can be counted separately for a viability count.

4. Move the haemocytometer to another set of 16 corner squares and carry on counting until all 4 sets of 16 corner squares are counted.

5. The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10^4 / ml.

6. Calculate the average no. of cells in 4 sets of 16 corners.

The total count from 4 sets of 16 corner = Average no. of cells/ml x 10^4 x 2

Where, 10^4 is conversion factor (Conversion of 0.1 mm^3 to ml) and 2 is dilution factor.

MTT solution

The MTT dye was dissolved in culture media (5 mg/ml). This stock solution was filtered through a 0.2µm filter and stored at 2-8°C in dark.

Solution Preparation of Plain drug, liposome and CPCL

The solution of plain drug (VBS), liposomal and CPCL formulation containing drug (100µM) was prepared in sterile PBS. This solution of drug and formulations containing drug filtered through 0.2µm filter and then 1 ml of these solutions pipette out, diluted up to 5 ml using DMEM to

obtain 20µM stock solutions of each (plain drug and formulations). From the above stock solution, aliquots of 10µl, 50µl, 100µl, 0.5 ml, 1 ml were accurately withdrawn with the help of micropipette and transferred to 2 ml eppendorf tube and the volume was made up to the mark using DMEM to give the final concentration of 0.1 µM,0.5 µM,1µM, 5µM, 10µM.

IN VITRO CYTOTOXICITY STUDY

The cytotoxicity of plain drug, Liposomal and CPCL formulation were evaluated by using methylthiazoletetrazolium (MTT) method.

- Briefly, 2.0 × 10^4 cells/well in its exponential growth phase were plated in 96-well flat-bottom tissue-culture plates.
- The cells were incubated at 37 ^{0} C in a 5% CO_2 incubator for 24 h, during which cells were attached and resumed to grow.
- The well was washed with sterile PBS and treated with 200µl medium containing plain drug, liposomal and CPCL formulation, respectively, at equal molar concentration ranging from 100nM to 10 µM.
- Control wells were treated with equivalent volumes of media.
- After additional 48 h incubation at 37 ˚C, 5% CO_2, each well was washed with sterile PBS and added with 20µl MTT solution and incubated for 4 hr.
- The unreduced MTT was then discarded. Each well was washed with 100µL of PBS and was replaced with 200µl DMSO to dissolve the blue formazan crystal converted from MTT. Plates were shaken for 20 min and absorbance was read at 595 nm using the microplate reader (ELISA Reader - Biorad).

Cell viability was determined using the formula in Eq. (1)

$$\% \text{ Viability} = \frac{\text{Mean Absorbance of Sample}}{\text{Mean Absorbance of Control}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots (1)$$

Where absorbance of sample and control cells represents the amount of formazan determined for cells treated with the different formulations and for control cells (nontreated), respectively. Cytotoxicity data were expressed as IC_{50} values, i.e., the inhibitory concentration of the drugs required for 50% reduction in cell population.

RESULT AND CONCLUSION

The cell viability of VBS and its formulations at different concentrations (µM) is shown below-

Table 2 Cell viability (%) of VBS and its formulation in H 1299 cells

Concentration (µM)	Cell viability (%)		
	Plain Drug(VBS)	LIPOSOMES	CPCL
0.1	97.67±0.28	96.89±0.17	93.12±0.43
0.5	92.45±0.48	86.13±0.45	78.37±0.15
1	75.23±0.33	69.34±0.52	52.39±0.68
5	59.15±0.39	48.23±0.25	31.78±0.37
10	35.56±0.57	27.15±0.34	9.23±0.19

