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Tamoxifen nanostructured lipid carriers: Enhanced *in vivo* antitumor efficacy with reduced adverse drug effects

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ABSTRACT

A novel approach of enhancing the Tamoxifen uptake via Intestinal Lymphatic System is executed by developing long chain lipid and oil based nanostructured lipid carrier system (Tmx-NLC). The aim was to achieve improved systemic bioavailability of Tamoxifen, prevent systemic and hepatotoxicity and enhance antitumor efficacy. Following the proof of concept achieved in cell culture experiments and *in vivo* pharmacokinetic and biodistribution study, the current work focuses on investigation of antitumor efficacy and treatment associated toxicity in murine mammary tumor mice model. The efficacy study demonstrated greater tumor suppression and 100% survival with 1.5 and 3 mg/kg Tmx-NLC compared to 3 mg/kg Tamoxifen suspension and Mamofen® (Khandelwal Pharmaceuticals, Mumbai, India). Tmx-NLC treatment for a month demonstrated improved systemic toxicity profile and no evidences of hepatotoxicity. Thus, developed Tmx-NLC could prove to be a promising delivery strategy to confer superior therapeutic efficacy and ability to address the biopharmaceutical and toxicity associated issues of drug.

1. Introduction

Breast cancer chemotherapy has relied on Tamoxifen citrate (Tmx), a non-steroidal anti-estrogen drug as the agent of choice for several years. The drug has demonstrated promising curative effect in estrogen positive (ER+) breast cancer patients and also been employed in preventive trials for healthy women at high risk of developing breast cancer (Fugh-Berman and Epstein, 1992; Vanchieri, 1992). However, some of the inherent limitations seen after oral administration of Tmx like poor biopharmaceutical profile and undesirable toxic effects at organ and systemic level need immediate attention. Several studies at preclinical and clinical levels have demonstrated high susceptibility of Tmx to liver metabolism and tendency to precipitate in gastric environment which consequently resulted in dramatically reduced systemic exposure (Hard et al., 1993; McVie et al., 1986; Tukker et al., 1986). This necessitates high dose administration which leads to dose dependent side effects and large inter-individual variation. Repeated oral administration of Tmx to patients showed higher incidence of liver abnormalities like toxic hepatitis, massive hepatic steatosis or multifocal hepatic fatty infiltration, sub-massive hepatic necrosis and even cirrhosis. Earlier work has reported more than 30% incidence of fatty liver in patients previously treated with Tmx (Elefsiniotis et al., 2004). Few reports have revealed that Tmx causes oxidative liver damage which is attributed to overproduction of free radicals associated with its major metabolism in liver, thus concluding it to be a hepatocarcinogen in rodents (Greaves et al., 1993; Han and Liehr, 1992; Karki et al., 2000). Moreover, chronic administration of Tmx renders patient highly vulnerable to endometrial cancer (Shin et al., 2006). Severe pancytopenia and thrombocytopenia are other grave concerns at systemic level which are linked to its erratic and abrupt drug exposure (Barbieri et al., 2013; Lerner et al., 1976; Spence et al., 2004). Besides, metastasis occurring via regional lymph nodes remains a prime reason of mortality in breast cancer

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patients (Chua et al., 2001). Lymphangiogenesis provocates the lymphatic endothelial progenitor cells and aggravates the migration of tumor cells into lymph node which further spreads to systemic circulation (Skobe et al., 2001). This scenario demands a smart delivery strategy which can take care of above-mentioned issues and improvise the efficacy of Tmx therapy with special priority to the circumvention of toxicity issues too. Accordingly in our previous research work we had developed Tmx loaded nanostructured lipid carrier system (Tmx-NLC) using long chain solid lipids and oils and further transformed it into freeze dried nanoparticles (Shete and Patravale, 2013). In this formulation, Tmx was molecularly dispersed in lipid matrix and was afforded rigorous stability to withstand the hostile environment of GI tract. The NLC showed superior antiproliferative activity against ER+ breast cancer cells by localizing in cytoplasmic and nuclear region. *In vivo* pharmacokinetic and mesenteric lymph node biodistribution studies demonstrated improved systemic drug profile and remarkable drug concentration in mesenteric lymph nodes (Shete et al., 2013). These results strengthened our hypothesis of targeting intestinal lymphatic system (ILS) and helped to anticipate the probable uptake pathway for Tmx-NLC. The present investigation is a step further in above mentioned research work wherein we have developed a murine mammary tumor model in BALB/c mice using 4T1 murine mammary cancer cells and evaluated the antitumor efficacy of Tmx and assessed the intrinsic effect of NLC on the improvement of antitumor activity to establish a proof of concept. The study also investigated the detailed post-treatment effect of Tmx-NLC on feed consumption, various vital organs and critical blood components for which Tmx is known to be damaging.

