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Original article

Etoposide loaded solid lipid nanoparticles for curtailing B16F10 melanoma colonization in lung

Rajani B. Athawale [a,*], Darshana S. Jain [a], Kamlinder K. Singh [a], Rajiv P. Gude [b]

[a] Department of Pharmaceutics, C.U. Shah College of Pharmacy, S.N.D.T Women's University, Juhu Tara Road, Santacruz (West), Mumbai 400049, India
[b] Gude Lab, Advanced Centre for Treatment, Research & Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai 410210, India

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ABSTRACT

Poor solubility of etoposide and associated poor bioavailability of the drug was circumvented by developing solid lipid nanocarrier system. The objective of the research work was to prepare etoposide loaded solid lipid nanoparticles (SLN) for improved efficacy and therapy of metastasized cancers. Entrapment of drug into nanoparticulate system modifies the pharmacokinetic and biodistribution profile of the drug with improved therapeutic efficacy. Solid lipid nanoparticles of various triglycerides were prepared using hot homogenization technique. Further, the process and formulation parameters viz. homogenization cycle and pressure, type of lipid were optimized. Developed nanoparticles were characterised for particle size, *in vitro* dissolution studies, DSC thermogram, surface morphology and cytotoxicity assay. Pharmacokinetic and biodistribution study were performed to assess the distribution of the drug *in vivo*. Modulation of the therapeutic activity of the drug was studied by performing antimetastatic activity on a B16F10 melanoma mouse model. The obtained results exhibited suitability of trimysristin for fabrication of nanoparticles. Characterisation of nanoparticles depicted formation of homogenous, spherical particles entrapping approximately 50% of the drug. The results for the performed MTT assay suggested that the developed nanoparticles exhibited cytotoxicity in a time- and concentration-dependent fashion. These findings concord with the results of the *in vitro* dissolution profile. Pharmacokinetic parameters demonstrated increase in area under curve (AUC), $t_{1/2}$ and mean residence time (MRT) for drug in plasma. Further there is enhancement in the ratio of the drug that reaches to the highly perfused organs (upon encapsulation into solid lipid nanoparticles). Generally, cancer cells metastasized through the blood or lymphatic system. Accumulation of the drug in the highly perfused organ suggests suitability of the developed nanoparticles for targeting metastasized tumors. This was proved by the findings of the *in vivo* B16F10 mouse melanoma model. Improvement in the tumoricidal activity and survival rate of the animals substantiates the application of nanoparticles for improved therapeutic activity of etoposide.

1. Introduction

Etoposide is a semi-synthetic podophyllotoxin derived from the root of *Podophyllum peltatum* [1]. It is used in the treatment of a variety of tumors including small cell lung cancer, testicular cancer, lymphomas and leukemias. Etoposide is one of the drugs in chemotherapy used against metastatic tumors. Etoposide has variable oral bioavailability ranging from 24–74% and has terminal half life of 1.5 hours by intravenous route. It is generally given by three weekly 24-hour infusions (600 mg/m^2) to patients with extensive-stage lung cancer [2]. Etoposide exhibits its action by inhibiting Topoisomerase II [3]. The conventional parenteral therapy causes inconvenience and pain to the patients as it has to be given through a continuous i.v. infusion over 24–34 h. The major limiting step in the formulation of etoposide is its lipophilicity and the conventional formulations available in market contain many solubilizers, which are associated with adverse side effects such as hypotension, anaphylaxis, bronchospasm, etc. [4].

Further, the conventional systemic administration of etoposide struggles with problems, such as short *in vivo* half-life and its toxic effects. These difficulties have urged the need for developing specialized systems which allow selective delivery of one or more chemotherapeutic agents to cancer cells, without affecting the physiology of normal cells. To meet these requirements, numerous targeted drug delivery systems have been proposed over the last two decades. In cancer chemotherapy, many type of carrier systems such as microspheres, microcapsules, polymeric nanoparticles, liposomes, cationic liposomes and emulsions have been

* Corresponding author. Tel.: +919892823040.
E-mail address: rajani.athawale@gmail.com (R.B. Athawale).

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investigated to find a means of delivering anti-tumor agent to target site [5].

Amongst the novel drug delivery strategies, nanoparticles are preferably and are receiving great attention for treatment of cancers. Angiogenic factors are not regulated properly in a tumor resulting in inadequate lymphatic system and blood vessels supplying the nutrients to cancer cells. Submicron-sized particulate matters tend to accumulate in tumor and retain there by the effect of "enhanced permeability and retention" (EPR). This kind of accumulation that is only related to particle size is called "passive targeting" and is advantageous. Nanoparticulate system such as polymeric nanoparticles, solid lipid nanoparticles etc. are prepared to achieve successful passive tumor targeting [6].

Lack of physical stability, difficulties in scale up and cytotoxicity of the polymers are major limitation of the polymeric drug delivery systems. Lipid based drug delivery systems have advantages of most other particulate carrier systems such as physical stability, protection of incorporated drugs from degradation, controlled release and excellent tolerability and at the same time minimization of drug associated adverse effects [7].

