Characterization and anticancer efficacy of tamoxifen citrate nanosuspension against MCF-7 breast cancer cells

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INTRODUCTION

Breast cancer (BC) is a common cause of cancer death in women worldwide. Risk is enhanced by late menopause, early menarche and obesity in postmenopausal women. Previously it was proved that high concentrations of endogenous oestradiol are associated with an increase in breast cancer risk (Waks and Winer, 2019). Many chemotherapeutic drugs are used for treating the breast cancer. The current anticancer drugs do not greatly distinguish normal and cancerous cells resulting in sys-
Polymeric nanoparticles play a significant role in delivering chemotherapeutic drugs in a controlled manner. Drug delivery via nanoparticles makes it easy to acquire the desired drug concentration in the particular site, thus reducing the adverse effects and minimizing the toxicity, dose dumping, etc (Hu et al., 2018). Polymeric nanoparticles have been established with the requirements of nanocarriers for hydrophobic chemotherapeutics and hormone regulators, including camptothecin, taxanes, cisplatin and tamoxifen (Chevalier et al., 2017). Tamoxifen citrate stops the actions of the hormone estrogen in breast tissue, which may help to keep breast cancer cells from growing (SreeHarsha et al., 2019).

Tamoxifen citrate, an anticancer agent, was loaded in the poly(lactic-co-glycolic acid) by various researchers. It is a drug used for treating certain types of breast cancer in women and men (Yu et al., 2020). Tamoxifen and its metabolites kill both ERα positive and negative breast cancer cells. It indicates an antiestrogenic action for breast cancer, and estrogenic effect on the uterus (Dehghani et al., 2017). Based on the concentration and dose several adverse effects like endometrial cancer for postmenopausal women were observed. Other adverse effect involves venous thrombosis, liver cancer, pulmonary emboli, and an ocular effect involves corneal opacities and retinopathy (Hanker et al., 2020).

Significant progress has been made to improve the breast cancer treatment in the past several years. Though, the current clinical approaches are aggressive, of low specificity and can produce moderate to severe side effects (Love, 1989). Nanotechnology as a quickly developing field, bring favorable opportunities to human cancer diagnosis and treatment. The utilization of nanoparticulate-based platforms overcome biological barriers and permit extended blood circulation time, simultaneous tumor targeting and increased drug accumulation in tumors (Wapnir et al., 2018).

There are still various barriers to overcome before extensive clinical use of new nanotherapies for human breast cancer and can become standard treatment protocols. The nanoparticles synthesized one is to increase the loading concentration and encapsulation efficiency of active agents. A therapeutic concentration of drug loaded nanoparticle or imaging contrast agents is necessary to achieve anticancer effects or quality imaging of tumors. The nanoparticles with high drug loading capacity, the quantities of matrix nanomaterials to be administered are decreased along with nanoparticle-related risks (Deng et al., 2018). Drug loaded nanoparticles were observed to be more cytotoxic than the free drug. Herein, we report for the first time the anticancer activity of the Tamoxifen citrate nanosuspension against breast cancer cells. Therefore, the present study was carried out to find the effectiveness of tamoxifen citrate loaded nanoparticle against breast cancer (MCF-7) cell lines.

**METHODOLOGY**

**Preparation of Tamoxifen citrate L-SNEDDS**

The tamoxifen citrate purchased from Sigma Aldrich Company, USA. Polyethylene Glycol (PEG), Tween 80 and cotton seed oil purchased from SD Fine Chemicals. About 10 mg of Tamoxifen citrate was dissolved in 100 mg of cotton seed oil, Tween 80 and PEG 400 mixture respectively. The prepared formulation was vortexed using vortex mixer. Various concentration of oil and surfactant mixture were selected to prepare Tamoxifen citrate nano suspension. 10mg/gm i.e. 1% w/w drug is loaded into the plain suspension formulations.

The separation of nanoparticles was done through centrifugation at 5,000 rpm for 5 minutes, the supernatant was re-centrifuged for 45 minutes at 15,000 rpm. The nanoparticles formed were frozen at -40°C and lyophilized (7–8 hours) to get a solid product. The lyophilized product obtained was stored in a desiccator for eliminating moisture, and then final product was kept in an airtight container at 4°C. The final formulations of suspension were examined for signs of turbidity and thermodynamic stability (Batool et al., 2020).

**Solubility test**

About 5ml of surfactant mixture and 100 mg of nanosuspension was added to the drug in test tube and kept for 24 hrs. The test tube was then covered with aluminum foil and sonicated for 10 min in bath sonicator and then Tamoxifen citrate was added to the solution. The procedure was continued for 3 days till saturation solubility was achieved. The solution was centrifuged at 5000 rpm and 4 ml of buffer was added to 1 ml of supernatant and absorbance was checked on UV Spectrophotometer at 268 nm (Pishnamazi et al., 2020).

