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RESEARCH ARTICLE

IN-VITRO CYTOTOXICITY ANALYSIS OF TAMOXIFEN CITRATE LOADED CROSS-LINKED GUAR GUM NANOPARTICLES ON JURKAT (HUMAN T-CELL LEUKEMIA) CELL LINE

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ABSTRACT

The present investigation was aimed to study the antiproliferative action of Tamoxifen citrate (TMX), a non steroidal antiestrogen, on a human T-cell leukemia cell line, Jurkat, as free drug and TMX loaded guar gum nanoparticles. For this we developed a new formulation containing chemically cross-linked guar gum nanoparticles (GG NPs) loaded with Tamoxifen citrate (TMX). Single step (oil in water) emulsion and in-situ polymer cross-linking technique was employed to prepare spherical and smooth surfaced nanoparticles in the size range of 200-300nm. Nanoparticle size and shape was confirmed from observation in transmission electron microscope (TEM) analysis. Cytotoxicity on Jurkat (human T-cell leukemia) cell lines as determined by cell growth inhibition after 48 hrs of incubation indicated that Tamoxifen citrate loaded guar gum nanoparticles were as efficient as the free drug when applied to the cancer cells. However, the crosslinked guar gum nanoparticles loaded with Tamoxifen citrate exhibited sustained release of the drug and delayed apoptosis over a long period of time making it suitable for cancer treatment.

Keywords: Nanoparticles, guar gum, Tamoxifen citrate, Jurkat (human T-cell leukemia) cell line, cytotoxicity, cell death, WST-1 assay.

INTRODUCTION

Cancer chemotherapy is challenged with the problem of delivering the required therapeutic concentration of the drug at the tumor site for the desired period of time without causing undesirable effects on other organs after systemic administration.[1,2,3] Targeted delivery of anticancer drugs to solid tumors, therefore, is necessary in order to achieve optimum therapeutic outcomes. Nanoscale devices has led to the development of biodegradable self-assembled nanoparticles, which are being engineered for the targeted delivery of anticancer drugs and imaging contrast agents capable of ferrying large doses of chemotherapeutic agents into malignant cells sparing the healthy cells.

Tamoxifen citrate, the nonsteroidal antiestrogen is the treatment of choice for patients with all stages of estrogen receptor positive breast cancer.[4,5] Tamoxifen belongs to a class of nonsteroidal triphenylethylene derivatives and is considered the first selective estrogen receptor modulator.[6,7] The drug exhibits anti-estrogenic activity by binding to the intracellular estrogen receptor.[8] The Tamoxifen-estrogen receptor complex binds with DNA and can alter or block subsequent mRNA transcription and lead to cellular apoptosis.[9,10]

Tamoxifen has some major side effects following long-term therapy in postmenopausal breast cancer patients, including higher incidence of endometrial cancer, liver cancer, thromboembolic disorders, and development of drug resistance.[11] Tamoxifen resistance has been shown in a variety of cells in vitro as well as in vivo.[12] These unwanted side effects of Tamoxifen, as well as various barriers to the delivery of the drugs to tumor, call for targeted delivery to the tumor site and enhanced uptake by the tumor cells. One approach to overcome the undesirable side effects of Tamoxifen includes the use of biodegradable polymeric nanoparticles for tumor-targeted drug delivery. Tamoxifen has been formulated in nanoparticulate carrier systems in the form of nanospheres such as poly-ε-caprolactone nanoparticles and long-circulating PEG-coated poly(MePEG cyanoacrylate-cohexadecylcyanoacrylate) nanoparticles in the form of free base.[13,14] It is known that the tumor vasculature is leaky and possesses an enhanced capacity for the uptake of macromolecules and colloidal drug carriers of up to 400 nm in diameter. This effect is known as the enhanced permeability and retention (EPR) effect.[15,16,17] Thus, such a delivery method could improve the selectivity of treatment by increasing the ratio of tamoxifen absorbed by the tumor

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to tamoxifen absorbed by other tissues, leading to a reduction in the systemic side effects.