Solid lipid nanoparticles (SLN) are good carriers for poorly water-soluble drugs [8]. Etoposide is a highly water insoluble anticancer agent, and has been selected for formulation of lipid based carrier systems. Tumor cells exhibit an increased uptake of low-density lipoproteins (LDL) owing to their high cholesterol need, which forms the basis of SLN as a drug carrier in cancer chemotherapy [9]. Entrapment of cytotoxic drug etoposide inside SLN as carrier improves drug specificity and reduces the toxicity to non-diseased cells. The solid lipid nanodispersions can be produced by a variety of techniques; solvent injection method, film hydration method, and high-pressure homogenization technique, etc. [8]. The production technique predominantly used for preparation of solid lipid nanoparticles is the high-pressure homogenization method, which reduces the number and size of large particles. The high-pressure homogenization method was employed to produce solid lipid nanodispersions of etoposide in the present study.

The present investigation was aimed at developing etoposide loaded biodegradable nanoparticles which would be a sustained release formulation and replace the conventional therapy of continuous intravenous administration. Further, the entrapment efficiency of etoposide was improved by preparation and optimisation of solid lipid nanoparticles. Literature search indicated that generally polymeric nanoparticles of Poly lactic–co-glycolic acid (PLGA) [10–12], Poly lactic acid (PLA) [13] and Polycaprolactone (PCL) [12] have been prepared for etoposide.

We have made an attempt to prepared and characterise solid lipid nanoparticles loaded with etoposide. The performance of these nanoparticles was assessed by performing *in vitro* as well *in vivo* studies.

2. Experimental

2.1. Materials and method

Etoposide of pharmaceutical purity was obtained as generous gift sample from Dabur Ltd., Ghaziabad and Neon Laboratories, Thane. Stearic acid (m.p. 54 °C) Glyceryl Behanate, Compritol ATO 888 (m.p. 62 °C) was received as generous gift sample from Gattefosse. Trimyristin (Dynasan 114) (m.p. 52 °C), Tripalmitin (Dynasan 116) (m.p. 54 °C), Tristearin (Dynasan 118) (m.p. 56 °C) were provided as a gift sample by Sasol, Germany. Soya lecithin (Epikuron 200) was provided as gift sample by Degussa Bioactives, Deutscland GmBH. All other chemicals and reagents used in the study were of the highest available grade.

2.2. Melanoma cells and inoculation

The B16F10 melanoma cell line was generated in Advanced Cancer Research Institute, Kharghar, Navi Mumbai. The cells were routinely maintained at 37 °C in humidified atmosphere, of 5% CO_2 in Iscove's minimum Dulbecod's medium GIBCO BRL, Maryland USA (IMDM) supplemented with 10% Fetal Calf Bovine Serum GIBCO USA obtained through Genetix, Mumbai. Antibiotics 100 units/ml penicillin and 100 μg/ml streptomycin were added to the cell culture medium. Cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cells were removed from the flask for sub-culturing with saline-EDTA solution.

2.2.1. Method of preparation for solid lipid nanodispersions

The solid lipid trimyristin, tripalmitin and tristearin and Compritol ATO 888 were melted at temperature 5 °C above the melting point of lipid. In the case of drug loaded batches, etoposide was dispersed in the lipid melt to obtain clear solution. The hydrophobic and hydrophilic surfactant along with solubilizer was added to the lipid and aqueous phase respectively. The aqueous dispersion medium containing Epikuron 200 surfactant was heated to the same temperature as that of lipid melt. The hot lipid phase was added to the hot aqueous phase and was kept under high speed stirring at 3000 rpm for 1 h. The particle size of the pre-emulsion was further reduced by high shear mixing using Ultra Turrax, T 25 for 2 min. The milky emulsions were further homogenized by high-pressure homogenizer (APV 2000 Model, Lab homogenizer). The homogenization step was repeated several times. The primary product of hot high-pressure homogenization was then rapidly cooled to about 10 °C by immersing the flask in cold ice bath. During cooling, nanoemulsion was continuously agitated at high speed by overhead stirrer to yield uniform solid lipid nanodispersions loaded with etoposide.

2.3. Characterization of drug loaded solid lipid nanodispersions

2.3.1. Total drug content

Assay of the drug loaded solid lipid dispersions was carried out by the developed HPLC method. One ml of solid lipid nanodispersion was transferred to a stoppered test tube; 5 ml of methanol was added to it. The mixture was centrifuged at 3000 rpm for one minute. Drug entrapped was extracted into methanol and lipids were allowed to settle down. One ml of above clear solution was transferred into another stoppered test tube and diluted it with one ml of mobile phase. 100 μl of this solution was subjected to HPLC analysis and chromatogram was recorded at 229 nm. The concentration of drug was determined from pre-developed standard calibration curve equation. The percent drug loading for each batch was calculated.

2.3.2. Mean particle size, particle size distribution and drug entrapment efficiency

The particle size analysis of the selected formulations was determined by laser diffraction technique using Beckman Particle size Analyzer. Nanodispersions were suitably diluted in a clean tube and placed in the cuvette [14]. The particle size distribution of selected drug loaded batches of solid lipid nanodispersions was measured and are given in Table 1.

Entrapment efficiency (%, mg/ml) was determined using high-speed centrifugation method. The sample (1 mg/ml) was suitably diluted with water to 10 ml. The diluted sample was centrifuged at high speed of 45,000 gy on a high speed centrifuging apparatus. The separated supernatant was collected in separate vacutainers and stored in deep freeze at −20 °C till further analysis. The free drug was estimated by analyzing the supernatant and the amount