**Transmission electron microscopy study**

The structure and morphological examination of L-SNEDDS were observed with transmission electron microscopy. The nanosuspension was diluted with distilled water in 1:50 ratio and mixed gently, a drop of diluted sample stained with phosphotungstic acid (1% w/v) solution and placed over the coated grid. For TEM experiment, the silver nanoparticles are coated on copper grids and the films on the TEM grids allowed standing for 2 min, after which the...
extra solution removed using a blotting paper and after drying the grid the nanoparticle structure and size was studied using CM 200 FEG Phillips transmission electron microscope at an accelerating voltage of 100 keV and photos taken using AMT XR41-B 4-megapixel (2048 x 2048) bottom mount CCD camera (Dang et al., 2017).

UV-spectrum study

The nanosuspension was centrifuged at 5000 rpm and 4 ml of phosphate buffer was added to 1 ml of supernatant and absorbance was checked on UV-Vis Scanning Spectrophotometer (Labomed) in the UV range of 200 – 500 nm to measure \( \lambda_{\text{max}} \) of Tamoxifen citrate suspension L-SNEDDS. The \( \lambda_{\text{max}} \) of Tamoxifen citrate suspension L-SNEDDS is 298 nm (Hoque et al., 2019).

In vitro anticancer study of Tamoxifen citrate L-SNEDDS

Cell lines breast carcinoma (MCF-7 cell line) were used in this study, procured from National Centre for Cell Sciences (NCCS), Pune, India.

Cell culture

The cells were centrifuged for 5 min at 3000 rpm to remove the cryopreservative agent, dimethylsulfoxide (DMSO). The cells were transferred into a T-25 falcon flask and cultured in DMEM medium together with 10% (v/v) fetal serum and 1% (v/v) penicillin-streptomycin to increase and stimulate cells survival and proliferation. The growth medium was supplemented with phenol red, a pH indicator. Cells were incubated in a CO\(_2\) incubator at 37 \( ^\circ \)C and supplemented with 5% CO\(_2\). The medium was replaced every alternate day until the cells were confluent and ready to be sub-cultivated. The media in the flask was discarded when the cells have reached its confluence. The cells were then washed several times with 2 ml of phosphate-buffered saline (PBS). Cells were detached by adding 500 \( \mu \)l of trypsin and incubated for 5 min. After viewing the cells under the microscope, 4 mL of 10% FCS-DMEM was added to the cells to inactivate the trypsin. The cell suspension was transferred to a 15 mL falcon tube and centrifuged at 3000 rpm for 5 min at 4 \( ^\circ \)C. Resuspension of the cells was done in the culture media. The medium was replaced every alternate day until the cells were confluent and ready to be sub-cultivated (Nosrati et al., 2018).

Preparation of test solutions

The Tamoxifen citrate L-SNEDDS were separately dissolved in medium supplemented with 2% inactivated FBS to obtain a stock solution of 1000 \( \mu \)m/ml concentration and sterilized by filtration. From this stock solution, five different lower dilutions (25-250 \( \mu \)m/ml) were prepared.

Cell Viability assay

Breast Cancer MCF-7 cells were pipetted into the well of microtiter plates at a density of \( 5 \times 10^4 \) cells/well and treated with Tamoxifen citrate L-SNEDDS nanosuspension with various concentration (25, 50, 100, 150, 200-250 \( \mu \)m/ml). The cells were permitted to adhere for 24 hours, and the growth medium (MEM) removed using micropipette and the monolayer of cells washed twice with MEM without FBS to remove dead cells and excess FBS. About 1 ml of medium (without FBS) containing different dilution of nanosuspension were added in respective wells; 20 \( \mu \)l of MTT (5 mg/ml in PBS) as added to each well, and the cells incubated for 6-7 hrs in 5% CO\(_2\) incubator. After removal of the medium, 1 ml of DMSO was added to each well. The cells were then exposed to the medium alone (as negative control). The drug containing nanosuspension (25 \( \mu \)m -250 \( \mu \)m) and a free drug (Tamoxifen citrate) were added to the respective wells. The supernatant was removed and 50 \( \mu \)l of propanol was added and the plates were mixed well to solubilize the formazan. The MTT enters the cells and transfers into the mitochondria where it is decreased to an insoluble, dark purple coloured formazan product. The plates were kept on a shaker for 15 min and the absorbance was measured on an ELISA reader (570 nm). Every experiment was carried out in triplicate and the half maximal inhibitory concentration (IC\(_{50}\)) of each test drugs were calculated as the percentage survival of the cells.