On the other hand, the coating with polymers provides sustained release along with protection of the active drug from gastric fluid.[18,19] Polysaccharides, the polymers of monosaccharides, retain their integrity in the upper gastrointestinal tract because they are resistant to the digestive action of gastrointestinal enzymes. The polysaccharide, guar gum, obtained from the seeds of *Cyamposis tetragonolobus*, is a low cost, easily available, non toxic, biodegradable polymer. It consists of linear chains of (1→4)β-D-manopyranosyl units with β-D-galactopyranosyl units attached by (1→6) linkages.[20] Guar gum is hydrophilic in nature and swells in cold water forming viscous colloidal dispersions or sols. This gelling property retards release of the drug from the dosage form which makes it an ideal carrier in drug delivery.[20,21,22,23]

We have reported earlier about the formulation development aspects of crosslinked guar gum nanoparticles containing tamoxifen citrate and characterized the nanoparticles for morphology, drug-polymer interaction, drug loading efficiency and drug release for finding its applications in drug delivery to cancer.[24,25] In the present investigation, we have studied the internal morphology and shape of nanoparticles in TEM and anticancer efficacy of the drug loaded guar gum nanoparticles on Jurkat (human T-cell leukemia) cell line. Citrate salt was preferred to Tamoxifen free base due to its higher efficacy and the fact that commercially marketed products are manufactured with Tamoxifen citrate.

MATERIALS AND METHODS

Materials

Central Drugs Laboratory, Kolkata, India generously supplied tamoxifen citrate as gift sample. Guar gum was purchased from Central Drug House, New Delhi, India. Span 80, and glycerol were procured from M/S Ranbaxy Ltd. Glutaraldehyde (25% aqueous solution) was procured from Sigma Chemicals, USA. All other solvents were of HPLC grade. Jurkat (human T-cell leukemia) cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. WST-1(Cell proliferation reagent) were purchased from Roche Diagnostics, Mannheim, Germany and RPMI 1640 (Roswell Park Memorial Institute medium 1640), Fetal Bovine Serum, penicillin-G, Streptomycin, Gentamycin were procured from Invitrogen, Carlsbad, CA.

Preparation of nanoparticles

Guar gum nanoparticles containing tamoxifen citrate were prepared by oil in water emulsion in-situ polymer crosslinking technique.[24] Briefly 5mg of the drug tamoxifen citrate was taken in 10ml of dichloromethane (DCM), this formed the oil phase. To this added 4 mg of Span 80 under stirring. The oil phase was then added to a 0.5% aqueous guar gum solution under constant magnetic stirring. After mutual saturation of the oil and the continuous phase, the mixture was rapidly stirred. Glycerol (as stabilizer) was then added followed by addition of 25% glutaraldehyde solution to affect crosslinking under continuous stirring. Nanosuspension was kept overnight for nanoparticle formation. Nanoparticles were obtained after centrifugation at 20,000 rpm for 30minutes, washed with 15 ml HPLC grade water and recentrifuged. The yielded nanoparticles were lyophilized, harvested in micro centrifuge tubes and preserved in vacuum desiccators.

CHARACTERIZATION OF TAMOXIFEN CITRATE LOADED GUAR GUM NANOPARTICLES

Size distribution in TEM

Transmission electron microscopy (FEI Technai 12 BioTwin, The Netherlands) with a CCD camera mega view II soft imaging system was employed to visualize and record the nature and the size distribution of NPs. A generalized protocol was used for TEM studies.[26] A drop of water suspension of the GG NPs was mounted on a carbon coated copper grid (CCG) and air-dried and micro graphed at 80-100kV (fig. 1).

