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RESEARCH ARTICLE

In Vitro Cytotoxicity Assessment of Imiquimod Loaded Liposomal Gel Formulation Using Basal Cell Carcinoma Cell Line: Part-II

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ABSTRACT:

This study was undertaken to address the efficacy of newly formed Imiquimod loaded liposomal gel formulation. The study evaluated the acute toxic effect of liposomal gel formulation intended to be used in skin cancers like basal cell carcinoma, superficial squamous cell carcinoma, some superficial malignant melanomas and actinic keratosis. Cytotoxicity study of Imiquimod loaded liposomal gel formulation 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. 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, liposomal gel formulation, MTT Assay method, Cell line.

INTRODUCTION:

Imiquimod is used to treat certain diseases of the skin, including skin cancers (basal cell carcinoma, Bowen's disease, superficial squamous cell carcinoma, some superficial malignant melanomas, and actinic keratosis) as well as genital warts (condylomata acuminata).

Liposomes are spherical vesicles composed of a bilayer membrane encapsulating an aqueous core. They can potentially be used as drug carriers. Some liposomal formulations have already been approved for clinical use and many more are currently under preclinical or clinical investigation. The hydrophobic property of the liposomal bilayer allows incorporation of hydrophobic chemotherapeutic agents and the hydrophilic property of the aqueous core allows encapsulation of water-soluble compounds. The liposomal delivery of these anticancer agents has been reported to improve solubility, prolong circulation time, alters biodistribution in-vivo and may reduce side effects for these compounds.

We developed an Imiquimod loaded liposomal gel formulation by thin film hydration technique and evaluated for different parameters. In this paper, we report an in-vitro cytotoxicity assessment of Imiquimod loaded liposomal gel formulation using basal cell carcinoma cell line.

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 liposomal 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 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.

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✓ **Protocol:**

- Cell lines were cultured in the absence of antibiotics prior to testing using 25cm^2 non-vented T flask.
- In case of adherent cell line, attached cells were bringing into suspension using a cell scraper. Suspension cell lines were tested directly.
- 1.5ml test sample (Cells) were Inoculated into two separate test tubes of each containing Thioglycollate Medium (TGM) and Tryptone Soya broth (TSB).
- 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			
	Liposomal gel formulation		Marketed Formulation	
	Vero	A375	Vero	A375
0	0	0	0	0
4	3.94	6.78	4.13	29.76
8	7.56	14.33	7.92	53.39
12	11.18	31.80	11.41	78.02
24	11.49	44.91	11.59	78.58
36	12.13	69.34	12.08	79.13
72	12.41	88.47	12.62	79.86

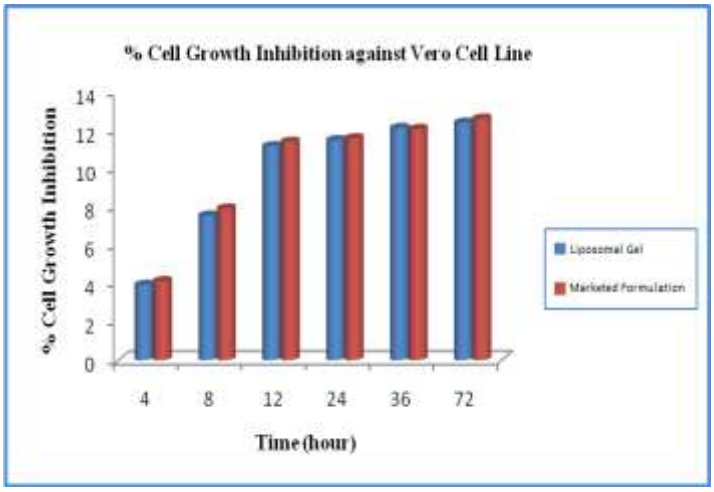

Figure 1: Comparison of % cell growth inhibition for liposomal gel and Marketed Formulation against Vero cell lines

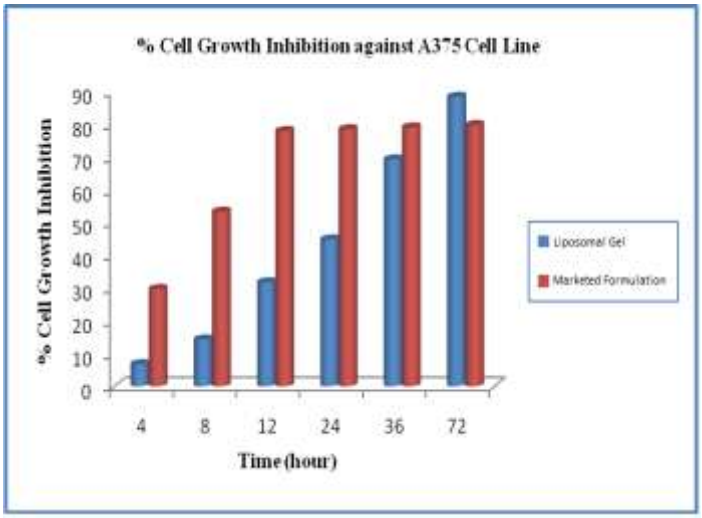

Figure 2: Comparison of % cell growth inhibition for liposomal gel and Marketed Formulation against A375 cell lines

CONCLUSION:

The cytotoxicity assessment discussed serves the determination of applicability of Imiquimod loaded liposomal 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. Thus, it can be concluded that the prepared Imiquimod loaded liposomal gel formulation passed cytotoxicity assessment.

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