2. Methods

2.1. Formulation preparation

Tmx-NLC were formulated by a method previously reported by our research group (Shete and Patravale, 2013). In brief, probe sonication aided solvent diffusion technique was employed to formulate Tmx-NLC. Glycerylmonosterate (GMS) [monoglyceride of stearic acid (C18)] (lot # 15320) as the solid lipid (Gattefosse, St-Priest, France) and Labrafil WL 2609 BS [ethoxylated polyglycolyzed glycerides of oleic acid (C18:1; 24–34%) and linoleic acid (C18:2; 53–63%)] (lot # 27898) as oil (Gattefosse, St-Priest, France) were used as lipid matrix components. The developed Tmx-NLC was further transformed into lyophilized product. Lyophilization of NLC was carried out on Labconco freeze dryer (FreeZone 4.5, USA) using 5% (w/v) of sucrose as cryoprotectants. The process includes freezing at −40 °C for 24 h, primary drying at 0 °C for 5 h followed by 10 °C for 2.5 h and 15 °C for 2 h and secondary drying at 25 °C for 2.5 h. The chamber pressure and cold trap temperature was maintained at 20 Pa and −50 °C for entire process.

2.2. In vivo *pharmacokinetic and mesenteric lymph node biodistribution studies*

The studies were executed by the approval of Institutional Animal Ethics Committee (IAEC) of Institute of Chemical Technology (Protocol no: ICT/IAEC/2012/P2) and conducted previously by our research group using High performance liquid chromatographic technique (Shete et al., 2013).

2.3. Cell culture and conditions

4T1 cell lines (ATCC, Rockville, USA) were employed to perform *in vivo* antitumor efficacy study in BALB/c mice. The cells were maintained at 5% CO_2 and humidified in 37 °C incubator in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco®, Carlsbad, USA). Cells after reaching the confluency of 100% were harvested by trypsinization using 0.05% Trypsin-EDTA (Gibco®, Carlsbad, USA). The cell counts were performed by staining the cell suspension with Trypan blue and counted using haemocytometer under the light microscope (BX41TF, Olympus Optical Co. Ltd., Tokyo, Japan) at 20X magnification in 4 different fields. The cell suspension was diluted suitably in complete medium to attain the cell count of approximately 2×10^5 cells/ml.

2.4. Animals

The animal study experiment protocol was reviewed and approved by Institutional Animal Ethics Committee of National Institute for Research in Reproductive Health (NIRRH) (protocol no. NIRRH/IAEC/23/12) where the study was executed in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), India. Seven weeks old, healthy female BALB/c mice with average body weight of 18–22 g were employed for *in vivo* antitumor efficacy study. Polypropylene cages were employed to house four animals per cage in institutional animal house. The animals were maintained at the controlled temperature of 23 ± 1 °C, humidity of 55 ± 5%, in a 14 h light/10 h dark cycle. The cages contained autoclaved corncobs as bedding that was replaced on a weekly basis. Throughout the study, the animals were provided with soy-free, in house-prepared mice pellets (consisting of crude protein, fibre, and nitrogen free extract) and water (purified by UV and reverse osmosis) ad libitum. The quality of food and water provided was routinely monitored by qualitative and quantitative analysis.

2.4.1. Tumor development protocol

One day prior to tumor implantation, hair on the right shoulder region of the mice was removed by shaving and chemical depilation. Injection site of the experimental mice was properly cleaned and sterilized with ethanol. The cell suspension (2×10^5 cells in 100 μl, phosphate buffer saline (PBS)) was drawn into 1 ml Tuberculin (TB) syringes without needle to minimize damage, lysis and death of the cells. The 100 μl cell suspension was injected subcutaneously into the right flank of mice slowly using a TB syringe (needle gauge 26). The area surrounding the injection site was washed with a generous amount of warm PBS to dissipate any cells that may have spilled or leaked. All animals were monitored on regular basis for the tumor development. Animals bearing measurable tumor size approximately 100 mm^3 were selected and divided into various treatment groups.

2.4.2. Treatment protocol

The tumor bearing mice were randomly assigned into various groups ($n = 8$; Table 1). The treatment was initiated on day 0 when the tumor volume reached approximately 100 mm^3. Antitumor activity of Tmx-NLC was evaluated at three different doses of Tmx namely 0.75, 1.5 and 3 mg/kg. Tumor bearing mice were administered respective doses of various formulations (Table 1) by oral gavage once in 3 days for 28 days.

Table 1
Summary of various groups and their treatment protocols used for *in vivo* antitumor efficacy study in tumor bearing mice.

Group ($n = 8$)	Dose of drug
Untreated control	Water
Placebo NLC	Equivalent to 3.0 mg/kg
0.75 mg/kg Tmx-NLC	Equivalent to 0.75 mg/kg Tmx
1.5 mg/kg Tmx-NLC	Equivalent to 1.5 mg/kg Tmx
3.0 mg/kg Tmx-NLC	Equivalent to 3.0 mg/kg Tmx
3.0 mg/kg Tmx-susp	Equivalent to 3.0 mg/kg Tmx
3.0 mg/kg Mamofen®	Equivalent to 3.0 mg/kg Tmx