In vitro cytotoxicity analysis of free tamoxifen citrate and tamoxifen citrate loaded guar gum nanoparticles on Jurkat (human T-cell leukemia) cell lines

Jurkat (human T-cell leukemia) cells (National Centre for Cell Science (NCCS), Pune, India) were cultured in RPMI 1640(Roswell Park Memorial Institute medium 1640) was used at pH 7.4, supplemented with 10% Fetal Bovine Serum and antibiotics (100U/ml penicillin-G, 100µg/ml-sterptomycin, 6µg/ml Gentamycin). The cells were incubated at 37^0C in a humidified atmosphere containing 5% CO_2 inside a CO_2 incubator.

WST-1 assay:

The antiproliferative assay was carried out using the commercially available colorimetric assay kit WST-1 (Cell proliferation reagent, Roche, Germany). The cells (5 $X10^4$ cells in 100 µl medium/well) were plated in 0.05% DMSO in media as control in 96-well plates. The cells as such or in the presence of varying concentration of free tamoxifen citrate and loaded NPs (**dissolved in 0.5% DMSO in media**) were incubated for 48 h. At the end of the treatments, each well was treated with WST-1 solution (10 µl), and after incubation for 2 h, the absorption at 450 nm was read with a microplate reader (BIOTEK; ELX 800, USA). The inhibition of cell proliferation by TMX and TMX loaded GG NPs was evaluated by calculating the IC_{50} values.

$$\%\text{Cell Death} = \frac{A_{control} - A_{treated}}{A_{control}} \times 100$$

Where $A_{control}$, $A_{treated}$ are the absorbances of control and free TMX and TMX loaded GG NPs.

RESULTS AND DISCUSSION

In the present study tamoxifen citrate loaded guar gum nanoparticles in the size range of 200-300nm were prepared via a oil in water emulsion insitu polymer crosslinking technique. Transmission electron microscopy analysis of drug loaded NPs were spherical with a dense TMX core (Fig. 1). Average TEM diameter was observed to be 205 nm. The antiproliferative action of free tamoxifen citrate and tamoxifen citrate loaded guar gum nanoparticles was investigated on Jurkat (human T-cell leukemia) cell line. For this purpose pure drug and drug loaded nanoparticles were applied on the cancer cells in-

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vitro against a control experiment with cancer cells in the culture media without the free drug and nanoparticles.

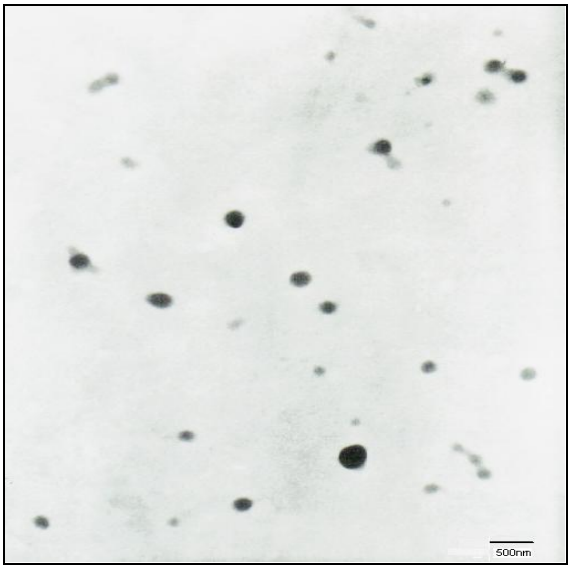

Figure 1: TEM micrograph of TMX loaded guar gum nanoparticles

The microscopic images of control cells without any drug exposure and cells incubated with empty guar gum nanoparticles did not show any cell death, where as the images of cells treated with encapsulated and free tamoxifen citrate exhibited cell death (Figure 3a-3b). A dose dependent cytotoxicity was observed with TMX and TMX loaded GG NPs (table1 and figure 2). The cell morphology was observed and no loss in cellular morphology was found when cells were treated with empty GG NPs but significant changes were found after treatment with free TMX and TMX loaded guar gum nanocarriers. Loss of aggregation and lysis of cells were observed under these conditions as presented in figure 3a-3b.

