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The effect of the oral administration of polymeric nanoparticles on the efficacy and toxicity of tamoxifen

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ABSTRACT

The present investigation reports on the conditions for preparation of tamoxifen loaded PLGA nanoparticles (Tmx-NPs) for oral administration. Tmx-NPs with >85% entrapment efficiency and 165.58 ± 3.81 nm particle size were prepared and freeze dried. Freeze dried Tmx-NPs were found to be stable in various simulated GIT media (pH 1.2, pH 3.5, pH 6.8, SGF & SIF). No significant changes in characteristics of Tmx-NPs were observed after 3 months accelerated stability studies. The cell viability in C127I cells was found to be relatively lower in Tmx-NP treated cells as compared to free Tmx treated cells. CLSM imaging reveled that nanoparticles were efficiently localized into the nuclear region of C127I cells. Oral bioavailability of Tmx was increased by 3.84 and 11.19 times as compared to the free Tmx citrate and Tmx base respectively, when formulated in NPs. *In vivo* oral antitumor efficacy of Tmx-NPs was carried out in DMBA induced breast tumor model and tumor size was reduced up to 41.56% as compared to untreated groups which showed an increase in tumor size up to 158.66%. Finally, Tmx-NPs showed the marked reduction in hepatotoxicty when compared with free Tmx citrate as evidenced by histopathological examination of liver tissue as well as AST, ALT and MDA levels. Therefore Tmx-NPs could have the significant value for the oral chronic breast cancer therapy with reduced hepatotoxicity.

1. Introduction

Breast cancer is the second leading cause of cancer deaths today after lung cancer and is the most common cancer among women [1]. For over a quarter of a century, tamoxifen (Tmx) has been prescribed to treat patients with advanced breast cancer. Tmx belongs to a class of non-steroidal triphenylethylene derivatives and is the first selective estrogen receptor modulator (SERM) [2]. The US Food and Drug Administration (FDA) approved Tmx for the treatment of advanced breast cancer in late 1998 [3]. Tmx shows its potential effects in patient who possess estrogen receptors (ER) positive cancer cells by competing with estrogen to bind with estrogen receptor in breast cancer cells [4].

As Tmx therapy is chronic one (3–5 years), oral delivery is the most preferred route of administration and its solubility problem in aqueous milieu has been overcome by forming its salt form, tamoxifen citrate (Tmx citrate). Commercially, Tmx is available only as tablet and oral solution containing Tmx citrate in a daily dose of 10–20 mg. However Tmx citrate also showed the poor oral bioavailability (20–30%) due to its precipitation as free base in the acidic environment of stomach and also due to extensive hepatic and intestinal first pass metabolism, so as to increase its does [5]. So in spite of a clinical choice in advanced and metastatic stages of breast cancer, it suffers from large inter subject variability and several dose and concentration dependent side effects [6–8]. It mainly causes oxidative stress mediated hepatotoxicity, i.e. toxic hepatitis, multifocal hepatic fatty infiltration, sub massive hepatic necrosis and cirrhosis [9]. Tmx is also having high risk of causing endometrial cancer which depends mainly upon treatment duration and dose accumulation [10]. Thus, existing therapy renders its difficult to administer in minimum effective dose, leading to liver toxicity. Thus an alternate delivery system is essential for optimal oral chronic therapy of Tmx with improved bioavailability and reduced side effects especially hepatotoxicity.

Biodegradable polymeric nanoparticles (NPs) have gained a considerable interest in this regard [11]. Amongst them poly (lactic-*co*-glycolic acid) (PLGA) is an approved biodegradable polymer with good biocompatibility and widely employed for loading and encapsulation of variety of anticancer drugs [12–14]. When polymeric NPs are administered by oral route, the M-cells

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(specialized cells staying over mucosa-associated lymphoid tissue) in Payer's patches uptake the nano/microparticle and transport them from the gut lumen to intra-epithelial lymphoid cells and afterward through the lymphatic system into the blood stream [15–17]. NPs follow this special pathway and thus enhance the bioavailability of encapsulated drug and also avoid the enzymatic degradation in enterocytes, first pass metabolism in liver thus decrease the dose and ultimately the drug related toxicity.