Statistical Analysis

The results are presented as mean \( \pm \) standard deviation of three experiments. Data were statistically analysed by ANOVA using the post hoc Dunnett’s test. The statistically significance level was measured at \( P < 0.05 \).

RESULTS AND DISCUSSIONS

Breast cancer (BC) is the uncontrolled growth and sudden proliferation of breast cells, initiating from the ducts or lobules, to other parts of the body. Breast cancer is the second leading cause of cancer-linked women fatality after lung cancer (Harbeck et al., 2019). Tamoxifen[(Z)-2-(4-(1,2-Diphenyl-1-butenyl)-phenoxy)-N,N-dimethylethanamine] (TAM) has been prescribed to treat patients with breast cancer. TAM is a “selective estrogen receptor modulator (SERM)”. This drug functions as anti-estrogen in breast tissue by interfering with the effect of estrogen: the female sex hormone that stimulates the progression of cancer cells in the
breast (Wang et al., 2019).

UV-Vis spectrophotometry includes measuring the quantity of visible or ultraviolet radiation absorbed by a substance in solution. Besides, assessment of drug by UV-Vis may be required in the following events: alterations in the synthesis of the drug substance; variation in the composition of the end product and difference in the analytical process (Wang et al., 2018). The wavelength of maximum absorption ($\lambda_{\text{max}}$) is normally selected. The theoretical Tamoxifen UV-Vis spectra for MCF 7 cell line has been shown in Figure 1. The $\lambda_{\text{max}}$ of Tamoxifen citrate suspension L-SNEDDS was observed at 298 nm.

Transmission electron microscopy (TEM) is a microscopy method in which a beam of electrons is passed through a specimen to form a photograph. The specimen is most frequently an ultrathin section below 100 nm thickness or a suspension on a grid (Marín-García et al., 2018). The size and morphology of nanoparticles acquired were observed by TEM. TEM images of the nanoparticles (Figure 2) indicate that there was homogeneous molecular dispersal of the drug in the polymer dependent nanoparticles and the drug was not distributed in the particular form. TEM images shows that the morphology of nanoparticle is almost spherical in shape with smooth edges. The average particle size for sample was calculated as 12 nm with an average size ranging from 8 and 20nm.

There was no diffraction of transmission of electrons via the particles and this is the reason why alike dark particles were observed devoid of spots. Spotted particles indicate the presence of a drug in particulate form rather than its dispersal in molecular form. Formation of rounded vesicles with distinctive bilayer structure can be seen in TEM. TEM images of the nanoparticles established their morphology that shows molecular and homogeneous drug distribution in the nanoparticles.

The cytotoxic effects of tamoxifen citrate L-SNEDDS were quantified in the MCF7 cell line using the MTT method. MCF7 cells were treated with different concentrations of tamoxifen citrate L-SNEDDS (25, 50, 100, 200, 250 $\mu$m/mL) for 24 h at 37 $^\circ$C. The viability rate of MCF7 cells treated with tamoxifen is shown in Figure 3, and it was found to be 82.7%, 61.3%, 46.7%, 41.7%, 37.4%, and 20.3% respectively. The cytotoxic effect of Tamoxifen citrate L-SNEDDS enhanced as the drug concentration rose from 25 $\mu$M to 250 $\mu$M.

The extend of MCF7 mitochondrial membrane permeability enhanced with enhancing tamoxifen concentration, probably to the fact that cells had undergone either necrosis or apoptosis along with variations in mitochondrial function. Such variations could result in the loss of mitochondrial membrane potential and secretion of cytochrome c from the mitochondria.

The toxic effect of Tamoxifen citrate L-SNEDDS remarkably reduced the cell viability from 100% to 20.3%, whereas nanosuspension without the drug showed cell viability of 99.5%.

A significant decrease in cell viability was found in Tamoxifen citrate free drug from 100% to 30.9%. Cell viability is a significant toxicity assay parameter and is directly connected with the toxic effects of a drug.

Tamoxifen induced a decrease in cell viability in MCF7 cells which is dependent on cell density and concentration of tamoxifen. Such activity may be attributed to cytostatic and/or cytocide actions that might down-regulate telomerase effect (Hassan et al., 2018).
CONCLUSION

In conclusion, tamoxifen citrate L-SNEDDS exhibited cytotoxic actions in MCF7 breast cancer cells. Tamoxifen citrate nano-suspension was transferred into the MCF-7 cells, indicating their suitability in anticancer treatment. The uptake efficiency of nanoparticles was more by tamoxifen citrate nanosuspension rather than the free form of tamoxifen citrate MCF-7 cells leads the cells to be more targets to the anticancer drug.

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Conflict of interest

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