ISSN: 2279 - 0594

Available Online at www.jbpr.in

Journal of Biomedical and Pharmaceutical Research 2 (3) 2013, 47-51

RESEARCH ARTICLE

# IN VITRO CYTOTOXICITY ASSESSMENT OF IMIQUIMOD LOADED SOLID LIPID NANOPARTICLES BASED GEL FORMULATION USING BASAL CELL CARCINOMA CELL LINE: PART-II

***Mr. Tanmay N. Patel[1], Dr. Madhabhai M. Patel[2]**

[1] Shree S. K. Patel College of Pharmaceutical Education and Research, Ganpat University

[2] Shankersinh Vaghela Bapu Institute of Pharmacy, Gujarat Technological University

**Received 05/04/2013; Revised 10 April 2013; Accepted 21 April 2013**

**ABSTRACT**

This study was undertaken to address the efficacy of newly formed Imiquimod loaded Solid Lipid Nanoparticles based gel formulation. The study evaluated the acute toxic effect of Solid Lipid Nanoparticles proposed for future use in skin cancers like basal cell carcinoma, superficial squamous cell carcinoma, some superficial malignant melanomas and actinic keratosis. Cytotoxicity study was carried out for Imiquimod loaded SLN based gel formulation against A375 cell line (basal cell carcinoma cell line) and Vero (african green monkey kidney cell line/normal cell line) by MTT Assay method. Investigation was carried out on A375 cell line to elucidate the mechanism of its cytotoxicity. The prepared gel formulation was noted to induce elevated levels of destruction of cancerous cell. Also the cell cultures were characterized for microbial contamination, cross contamination, % cell viability, population doubling time and pH.

**KEYWORDS:** Imiquimod, Solid Lipid Nanoparticles, MTT Assay method, Cell line.

## INTRODUCTION:

With the rapidly growing interest in nanoparticle research, the toxicity of nanoparticles is becoming an increasingly important issue in nanotechnology. The reason for the growing concern for nanoparticle toxicity is the rise in both the number and types of nanoparticles being encountered today. Engineered nanoparticles are one of the leading materials under investigation in the various fields of nanotechnology. The use of nanoparticles for biomedical applications, such as drug and gene delivery, biosensors, cancer treatment and diagnostic tools, has been extensively studied throughout the past decade. The commercialization of nanoparticles for nanomedicine is also in progress with a significant growth rate. The National Science Foundation has proposed that market size for pharmaceutical nanoproducts will reach approximately US$180 billion per year between 2010 and 2015.

Due to the increased production and use of nanoparticles in various fields, the unintended adverse effect of nanoparticles is an emerging and growing concern both academically and socially. Several studies have investigated the toxicity of nanoparticles based on various characteristics, such as shape, size, surface chemistry, chemical composition, surface activity and solubility. However, a more rigorous evaluation of toxicity is needed in order to confidently regulate the safe use of all forms of nanoparticles.

## MATERIALS AND METHODS:

### MATERIALS:

Imiquimod was received as a gift sample from Glenmark Pharmaceuticals Ltd, Mumbai Maharashtra, India. The A375 cell line and Vero cell line were procured from NCCS (National Center for Cell Sciences), Pune, Maharashtra, India.

### METHOD:

Anticancer study was carried out for Imiquimod loaded SLN based gel formulation against A375 cell line (basal cell carcinoma cell line) and Vero (african green monkey kidney cell line/normal cell line) by MTT Assay method. Both the cell cultures were characterized for microbial contamination, cross contamination, % cell viability, population doubling time and pH.

### CHARACTERIZATION OF CELL LINES AND CULTURE MEDIA:

Characterization is essential not only when deriving new lines, but also when a cell line is obtained from a cell bank or other laboratory. Cultures are examined under an inverted phase microscope before start of experiments and



*Corresponding author: Tanmay N. Patel | Email: pr.tanmaypatel@yahoo.com

frequent assessments are made of the viability of the cell population throughout the experimental periods.

