Journal of Photochemistry & Photobiology, B: Biology 165 (2016) 182–188

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Development and evaluation of topotecan loaded solid lipid nanoparticles: A study in cervical cancer cell lines

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ARTICLE INFO

Article history:
Received 6 October 2016
Accepted 17 October 2016
Available online 18 October 2016

Keywords:
Cervical cancer
Topotecan
Solid lipid nanoparticles
Chemotherapy

ABSTRACT

The study aims at statistical development of solid lipid nanoparticles (SLNs) loaded with topotecan hydrochloride for avoiding the drawbacks of conventional drug therapies used in cervical cancer. Twenty SLN batches were prepared using organic solvent evaporation method to provide response surface curves. Thereafter, optimized SLNs were obtained using numeric method based on desirability functions providing maximum drug loading and appropriate particle size. Physical characterization of optimized TPH loaded SLNs was performed in terms of particle size, zeta potential, transmission and scanning electron microscopic evaluation. Cytotoxicity studies were performed against cervical cancer cell lines, including cervical squamous cell carcinoma cell line (HeLa) and human squamous cell carcinoma cell line (SiHa). Also, Swiss mouse embryo fibroblast cells (3T3-L1) and African green monkey kidney epithelial (Vero) cells were used to evaluate biocompatibility in normal cells. As pronounced from the results, optimized SLNs may provide an attractive alternative to conventional cervical cancer drug products.

1. Introduction

Cervical cancer is among the most common malignant disorders occurring in women globally, with nearly around 0.5 million cases occurring each year, approximately half of which turn into terminal malignancies [15]. The incidence of this disorder is particularly high in developing nations, which are not having adequate healthcare measures for screening and immunizations. Despite relentless efforts from academic scientists and pharmaceutical industries around the world, efforts for combating cervical cancer seem insufficient. In addition to synthesis of novel drugs against cervical cancer, there is a prominent need to improve the currently available therapies using novel or unconventional drug delivery vehicles [4,12].

Topotecan hydrochloride (TPH) is a semisynthetic anticancer drug, approved in most nations about two decades back for treatment of cervical cancer [15]. It is a cytotoxic agent acting by inhibiting *via* covalent linkage, an enzyme topoisomerase, which is involved in DNA replication. Consequently, this DNA damage results in apoptotic cell death particularly in rapidly dividing cancerous cells. Conventionally available topotecan products are sold under a brand Hycamtin®, and are available as oral capsules or powder for intravenous solutions. Despite being the most commonly used drug in cervical cancer, Hycamtin® suffers from certain disadvantages [19]. For instance, the oral capsules are known to have variable and erratic absorption. This can lead to incomplete drug efficacy and increased inter-person variability in bioavailability. Moreover, the injectable formulation in addition to the usual limitations of poor patient compliance is also associated with severe adverse reaction and undesired side effects [17,19].

This instigated us to prepare a novel drug carrier system of TPH, which could simultaneously address the problem of poor bioavailability and at the same time reducing the toxicity profile of the drug. Solid lipid nanoparticles (SLNs) are hailed as novel solid nano-cores prepared using lipids and surfactants, which in addition to efficiently encapsulating the miscellaneous drugs also enhance their bioavailability and safety profile. Recent studies have exhibited their potential in delivery of hydrophobic, hydrophilic as well as amphiphilic drugs [2].

SLNs of TPH were developed using a statistical design of experiment based approach, which involved designing an experimental matrix consisting of 20 experimental runs. It aided to optimize the SLNs with respect to their particle size and percent drug loading (PDL%). The optimized formulations were characterized for various formulation attributes including particle morphology, polydispersity index, zeta potential and *in vitro* release studies. The *ex vivo* efficacy of optimized SLNs was evaluated using cytotoxicity studies in cervical cancer cell line (HeLa and SiHa) studies.

2. Materials and Methods

2.1. Materials

Topotecan hydrochloride (TPH), Eagle's minimal essential medium (EMEM), RPMI-1640 tissue culture medium, foetal bovine serum

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http://dx.doi.org/10.1016/j.jphotobiol.2016.10.019
1011-1344/

(FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Shanghai, China). Various lipid excipients and surfactants used were obtained from XYZ chemical company (Shanghai, China). Other reagents used were of analytical grade. The American Type and Culture Collection (ATCC) cancer cell lines and normal cell lines were procured *ex gratis* from Fuda Cancer Hospital (Guangzhou, China).

2.2. Methods

2.2.1. Preparation of SLNs

The first step was to carry out solubility studies of the drug into several lipids to ensure maximum drug loading capacity. Weighed amount of TPH (5 mg) was placed in screw capped vials and different lipids, separately heated approximately 5 °C above their melting point were added with vortex mixing. The amount of molten lipid required for complete solubilization of TPH was visually observed.

Thereafter, SLNs were prepared using organic solvent evaporation technique with modifications [7]. Briefly, the drug along with lipid (cetyl palmitate) was dissolved in a 1:1 (v/v) mixture of chloroform and methanol, and the solution was warmed to 65 ± 2 °C. The surfactant (Tween 80) was dissolved in water, and the alcoholic solution of previous step was added as a thin stream with the help of a hypodermic needle into the preheated with vigorous stirring to it. The suspensions were then probe-sonicated for 5 cycles of 1 min to obtain a dispersion containing SLNs.