In the present work tamoxifen loaded PLGA nanoparticles (Tmx-NPs) have been prepared, characterized and freeze dried. The freeze dried Tmx-NPs were evaluated for *in vitro* release characteristics, GIT stability and accelerated stability study. *In vitro* antitumor activity was evaluated on mouse breast cancer cells C127I [18]. Pharmacokinetics, *in vivo* antitumor efficacy and hepatotoxicity were also evaluated after oral administration.

2. Materials and methods

2.1. Materials

PLGA 50/50 (inherent viscosity 0.41 dl/g in chloroform at 25 °C) was used from Boehringer Ingelheim (Ingelheim, Germany). Tamoxifen (Z)-2-[4-(1, 2-diphenyl-1-butenyl)phenoxy]-N,N dimethylethylamine (free base and citrate salt), Didodecyldimethylammonium bromide (DMAB) (98%), Polyvinyl alcohol (PVA) (M_W. 30000–70000), Pluronic F-68, 7, 12-dimethylbenz[a]anthracene (DMBA), Trypsin-EDTA, MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), coumarin-6, triton X-100 and propidium iodide (PI) were obtained from Sigma, USA. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (Antibiotic–antimycotic solution) and Hanks's balanced salt solution (HBSS) were purchased from PAA, Austria. Tissue culture plates and 8-well culture slides were procured from Tarsons and BD Falcon, respectively. Ethyl acetate (LR grade), Acetonitrile (HPLC grade), methanol (HPLC grade) were purchased from Ranchem Fine Chemicals, India. Ultra pure water (SG water purification system, Barsbuttel, Germany) was used for all the experiments. All other reagents used were of analytical grade.

2.2. Preparation of Tmx loaded nanoparticles

Tmx-NPs were prepared by emulsion diffusion evaporation method as reported earlier in literature [19] with slight modification according the laboratory conditions. Briefly, 50 mg of PLGA along with 5 mg of Tmx were dissolved in 2.5 ml of ethyl acetate (EA) at room temperature. The organic phase was then added to 5 ml of an aqueous phase containing the stabilizer. The resulting o/w emulsion was stirred at 1000 rpm for 20 min. The droplet size reduction of resulting emulsion was carried out either by homogenization (high-speed homogenizer, Polytron PT 4000, Switzerland) or sonication (Misonix, USA). The resulting emulsion was poured into 25 ml of water with constant stirring to diffuse and finally evaporating the organic solvent. This resulted in nanoprecipitation and formation of NPs. The NPs suspension was then centrifuged and washed repeatedly to remove the excess surfactant and finally dispersed in 2 ml distilled water and freeze dried (FD).

2.3. Optimization of process variables

2.3.1. Effect of droplet size reduction process

Screening of the droplet size reduction processes (i.e. either homogenization or sonication) was carried out to get the optimum size (below 200 nm). For this, NPs were prepared following the above described process keeping other experimental parameters like aqueous to organic phase ratio 1:2, final volume of dilution 25 ml and stabilizer concentration (2% w/v PVA) constant. Different homogenization speeds and sonication (60% amplitude for 1 min) were employed to prepare NPs dispersion. Finally particle size and PDI of NPs dispersion was measured using zeta sizer (Nano ZS, Malvern, UK).

2.3.2. Screening of suitable stabilizer

Tmx-NPs were prepared by using different type and concentration of stabilizers like DMAB, PVA and Pluronic F-68. The best suitable stabilizer was identified based on the optimum particle size, zeta potential and entrapment efficiency.

2.3.3. Screening of optimum concentration of stabilizer

The best suitable stabilizer identified as above was then screened for the optimum concentration of the stabilizer required for the preparation of Tmx-NPs. The optimum concentration of stabilizer was determined on the basis of particle size, size distribution and encapsulation efficiency.