**1. TESTING FOR MICROBIAL CONTAMINATION:**

Two methods generally used to check for bacterial and fungal contamination. Detection carried out using special media like Fluid thiogycolate media (TGM) and Tryptone Soya broth (TSB) and direct observation using Grams stain. Contamination by bacteria, yeast or fungi was detected by an increase in turbidity of the medium and/or a decrease in pH (yellow in media containing phenol red as a pH indicator). Cells were inspected daily for presence or absence of microbial growth.

✓ **PROTOCOL:**

**1.** Cell lines were cultured in the absence of antibiotics prior to testing using 25cm$^2$ non-vented T flask.

**2.** In case of adherent cell line, attached cells were bringing into suspension using a cell scraper. Suspension cell lines were tested directly.

**3.** 1.5ml test sample (Cells) were Inoculated into two separate test tubes of each containing Thioglycollate Medium (TGM) and Tryptone Soya broth (TSB).

**4.** 0.1ml E.Coli, 0.1ml B. subtilis and 0.1ml C. sporogenes inoculated into separate test tubes (duplicate) containing (TGM) and (TSB). These were act as positive controls where as two separate test tubes of each containing (TGM) and (TSB) un-inoculated as negative controls.

Broths were incubated as follows:

- For TSB, one broth of each pair were incubated at 32ºC the other at 22ºC for 4 days.
- For TGM, one broth of each pair were incubated at 32ºC the other at 22ºC for 4 days.
- For the TGM inoculated with C.sporogenes incubate at 32ºC for 4 days.

**Note:** Test and Control broths were examined for turbidity after 4 days.

**CRITERIA FOR A VALIDITY OF RESULTS:**

Control broths show evidence of bacteria and fungi within 4 days of incubation in all positive control broths and the negative control broths show no evidence of bacteria and fungi.

- **CRITERIA FOR A POSITIVE RESULT:** Test broths containing bacteria or fungi show turbidity.
- **CRITERIA FOR A NEGATIVE RESULT:** Test broths should be clear and show no evidence of turbidity.

**2. PREPARATION OF MEDIA:**

✓ **PREPARATION OF DMEM:**

10.7gm of DMEM powder was added into 1litre of distilled water and then it was stirred continuously until clear solution formed. To this, $NaHCo_3$ was added to maintain pH 7.0-7.2 and then solution was filtered using membrane filtration assembly. It was stored in reservoir bottle under room temperature.

✓ **PREPARATION OF THE TRYPSIN DILUTION:**

5ml of Trypsin solution was pipette out in to 50ml falcon tube containing 45ml of PBS using 10ml pipette.

**3. DETERMINATION OF CELL VIABILITY AND POPULATION DOUBLING TIME**

The quantification of cellular growth, including proliferation and viability has become an essential tool for working on cell-based studies.

✓ **CELL VIABILITY BY TRYPAN BLUE DYE EXCLUSION METHOD:**

The viability of cells was determined by the Trypan Blue dye exclusion method. It takes advantage of the ability of healthy cells with uncompromised cytoplasmic membrane integrity to exclude dyes such as trypan blue.

✓ **HAEMOCYTOMETER CELL COUNT:**

1. Haemocytometer and cover slip were cleaned and wiped with 70% alcohol. Then cover slip was placed on haemocytometer.
2. In separate 2ml centrifuge tube, cell suspension (cells in culture media) was added. Than two fold dilution of reaction mixture was prepared by mixing aliquot of 0.1 ml cell suspensions with 0.1 ml trypan blue.
3. Afterwards 0.1ml of Cell suspension was then placed in chamber of haemocytometer.
4. By using a Lieca inverted microscope, numbers of cells were counted in 1mm$^2$ area with use of 10X objective.
5. Viable and non-viable cells were counted in both halves of the chamber.

**CALCULATION:**

(1) Total number of viable cells = $A \times B \times C \times 10^4$

(2) Total dead cell count = $A \times B \times D \times 10^4$

Where,

A = Volume of cell solution (ml)

B = Dilution factor in trypan blue

C = Mean number of unstained cells

D = Mean number of dead/stained cells

$10^4$ = Conversion of 0.1 mm$^3$ to ml

(3) Total cell count = Viable cell count + dead cell count

**% Cell Viability = (Viable cell count/Total cell count) × 100**

✓ **POPULATION DOUBLING TIME (PDT):**

It is the time expressed in hours, taken for cell No. to get double. Population doubling time can be determined as follows.

