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Development and evaluation of long-circulating nanoparticles loaded with betulinic acid for improved anti-tumor efficacy

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ABSTRACT

The clinical application of betulinic acid (BA), a natural pentacyclic triterpenoid with promising antitumor activity, is hampered due to its extremely poor water solubility and relatively short half-life in the systemic circulation. In order to address these issues, herein, we developed betulinic acid loaded polylactide-co-glycolide-monomethoxy polyethylene glycol nanoparticles (PLGA-mPEG NPs). The PLGA-mPEG co-polymer was synthesized and characterized using NMR and FT-IR. BA loaded PLGA-mPEG NPs were prepared by an emulsion solvent evaporation method. The developed nanoparticles had a desirable particle size (~147 nm) and exhibited uniform spherical shape under transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The PLGA-mPEG NPs were able to decrease the uptake by macrophages (*i.e.* J774A.1 and Raw 264.7 cells) as compared to PLGA nanoparticles. *In vitro* cytotoxicity in MCF7 and PANC-1 cells demonstrated enhanced cytotoxicity of BA loaded PLGA-mPEG NPs as compared to free BA. The cellular uptake study in both the cell lines demonstrated time dependent uptake behavior. The enhanced cytotoxicity of BA NPs was also supported by increased cellular apoptosis, mitochondrial membrane potential loss, generation of high reactive oxygen species (ROS) and cell cycle arrest. Further, *intravenous* pharmacokinetics study revealed that BA loaded PLGA-mPEG NPs could prolong the circulation of BA and remarkably enhance half-life by ~7.21 folds. Consequently, *in vivo* studies in Ehrlich tumor (solid) model following intravenous administration demonstrated superior antitumor efficacy of BA NPs as compared to native BA. Moreover, BA NPs treated Ehrlich tumor mice demonstrated no biochemical, hematological and histological toxicities. These findings collectively indicated that the BA loaded PLGA-mPEG NPs might serve as a promising nanocarrier for improved therapeutic efficacy of betulinic acid.

1. Introduction

Cancer is one of the most devastating diseases and chemotherapy remains as the widely used approach among various therapies such as different surgical procedures, antibody therapy, and radiotherapy (Saneja et al., 2014a). However, major reasons for the failure of chemotherapy are poor water solubility (causing difficulty in drug administration), severe side effects (due to poor penetration into tumors) and efflux transporter specificity (conferring drug resistance to cancer cells and result in poor pharmacokinetics) of chemotherapeutic agents (Saneja et al., 2014a,b, 2017a). It is also estimated that more than half of the common chemotherapeutic drugs are of natural origin or their synthetic derivatives (Saneja et al., 2017b; Watkins et al., 2014). Betulinic acid (BA), a naturally occurring plant-derived pentacyclic triterpenoid, was discovered as the most promising anticancer agent in a screen of 2500 plant extracts in the 1990s (Pisha et al., 1995). The capacity of BA to induce tumor cell death has been demonstrated *in vitro* in various cancer cells, including pancreatic, breast, hepatoma, glioma, leukemia, ovarian, cervix, prostate, lung, and colorectal cancers (Saneja et al., 2017b; Soica et al., 2014; Zhang et al., 2016). The mechanism of action of BA to induce cancer death has been investigated comprehensively. Several mechanisms have been postulated such as

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mitochondrial membrane permeabilization with the release of factors like cytochrome c, Smac or apoptosis inducing factor (AIF) in a permeability transition pore-dependent manner, decreased expression of vascular endothelial growth factor, suppression of STAT3 activation, inhibition of topoisomerases, formation of reactive oxygen species (ROS), activation of caspases and nuclear fragmentation (Dai et al., 2014a; Fulda and Kroemer, 2009; Mullauer et al., 2011; Saneja et al., 2017b). In spite of tremendous biological activity of BA, it has been associated with some drawbacks such as poor aqueous solubility and short half-life *in vivo* which hamper its clinical efficacy (Dai et al., 2014a; Saneja et al., 2017b). Recently, to tackle these challenges, much work has been focused on enhancing its aqueous solubility, half-life and efficacy by using nanoscale drug delivery vehicles. These nanoscale drug delivery vehicles have the ability to accumulate more easily and selectively in the tumor tissues, due to lack of well-defined lymphatic system, as compared to the native drug by the phenomena of enhanced permeation and retention (EPR) effect (Arora and Jaglan, 2016; Noble et al., 2014). Several nanoscale drug delivery vehicles of BA such as nanoparticles (Dai et al., 2015a,b,c, 2014b; Hussein-Al-Ali et al., 2014), liposomes (Liu et al., 2016; Mullauer et al., 2011), polymeric conjugates (Dai et al., 2014a; Lomkova et al., 2016), carbon nanotubes (Tan et al., 2014) and cyclodextrins (Dai et al., 2017; Soica et al., 2014; Sun et al., 2013) have been developed and exhibited their superior efficacy as compared to native BA.

Among the wide range of nanoscale drug delivery vehicles used by the researchers, poly(lactic-co-glycolic acid) (PLGA) based nanoformulations have been most attractive because of its biodegradability, biocompatibility and approval by Food and Drug Administration (FDA) for the systemic administration (Dubey et al., 2016; Saneja et al., 2017a). The usefulness of PLGA based formulations can be supported by the approval of various PLGA based formulations by the FDA such as Arestin®, Trelstar®, Profact®, Decapeptyl® *etc*. (Jain et al., 2016). In addition to this, the surface modification of PLGA based nanocarriers with stealth polymer such as polyethylene glycol (PEG), could prolong the systemic circulation time and reduces protein opsonization which decreases nonspecific uptake in normal tissues (Garinot et al., 2007; Li and Huang, 2010). Further, pegylated PLGA NPs comprising hydrophobic PLGA core and encircled by a hydrophilic PEG layer are reported to be one of the best controlled release systems for targeted drug delivery (Kamaly et al., 2012).