2.3.4. Optimization of drug loading

Finally, Tmx-NPs were prepared using different Tmx loading i.e. 5%, 10% and 15% w/w of polymer and its effect on particle size and entrapment efficiency was studied. The other experimental parameters like sonication time (1 cycle at 60% of amplitude for 60 s), stabilizer concentration (2% PVA) and aqueous to organic phase ratio 1:2 were kept constant.

2.4. Characterization of nanoparticles

2.4.1. Particles size and zeta potential measurement

Tmx-NPs were evaluated for their mean particle size and polydispersity index (PDI) by using Zeta Sizer (Nano ZS, Malvern Instruments, UK). All the values were taken by the average of 6 measurements. Zeta potential was estimated on the basis of electrophoretic mobility under an electric field, as an average of 30 measurements. Zeta potential was also determined by using Zeta Sizer (Nano ZS, Malvern Instruments, Malvern, UK).

2.4.2. Entrapment efficiency

The percentage of drug encapsulated in PLGA NPs was determined by using a validated HPLC method reported in literature with slight modifications [20]. Briefly, Tmx-NPs suspension was centrifuged and the obtained pellet was dissolved in acetonitrile furthermore analyzed by Waters high-performance liquid chromatography (HPLC) system consisting of 996 Photodiode Array Detector and dVR Agilent Technologies Lichrospher® 100 RP-18e end capped 5 μm column (Lot No. L 54921633) (Germany). Acetonitrile and methanol (containing 0.02% triethylamine) (70:30) were used as the mobile phase with a flow rate of 0.7 ml/min. The injection volume was 10 μl and retention time of Tmx was found to be 5.1 min. The detection wavelength (λ_{max}) for Tmx was 281 nm.

2.4.3. Morphology of nanoparticles

The surface morphology of nanoparticles was analyzed by atomic force microscope (Veeco Bioscope II, USA). The nanoparticles suspension were placed on the silicon wafer with the help of a pipette and allowed to dry in air. The microscope is vibration damped and measurements were made using commercial pyramidal Si_3N_4 tips (Veeco's CA, USA). The cantilever used for scanning was having length 325 μm and width 26 μm with a nominal force constant 0.1 N/m. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded.

2.5. Freeze drying of NPs

Tmx-NPs were freeze dried (Vir Tis, Wizard 2.0, New York, USA freeze dryer) following an optimized freeze dried cycle (Table 1) [21]. The condenser temperature was −60 °C and pressure applied in each step was 200 Torr. 2 ml of washed NPs suspension was filled in 5 ml glass vials and subjected to freeze drying using 5% w/v of trehalose. After freeze dying the Tmx-NPs were characterized for the appearance of the cake, reconstitution time, size after freeze drying, entrapment efficiency, nature of drug in nanoparticles using DSC and XRD analysis.

2.6. DSC analysis

Differential scanning calorimetry (DSC) thermogram of the freeze dried Tmx-NPs, physical mixture, pure tamoxifen and trehalose was carried out using a Mettler Toledo differential scanning calorimeter calibrated with indium standards. Measurements were performed at heating rate of 10 °C/min from 0 to 200 °C.

2.7. XRD analysis

The X-ray diffraction patterns of pure tamoxifen, PLGA, blank nanoparticles, drug loaded freeze dried nanoparticles were obtained using the X-ray diffractometer

Table 1
Optimized freeze drying cycle.

Thermal treatment			Primary drying		
Step	Temperature (°C)	Time (Min)	Step	Temperature (°C)	Time (Min)
1	20	30	1	−45	60
2	15	60	2	−30	360
3	10	60	3	−20	360
4	−5	120	4	−10	420
5	−15	60	5	−5	360
6	−25	60	6	0	180
7	−45	30	7	5	120
Secondary drying			8	10	60
1	25	120	9	15	60
			10	20	30