**POPULATION DOUBLING TIME = (X/Y) x 24 hrs.**

Where,

X= (cell number at harvest/cell number initially plated)/2

Y= Total number of days



 

**4. % CELL GROWTH INHIBITION BY MTT ASSAY:**

**PROTOCOL:**

**1.** Cells were pre-incubated at a concentration of 1× $10^6$ cells/ml in culture medium for 3 h at 37°C and 6.5% CO2.

**2.** Cells were seeded at a concentration of 5× $10^4$ cells/well in 100 µl culture medium and various amounts of formulation (final concentration *e.g.* 100µM - 0.005µM) were added into microplates (tissue culture grade, 96 wells, flat bottom).

**3.** Cell cultures were incubated for 24 h at 37°C and 6.5% CO2.

**4.** 10 µl MTT labeling mixture was added and incubate for 4 h at 37°C and 6.5% CO2.

**5.** 100 µl of solubilization solution was added to each well and incubate for overnight.

**6.** Absorbance of the samples was measured using a microplate (ELISA) reader.

**7.** From the absorbance, % cell growth inhibition was calculated using following formula.

$$\% \text{ Cell Growth Inhibition} = 100 - \left( \frac{\text{Mean absorbance of individual test group}}{\text{Mean absorbance of control group}} \right) X\ 100$$

**RESULT & DISCUSSION:**

**1. Characterization of cell lines and culture media**

Characterization of cell lines was performed for detection of microbial and cross contamination. Cell lines used in our experiments were free from any kind of microbial or fungal contamination

**Table 1: Result for characterization of cell lines and culture media**

| Cell line | % Viability | PDT (hr.) | Microbial contamination | Cross contamination | pH |
|---|---|---|---|---|---|
| VERO | 71.91 | 27.9 | NO | NO | 7.5 |
| A375 | 87.20 | 24.5 | NO | NO | 7.0 |

Culture media were also tested for microbial contaminations. To prevent microbial contamination, 2.5% Amphotericin B25 (µg/ml) was supplemented to media which act as working concentration. Bacterial contamination was prevented by addition of 1 % of Antibiotic, 100X (10000U/ml Penicillin G, 10000µg/ml Streptomycin) into culture medium.

Cross contamination of cell line was tested by direct observation of particular cell line under inverted microscope. From viability studies and PDT, we have concluded that the cell lines derived from NCCS, Pune were initially free from cross contamination.

**2. % CELL GROWTH INHIBITION BY MTT ASSAY:**

The in-vitro cytotoxicity study of optimized batch was carried out against A375 cell line (basal cell carcinoma cell line) and Vero (african green monkey kidney cell line/normal cell line) by MTT Assay method. The results showing % cell growth inhibition at different time interval were mentioned below.

**Table 2: % Cell growth Inhibition against different cell line**

| Time (hrs.) | % Cell growth inhibition | | | |
|---|---|---|---|---|
| | SLN based gel formulation | | Marketed Formulation | |
| | Vero | A375 | Vero | A375 |
| **0** | 0 | 0 | 0 | 0 |
| **4** | 4.11 | 7.16 | 4.13 | 29.76 |
| **8** | 7.96 | 16.07 | 7.92 | 53.39 |
| **12** | 11.26 | 34.15 | 11.41 | 78.02 |
| **24** | 11.89 | 45.73 | 11.59 | 78.58 |
| **36** | 12.16 | 70.34 | 12.08 | 79.13 |
| **72** | 12.71 | 89.71 | 12.62 | 79.86 |



 