To the best of our knowledge, BA loaded PLGA-mPEG nanoparticles have not been reported yet and extensively characterized. However, in a recent study, BA loaded PLGA nanoparticles have been developed, which demonstrated the improved efficacy in lung cancer cells (Das et al., 2016). In our study, we have developed betulinic acid loaded PLGA-mPEG nanoparticles in order to prolong its circulation time. The developed nanoparticles (BA NPs) were characterized for various parameters such as particle size, surface charge, morphology, physical state, drug loading, hemolytic activity and *in vitro* release profile. The macrophage uptake study using J774A.1 and RAW 264.7 cells support the long circulatory behavior of BA NPs, which was further confirmed by the pharmacokinetic profile of BA NPs. The *in vitro* cytotoxicity of the developed formulation was investigated using MTT assay on MCF7 (human epithelial mammary gland carcinoma) and PANC-1 (human pancreatic carcinoma) cells. To investigate the mechanism of cellular cytotoxicity of the developed formulation, various assays such as apoptosis, mitochondrial membrane potential (MMP) loss, cell cycle analysis and reactive oxygen species (ROS) generation were performed. The *in vivo* anticancer potential of developed formulation was investigated in Ehrlich tumor (solid) model and finally, various hematological and biochemical parameters were investigated to assess betulinic acid induced toxicity, if any.

2. Materials and methods

2.1. Materials

PLGA (Resomer RG502 H, inherent viscosity 0.21dl/g in 0.1% $CHCl_3$) was purchased from Evonik Industries (Germany). Monomethoxy poly(ethylene glycol) (mPEG, MW 2 kDa), *N*-Hydroxysuccinimide (NHS), *N,N*-dicyclohexylcarbodimide (DCC), coumarin-6, betulinic acid (BA), dimethyl sulfoxide (DMSO), anhydrous dichloromethane (DCM), polyvinyl alcohol (PVA, MW 30–50 kDa, 98–99% hydrolyzed), branched polyethylenimine (PEI; average MW 25 kDa), phosphate-buffered saline (PBS), 2′,7′-dichlorofluorescein-diacetate (DCFDA), and paraformaldehyde was purchased from Sigma–Aldrich, India. Diethyl ether and triethylamine was purchased from Fischer Scientific, India. Human epithelial mammary gland carcinoma (MCF7) and human pancreatic carcinoma (PANC-1) cells were purchased from European Collection of Cell Culture (ECACC). Murine macrophage cell lines J774A.1 and RAW264.7 cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in RPMI 1640/DMEM containing 10% (v/v) Fetal Bovine Serum (FBS), 100 mg/mL streptomycin, 2 mM glutamine and 100 U/mL penicillin and were incubated at 37 °C under an atmosphere of 5% CO_2 and 90% relative humidity. All other solvents and reagents, unless otherwise stated, were purchased locally and were of highest purity grade.

2.2. Preparation of PLGA-mPEG nanoparticles

2.2.1. Synthesis and characterization of PLGA-mPEG co-block polymer

The PLGA-mPEG co-polymer was prepared using standard carbodiimide chemistry as described in our previous work (Khare et al., 2016; Saneja et al., 2017a). Briefly, PLGA (1 equivalent), *N,N*-dicyclohexylcarbodimide (5 equivalents), and *N*-hydroxysuccinimide (5 equivalents) were dissolved in 10 mL DCM and stirred for 24 h with continuous magnetic stirring at room temperature (RT) under nitrogen environment to convert PLGA-COOH to PLGA-NHS. The obtained solution was filtered in order to remove by-product dicylohexylurea (DCU) using 0.45 μm PTFE syringe filter (Millipore). Thereafter, activated PLGA (PLGA-NHS) was isolated by precipitation with ice cold diethylether, washed 3 times and dried under reduced pressure. The activated PLGA (1 equivalent) was dissolved in DCM and added dropwise to the excess of methoxy PEG amine (5 equivalents) dissolved in 5 mL of DCM. Subsequently, after the addition of triethylamine (50 μL), the reaction mixture was kept on stirrer for 24 h at RT under nitrogen environment. The reaction mixture was precipitated in ice cold diethyl ether to obtain the resultant product (PLGA-mPEG). The obtained PLGA-mPEG was washed with ice cold methanol for three times to remove unreacted PEG and dried under reduced pressure. The copolymer was further purified by dissolving it in DMSO and dialyzing against water for 48 h using Pur-A-lyzer (MWCO 3.5 kDa). The product was then freeze dried and stored at −20 °C until further use (characterized by ^{1}H NMR, and FTIR, see Supplementary information).

2.2.2. Preparation of nanoparticles

BA loaded PLGA-mPEG nanoparticles were prepared using emulsion-solvent evaporation method. Briefly, PLGA-mPEG (40 mg) and BA (4 mg) were dissolved in DCM (4 mL) by mild stirring and sonicated under water bath to form clear solution of oil phase. Then the oil phase was added to 6 mL of 1% polyvinyl alcohol (PVA) in an ice-bath using ultrasonic homogenizer (Labsonic Sartorius Stedim, Germany) for 3 min at 40% amplitude. The oil-in-water (o/w) emulsion thus formed was rapidly poured into 10 mL water kept under magnetic stirring until complete removal of DCM. The resultant nanoparticles suspension was then centrifuged at 29,068*g* (Sigma 3–30 K, Germany) for 20 min and washed thrice with deionized water to remove excess of PVA. In order to investigate the cellular uptake behavior, coumarin-6 loaded