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Targeted poly (L-γ-glutamyl glutamine) nanoparticles of docetaxel against folate over-expressed breast cancer cells

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ABSTRACT

A novel folate (FA) conjugated poly(L-γ-glutamyl glutamine) (PGG) nanoparticle loaded with docetaxel (DTX) was prepared to take advantage of both targeted drug delivery in breast cancer and reducing the overall side effects due to the adjuvant free formulation in comparison with Taxotere®. Nano-precipitation method was employed to prepare nanoparticles (NPs). The chemical structure of PGG synthesized polymers and PGG-FA conjugates and polymeric nanoparticles were characterized by H NMR, FTIR spectroscopy, field emission scanning electron microscopy, and laser scanning confocal microscopy. The average size of optimized nanoparticles with the aid of Box–Behnken experimental design was 131.96 ± 5.34 (nm) with polydispersity of 0.089 ± 0.019, zeta potential of −25.8 ± 2.21 (mV), and entrapment efficiency of 67.83 ± 3.29(%). *In vitro* cytotoxicity of the designed NPs was investigated by MTT assay against three chosen cell lines of MCF7, 4T1, and A549 based on their folate receptor expression capacity and was compared with Taxotere®. Moreover, PGG-FOL NPs were loaded with 6-coumarin for cellular uptake investigation. In order to assess the antitumor efficacy and biodistribution of targeted NPs, 4T1 murine breast tumors were established on the balb/c mice and *in vivo* studies were performed. The obtained results showed that the novel designed system was highly effective against tumor cells and successfully localized in the tumor site.

1. Introduction

It is difficult to disguise the fact that nanotechnology, particularly nanoparticles, open a new route in drug administration and exclusively in cancer treatment strategies (Doane and Burda, 2013). There are several types of nanoparticle systems such as micelles, carbon based nanotubes, protein nanoparticles, liposomes, dendrimers and polymeric nanoparticles (Couvreur, 2013; Hu and Zhang, 2012; Couvreur and Vauthier, 2006; Kopeček, 2013; Tekade et al., 2009; Bangham et al., 1965). The advantages of nano sized drug delivery systems are countless. Namely, less side effects with higher drug efficacy and desirable distribution, passive targeting due to the leaky tumor vascular, enhanced permeability and retention (EPR) effect and desirable biodistribution is amongst NPs drug delivery systems benefits (Zhang et al., 2013). On the other hand, targeting polymeric nanoparticles has received a great deal of attention particularly in cancer treatment (Brannon-Peppas and Blanchette, 2012; Byrne et al., 2008; Shapira et al., 2011; Esmaeili et al., 2008; Taheri et al., 2011). Folate (FOL) receptors are mostly noted due to their over-expression in tumors, *e.g.* breast cancer (Liu et al., 2010; Wang et al., 2011a). Employing folic acid as a targeting moiety is a promising strategy to enhance the polymeric nanoparticles delivery to cancerous cells. Moreover, reduced side effects and a lower damage to normal cells will occur due to their specificity and high local concentration in tumor cells (Werner et al., 2011; Zhao et al., 2011).

Poly-L-glutamic acid has been widely used to prepare polymeric nanoparticles during the recent years (Lee, 2006) and some of

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these newly developed nanoparticulate formulations such as a paclitaxel (PTX) conjugated poly-L-glutamic acid formulation (CT-2103) have achieved great fortune in clinical trials (Wang et al., 2011b; Feng et al., 2010; Lee et al., 2013). There are only few studies available on poly (L-γ-glutamyl glutamine (PGG) polymers properties and its characterization (Van et al., 2010; Yang et al., 2012a). PGG is prepared by a modification on poly(L-glutamic acid) (PGA) polymer by addition of glutamic acid moieties on PGA side chains (Yang et al., 2011a). PGG polymer in conjugation with paclitaxel represented paramount therapeutic and pharmacokinetic results in comparison to Abraxane® as reported by Van et al. (Van et al., 2010). Additionally, desirable water solubility and stability of PGG-PTX nano-conjugates and their appropriate biocompatibility has been demonstrated by Yang et al. (2011a,b). The anti-tumor activity and biodistribution of paclitaxel conjugated with PGG was also represented in Wang et al. study; indicating that the prepared nanoparticles displayed both prolonged release and favorable antitumor efficacy (Wang et al., 2010). Moreover, a docetaxel (DTX) conjugated PGG has been also designed to act against human non-small cell lung cancer cell line (Yang et al., 2012b). The molecular weight is amongst the parameters that play a major role in physicochemical properties of the polymer based systems. Therefore, the effect of PGG-PTX molecular weight on both toxicity and efficacy has been evaluated in a report by Yang et al. (2012a).