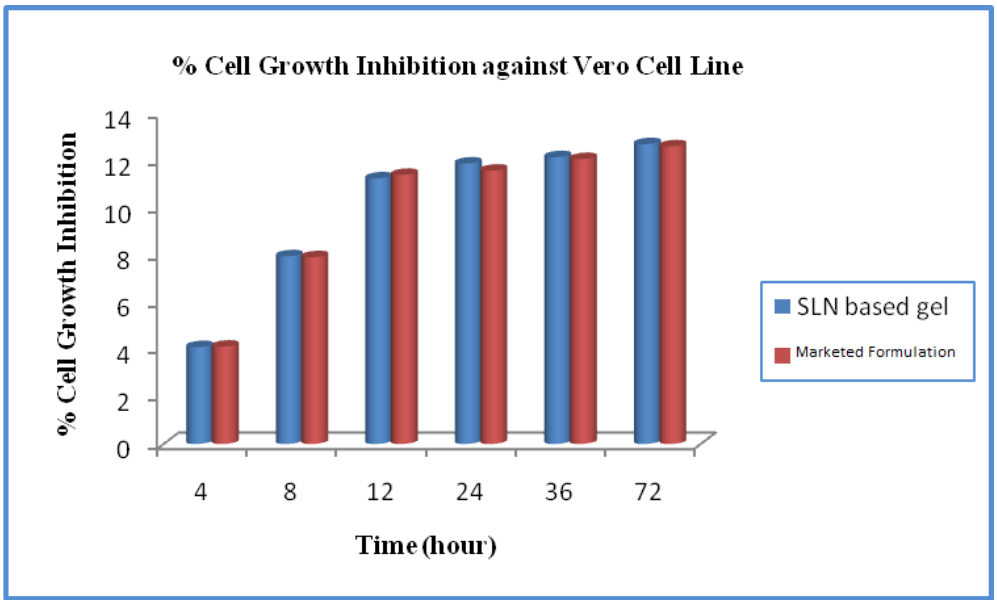


**Figure 1: Comparison of % cell growth inhibition for SLN based gel and Marketed Formulation against Vero cell lines**

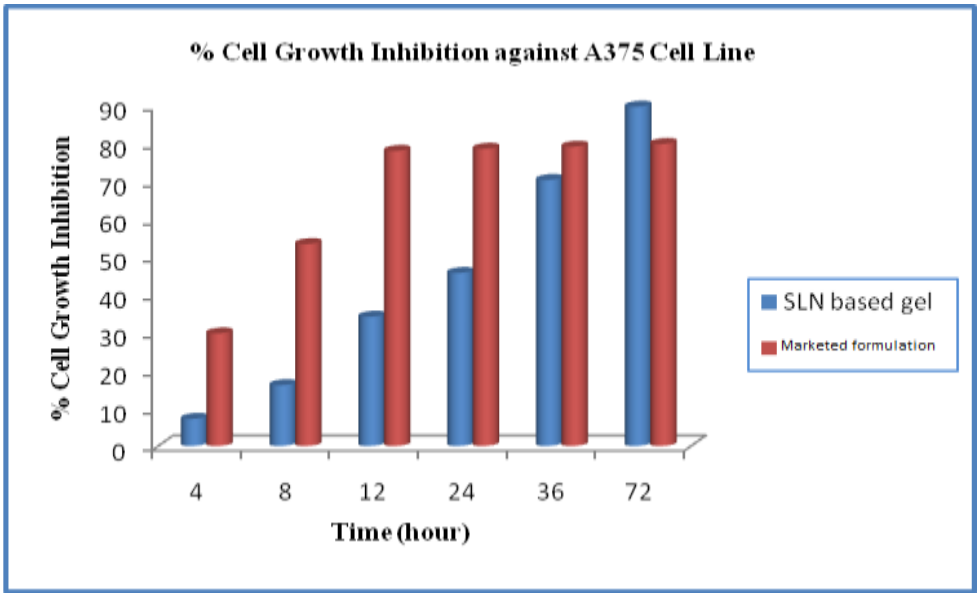


**Figure 2: Comparison of % cell growth inhibition for SLN based gel and Marketed Formulation against A375 cell lines**

**CONCLUSION:**

The cytotoxicity assessment discussed serves the determination of applicability of Imiquimod loaded Solid Lipid Nanoparticles based gel formulation proposed for use in skin cancers like basal cell carcinoma, superficial squamous cell carcinoma, some superficial malignant melanomas and actinic keratosis. In present study, prepared gel formulation was assessed for its anticancer property against A375 cell line (basal cell carcinoma cell line) and Vero (African green monkey kidney cell line/normal cell line) by MTT Assay method. The prepared gel formulation was noted to induce elevated levels of destruction of cancerous cell of A375 cell line. Also the cell cultures were characterized for microbial contamination, cross contamination, % cell viability, population doubling time and pH.

**REFERENCES:**

1. Adams, L. K., Lyon, D. Y, and Alvarez, P. J. J. (2006) Comparative eco-toxicity of nanoscale TiO2, SiO, and ZnO water suspensions, Water Research, 40, 3527-3532.
2. Bakand, S., Hayes, A., Winder, C., Khalil, C., and Markovic, B. (2005) In-vitro cytotoxicity testing of air borne formaldehyde collected in serum free culture media, Toxicology and Industrial Health, 21, 147-154.



 

3. Bakand, S., Winder, C., Khalil, C., and Hayes, A. (2005) Toxicity Assessment of Industrial Chemicals and Airborne Contaminants: Transition from In Vivo to In Vitro Test Methods: A Review, Inhalation Toxicology, 17, 775 - 787.
4. Bakand, S., Winder, C., Khalil, C., and Hayes, A. (2006a) An experimental in vitro model for dynamic direct exposure of human cells to airborne contaminants, Toxicology Letters, 165, 1-10.