Since, guar gum is nontoxic and is approved by FDA for pharmaceutical applications, it is expected that the drug loaded guar gum nanoparticles should be biocompatible, biodegradable and potentially safe as a drug carrier for clinical use. *In vitro* cytotoxicity assay proved that empty nanocarriers showed no cytotoxicity and TMX loaded nanoparticles was as effective as free TMX. It is observed that there is significant change in morphology of the cells after treatment with TMX and TMX loaded nanoparticles suggesting effectiveness of TMX loaded guar gum nanoparticles on the experimental cell line and eliminating the probability of plain guar gum induced cytological changes in the present investigation.

Table 1: Percent death of Jurkat cells with free TMX and TMX loaded GG NPs after 48 h incubation in RPMI-1640 medium.

S. No	Concentration µM	$A_{control}$ (only cell in media)	$A_{treated}$ Cell + TMX	$A_{treated}$ Cell + NPs	%Cell Death Free TMX	%Cell Death TMX loaded NPs
1	10	2.0	0.545	1.827	41	10
2	25	2.0	0.227	0.293	83.21	77.45
3	50	2.0	0.214	0.289	89.46	85.76
4	100	2.0	0.184	0.331	90.94	83.69
5	150	2.0	0.171	0.369	91.45	81.82

A stands for Absorbance

It is evident from table1 and figure 2 that the trend of % cell death tends to decrease at nanoparticle dose of 50 µM and more. One possible explanation for this fact may be a progressive saturation of the endocytotic process with time suggesting a saturable kinetics for the transport of nanoparticles.[27] The cellular uptake of guar gum nanoparticles may be assumed to be mediated by non specific endocytotic process rather than receptor mediated. Similar results were observed by Amiji *et al*.[28] The inhibitory IC50 for both free TMX and TMX loaded GG NPs was found to be below 25µM. Based on the saturation of the endocytotic process for the nanoparticles, it may be assumed that at higher doses of nanoparticles (50µM and more) the intercellular Tamoxifen concentration was not as high as it should be resulting in the reduced rate of cell death of the cells. The cellular uptake of free drug however can occur either by passive diffusion and pinocytosis.,[29] Pinocytosis mainly performs the capture of macromolecules adherent to the surface membrane. The size of an average pinocytic vesicle is about 100 to 200 nm.[30] It may further be assumed that the free drug solution will be distributed all over the body whereas the NPs (200-300nm) may be preferentially distributed in the tumor tissue because of the EPR effect. Leaky vasculature and lack of effective lymphatic drainage from the tumour results in the extravasation and accumulation of particulates, plasma proteins and other macromolecules.[31] This trapping of macromolecules has been termed the "enhanced permeability and retention" (EPR).[32,33] EPR effect is now thought to contribute to the effects of many anticancer drugs delivered as conjugates with synthetic polymers[34,35] or in liposomes.[36] Mechanism of action for

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highly protein bound drugs (anthracylcines, paclitaxel, etoposide) also seems to be mediated through the EPR.[37] However, the cut-off size of the permeable vasculature varies from tumour to tumour, and the size of a drug carrying particle may be used to control the passive drug delivery.[38] Doxorubicin incorporated into long circulating pegylated liposomes showed excellent tumour accumulation through EPR and reduced side effects of doxorubicin.[39,40] With the support of the established literature we may assume that the prepared guar gum nanoparticles of 200-300nm size are preferentially accumulated on the Jurkat cells by EPR effect and is expected to increase the concentration of drug inside the tumor cells as a result of a nonspecific endocytotic process, followed by a gradual release of the drug and binding of tamoxifen to the estrogen receptor around the nucleus to produce the desired antitumor effect.