2.2.2. Characterization of SLNs

The particle size, polydispersity index and zeta potential were determined using Malvern Zetasizer (Malvern Instruments Ltd., Worcestershire, UK). In addition, the morphology of optimized SLNs was also studied using transmission electron microscopy (H-7500, Hitachi, Japan) after depositing the SLNs sample on a film coated 200-mesh gold specimen grid. Scanning electron microscopic (SEM) images of freeze dried SLNs was also carried out using Jeol scanning microscope (Jeol Inc., Osaka, Japan). The sample surface was made electrically conductive in a sputtering apparatus (Jeol Fine Coat, ion sputter, JFC-1100, Japan) by evaporation of gold.

The PDL% of the prepared SLNs was determined using ultracentrifuge technique. The PDL% achieved by the SLN dispersions was determined using centrifugation method. The nanoparticulate suspensions comprising 10 mg of drug were cold centrifuged (4 °C) for a period of 60 min at 10,000 rpm. The supernatant liquid was collected and filtered to measure the free drug concentration after suitable dilution with ethanol. The drug quantification was performed using a calibration plot prepared in ethanol (λ_{max} 382 nm). The PDL% values were obtained using the Eq. (1).

$$\text{PDL}\% = \frac{\text{Drug}_{in}}{\text{Drug}_{T}} \times 100 \quad (1)$$

where, Drug_{in} = Amount of drug incorporated
Drug_{T} = Total drug used

2.2.3. Statistical Design of Experiments

The SLNs were developed as per a face-centered 3^3-factorial experimental design using statistical software Design Expert® 6.0.10 (Stat-Ease Inc., Minneapolis, USA). Following the preliminary studies (data not shown), 3 factors namely, lipid amount (cetyl palmitate; X_1), surfactant (X_2; Tween 80) amount and ethanol volume (X_3) were studied for attaining a formulation having most desirable features. The low-, mid- and high-level of X_1, X_2 and X_3 were determined during preliminary experimental runs. Explicitly, the levels were: 3, 4.5 and 6% (w/w); 1, 2 and 3% (w/w); and 20, 30 and 40 mL for X_1, X_2, and X_3, respectively. The design matrix involved 20 experimental runs with center points and 14 axial points (Table 1). Software generated quadratic models were constructed, which provided the correlation among experimental factors and formulation attributes. The statistics of quadratic models depicted high level of regression and show high regression and model precision values (Table 2).

The response surface methodology was constructed and final optimization was performed numerically with an aim of obtaining highest desirability function. The optimization target was to attain maximum DPL% and particle size in desired range (between 200 and 300 nm).

2.2.4. In vitro Drug Release Study

The *in vitro* drug release profile of prepared formulations was studied using dialysis pouch technique. Briefly, weighed amount of SLNs dispersion (equivalent to 10 mg of drug) was taken in a dialysis pouch, which was sealed using cotton thread. Thereafter, the sealed pouch was placed in a 500 mL beaker containing 300 mL of phosphate buffet saline (pH 7.4). Gentle stirring at 50 rpm was performed at 37 ± 1 °C using magnetic bars using a digital magnetic stirrer IKA C-MAG (Staufen, Germany). At predetermined intervals, aliquots were withdrawn (with replacement) from the beaker. Finally, the drug content spectrophotometrically determined using previously described method, to calculate the amount of TPH released from the nanoparticles.

2.2.5. Cytotoxicity of SLNs in Cervical Cancer Cell Lines

The human cervical squamous cell carcinoma cell line (HeLa) was grown in RPMI-1640 culture medium, whereas human squamous cell carcinoma cell line (SiHa) was maintained using EMEM. Additionally, the inoculated cultures were supplemented with FBS (10%, w/v). To prevent any microbial growth, penicillin and streptomycin (100 IU/mL and 100 μg/mL, respectively) were added and the cell lines were maintained at constant temperature of 37 °C.

Varying strengths of SLNs dispersions (3.125 to 100 mM) were added to a 96-well plate for 24, 48, and 72 h. Similarly, untreated cells were also included as control sample. Thereafter, MTT solution (5 mg/mL) was added to each well and the well plate was incubated for 3 h. Dimethyl sulfoxide was then added to solubilize the dark-blue formazan crystals. The absorbance at 570 nm and the reference wavelength of 630 nm were measured with a SpectraMax microplate reader (Beijing, China). Cell viability was determined using Eq. (2).

$$\text{Cell viability} = \frac{\text{OD}_{\text{treated}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \times 100 \quad (2)$$

Table 1
A 3^3 factorial design matrix used for optimizing TPH loaded SLNs (*italicized runs depict replicate center points).

Formulation code	Factor X_1 (%, w/w)	Factor X_2 (%, w/w)	Factor X_3 (mL)	Particle size (nm)	DPL (%)
TPH1	3	1	20	228.5	53.62
TPH2	6	1	20	318.4	86.23
TPH3	3	3	20	185.5	60.85
TPH4	6	3	20	290.7	92.25
TPH5	3	1	40	210.6	55.29
TPH6	6	1	40	296.6	87.53
TPH7	3	3	40	176.6	64.36
TPH8	6	3	40	279.4	93.95
TPH9	3	2	30	203.9	59.63
TPH10	6	2	30	277.2	90.15
TPH11	4.5	1	30	233.5	60.36
TPH12	4.5	3	30	210.9	88.36
TPH13	*4.5*	*2*	*20*	*268.6*	*83.36*
TPH14	*4.5*	*2*	*40*	*236.5*	*85.45*
TPH15	*4.5*	*2*	*30*	*250.3*	*83.26*
TPH16	*4.5*	*2*	*30*	*262.9*	*80.32*
TPH17	*4.5*	*2*	*30*	*255.7*	*78.63*
TPH18	*4.5*	*2*	*30*	*261.2*	*82.5*
TPH19	*4.5*	*2*	*30*	*259.4*	*80.06*
TPH20	*4.5*	*2*	*30*	*260.6*	*79.36*