5. Bakand, S., Winder, C., Khalil, C., and Hayes, A. (2006b) A novel in vitro exposure technique for toxicity testing of selected volatile organic compounds, Journal of Environmental Monitoring, 8, 100-105.
6. Cai, R., Hashimoto, K., Itoh, K., Kubota, Y., and A., F. (1991) Photokilling of malignant cells with ultrafine TiO powder, Bulletin of the Chemical Society of Japan, 64, 1268-1273.
7. Colvin, V. L. (2003) The potential environmental impact of engineered nanomaterials, Nature Biotechnology, 21, 1166-1170.
8. Cook, J.A., Mitchell, J.B., 1989. Viability measurements in mammalian cell systems. Anal. Biochem. 179, 1–7.
9. Crouch, S., 2000. Biocompatibility testing ATP bioluminescence. Med. Device Technol. 11, 12–15.
10. Crouch, S., Slater, K., 2000. High-throughput cytotoxicity screening: hit and miss. DDT 6.
11. Crouch, S.P., Kozlowski, R., Slater, K.J., Fletcher, J., 1993. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J. Immunol. Methods 160, 81–88.
12. Decker, T., Lohmann-Matthes, M.L., 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J. Immunol. Methods 115, 61–69.
13. Deepalakshmi, P.D., Parasakthy, K., Shanthi, S., Devaraj, N.S., 1994. Effect of chloroquine on rat liver mitochondria. Indian J. Exp. Biol. 32, 797–799.
14. DeRenzis, F.A., Schechtman, A., 1973. Staining by neutral red and trypan blue in sequence for assaying vital and nonvital cultured cells. Stain Technol. 48, 135–136.
15. Dufour, E. K., Kumaravel, T., Nohynek, G. J., Kirkland, D., and Toutain, H. (2006) Clastogenicity, photo-clastogenicity or pseudo-photoclastogenicity: Genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells, Mutation Research, 607, 215-224.
16. Inomata, K., Tanaka, H., 2003. Protective effect of benidipine against sodium azide-induced cell death in cultured neonatal rat cardiac myocytes. J. Pharm. Sci. 96, 1–8.
17. Isobe, I., Michikawa, M., Yanagisawa, K., 1999. Enhancement of MTT, a tetrazolium salt, exocytosis by amyloid beta-protein and chloroquine in cultured rat astrocytes. Neurosci. Lett. 266, 129–132.




 