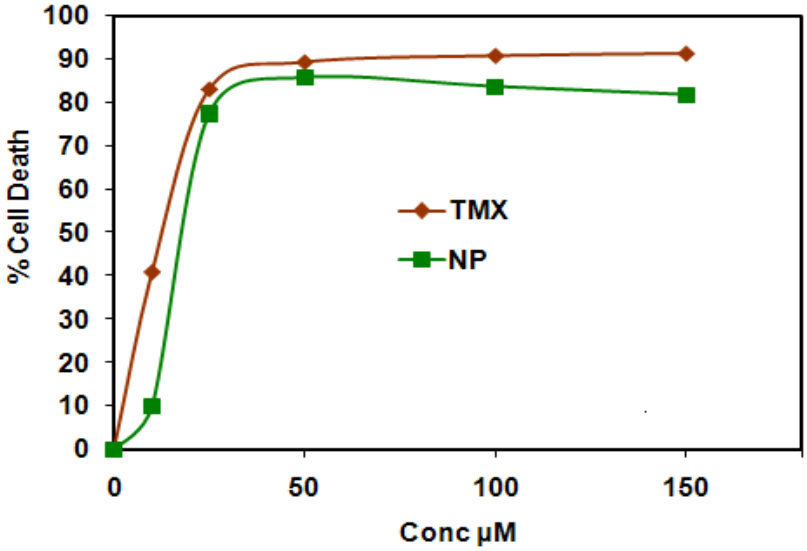

Figure 2: Showing cell death with free TMX and TMX loaded guar gum nanoparticles after 48 h incubation in RPMI-1640 medium

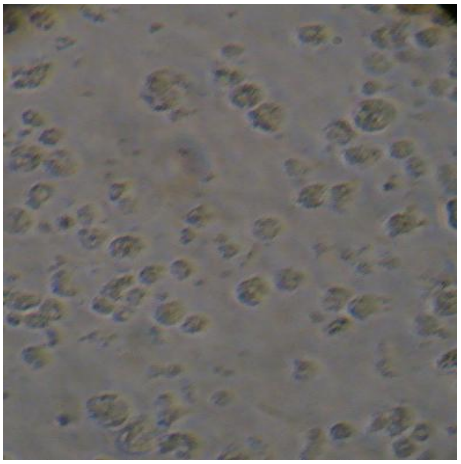

Figure 3a: TMX loaded GG NPs x 48 hrs(Jurkat Cells)

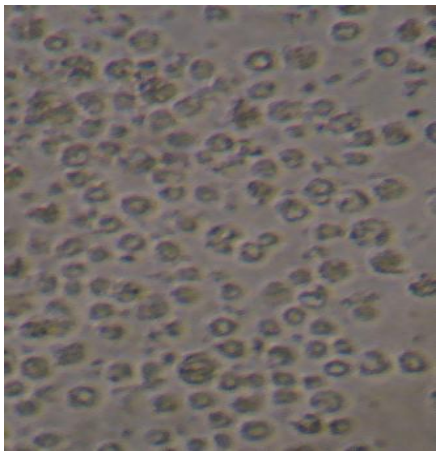

Figure 3b: TMX x 48 hrs (Jurkat Cells)

Under these conditions the formulations of predesigned nanoparticles with calculated process parameter may be very efficient tool for intercellular delivery of anticancer drugs like tamoxifen citrate. These results demonstrated that this delivery system could be a promising choice for administering tamoxifen citrate without loss of its therapeutic efficacy.

CONCLUSION

Nanoparticles by virtue of its size and properties are potential tools for treating cancer. Guar gum nanoparticles containing tamoxifen citrate under the present investigation exhibited the benefits of nanoencapsulation for highly protein binding drug tamoxifen citrate in passive targeting of cancer. The drug loaded nanoparticles were as efficient as the free drug when applied to the cancer cells. However, the crosslinked guar gum nanoparticles loaded with tamoxifen citrate exhibited sustained release of the drug and delayed apoptosis over a long period of time making it suitable for cancer treatment.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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