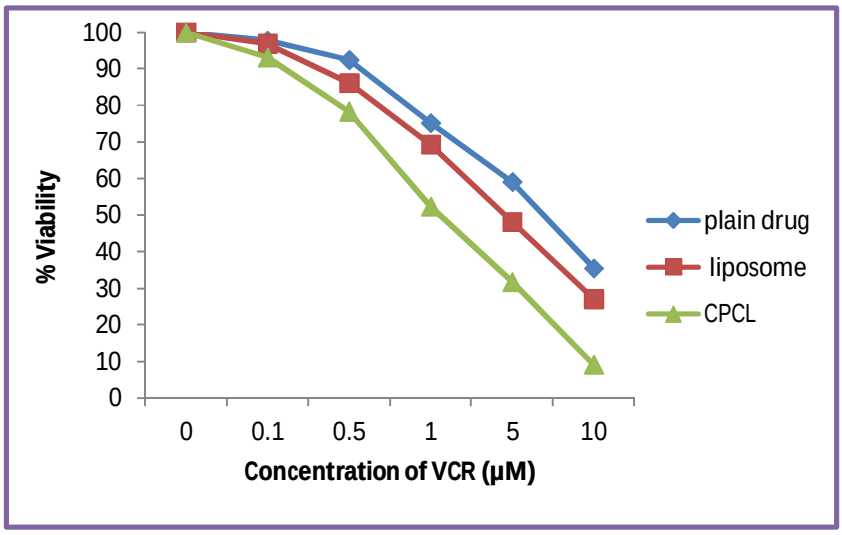

Fig 2 Cytotoxic effects of VBS and its formulation against H 1299 cells

With increase in concentration, % cell viability is decreasing. There was no cytotoxic effect found in H1299 cells treated with blank Liposome.

The IC50 values (*i.e.*, concentration resulting in 50% growth inhibition) of VBS and its formulations were graphically calculated from concentration-effect curves, considering the optical density of the control well as 100% (Sharma et al., 1996).

Table 3 IC50 values of VBS and its formulation against H1299 cells determined by MTT assay

Formulation	IC_{50} value (µM)
VBS	6.75
LIPOSOMES	4.25
CPCL	1.5

Cell cytotoxicity of VBS, Liposomes and CPCL for H1299 cells were investigated as shown in Fig.8.2 and tabulated in table 8.2. It should be noted that a relatively short incubation period used in the MTT based cytotoxicity assay (48 h) was not enough to determine a long-term cytotoxicity of calcium phosphate coated liposome. For H1299cells, after 48 h incubation, calcium phosphate coated liposome (IC_{50}=1.5µM) exhibit superior cytotoxic activities to liposome (IC_{50}=4.25µM) and free VBS (IC_{50}=6.75µM). Liposomal formulation show higher cytotoxicity compared to free VBS. However, calcium phosphate coated liposome exhibit higher cytotoxicity than liposome and free VBS. The results showed that calcium phosphate coated liposome had approximately three and four times lower IC_{50} value compared to that of liposome and free VBS respectively. This indicate that calcium phosphate coating on liposome played an important role in enhancing cytotoxic effect by increasing their intracellular uptake as a result of the clathrin coated receptor mediated endocytosis.

As a greater amount of VBS could be intra-cellularly delivered into cells in the form of nanosized CPCL by endocytosis, the cells were more vulnerable to the cytotoxic effect of VBS. Free VBS shows less cytotoxicity compared to liposome and CPCL, resulting from the reduced cellular uptake of VBS. For free VBS, a multi-drug resistant effect, out-fluxing VBS through the p-glycoprotein pump, might play an additional role in decreasing the intracellular concentration of VBS. Therefore, calcium phosphate coated liposomal intracellular delivery approach of VBS by using calcium phosphate coating is an attractive way in circumventing the MDR effect as well as in increasing the cytotoxic effect. Thus, the higher cytotoxic result for calcium phosphate coated liposome can be attributed to the increased cellular uptake of VBS.

REFERENCES:

1. Bradshaw DM, Arceci R. “Clinical Relevance of transmembrane drug efflux as a mechanism of multidrug resistance” J Clin Oncol 1998;16;11;3674-90

2. Campos L, Guyotat D, Archimbaud E, et al. “Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis” Blood1992;79:473-76.

3. Eagle H and Foray GE. “The cytotoxicity action of carcinolytic agents in tissues culture” Am. J Med.1956; 21: 739-745.

4. Freshney. R. I., “Culture of Animal Cells A Manual of Basic Technique”, Fifth Edition.

5. Gottesman MM, Pastan I. “Biochemistry of multidrug resistance mediated by the multidrug transporter” Ann Rev Biochem 1993;62:385-427.

6. Harmon BV, Takano YS, Winterford CM, Potten CS. "Cell death induced by vinblastine in the intestinal crypts of mice and in a human Burkitt's lymphoma cell line" *Cell Prolif.* 1992;25:523–536.

7. Phillips JC, Gibson WB, " Survey of the QSAR and in vitro approaches for developing non animal methods to supersede the in vivo LD50 test" Food Chem toxicol.1990; 28; 375-394.

8. Twentyman, P.R., Luscombe, M., 1987. "A study of some variables in a tetrazoliumdye (MTT) based assay for cell growth and chemosensitivity" Br. J. Cancer 56, 279–285.