DTX is a semisynthetic analogue of paclitaxel, a well-known lipophilic anti-cancer agent that is obtained from the European yew tree, *Taxus baccata* (Fite et al., 2007; Yousefi et al., 2009). DTX seems to be a better choice in comparison with paclitaxel due to its higher microtubules assembly promotion; however, neuro and musculoskeletal toxicity due to the presence of tween 80 and ethanol in Taxotere(r) formulation is still regarded as a matter of concern, especially with considering the risk of extravastion and hypersensitivity reactions and also the incompatibility of Taxotere formulation with common administration sets (Liu et al., 2012). The goal of the present study was to design a novel folate targeted PGG polymeric nanoparticle free from tween 80 and ethanol that could deliver DTX to breast cancerous cells not only with higher specificity and efficacy but also with lower overall side effects. Based on this knowledge we hypothesized that PGG nanoparticles loaded by DTX and targeted by folic acid are more potent with a higher efficacy on breast cancerous cell models in comparison with Taxotere(r) especially with considering the overexpression of folate receptors in tumor tissues and in cancerous cells (Esmaeili et al., 2008). The objective of the current study was to prepare a new surfactant and ethanol free formulation to prevent these two excipients related side effects and in the same time develop a targeted system (by targeting the over expressed folate receptors in tumor tissues) and to specifically deliver the NPs to the targeted tissue to enhance specificity and efficacy of drug formulation and to reduce the overall side effect and to provide a better alternative for the patients' chemotherapy regimen. Here, we report the applied method for PGG synthesis and its conjugation with folate. We employed the straight forward precipitation technology for manufacturing the PGG-FA nanoparticle loaded with DTX, to overcome the poor aqueous solubility of DTX (Bilati et al., 2005). Additionally, the Box–Behnken experimental design was employed and the obtained optimum formulation was further investigated by various methods of nanoparticle characterization (Gazori et al., 2009). Moreover, *in vitro* cytotoxicity of the optimized PGG-FA nanoparticles loaded with DTX was compared with Taxotere® through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on three different cell lines including MCF7 (high folate receptors over expression), 4T1 (moderate folate receptors over expression) and A549 (control cell line without folate receptors over expression) which were differentiated by their folate expression capacity (Mi et al., 2011; Watanabe et al., 2012).

Determination of the *in vivo* performance of NPs is essential for predicting their therapeutic response. In this study, 4T1 breast tumor was established on balb/c mice as a tumor-bearing model. Furthermore, the optimized NPs were evaluated *in vivo* by survival, antitumor efficacy and biodistribution studies. Concisely, tissue distribution and tumor accumulation of Taxotere® and targeted NPs were compared and analyzed.

2. Material and methods

2.1. Materials

Poly(L-glutamic acid) sodium salt (MW 20–30 kDa), *N*-(3-dimethylamino propyl)-*N*′-ethylcarbodiimide (EDC), L-glutamic acid di-*tert*-butyl ester hydrochloride, *N*-hydroxysuccinimide (NHS), coumarin-6, MTT, fetal bovine serum (FBS), penicillin and streptomycin were all obtained from Sigma–Aldrich (St. Louis, MO, USA). Docetaxel (anhydrous) and folic acid were purchased from Cipla (Mumbai, India). Roswell Park Memorial Institute 1640 (RPMI) was acquired from life technologies (Grand Island, NY, USA). MCF7 human breast adenocarcinoma cells, 4T1 murine breast cancer cells and A549 human pulmonary adenocarcinoma cells were obtained from national cell bank of Iran (NCBI). Annexin V-FITC/PI apoptosis kit was from Biovision (Germany, Lorrach). The water used was pretreated with the TKA-GenPure water purification system. *N*,*N*′-Dicylohexylcarbodiimide (DCC), ethylenediamine, ethyl acetate, dichloromethane (DCM), acetonitrile (ACN), dimethyl sulfoxide (DMSO) and methanol were purchased from Merck (Darmstadt, Germany). All reagents used for HPLC analysis were of HPLC grade.

2.2. Synthesis

2.2.1. Activation of PGA carboxyl group

Poly(L-glutamic acid) sodium salt was first dissolved in DCM, and its carboxyl groups were activated by the addition of DCC and NHS (1:9:10 molar ratio). The reaction was maintained under nitrogen atmosphere and was stirred at room temperature for 24 h. The reaction mixture was filtered and precipitated by adding diethyl ether. The solvent was evaporated under reduced pressure and was used for the subsequent step.

2.2.2. Synthesis of PGG

The activated PGA polymer was conjugated with L-glutamic acid di-*tert*-butyl ester hydrochloride monomers (1:4 molar ratios) by adding NHS, DCC, and DCM under constant stirring at room temperature. Subsequently, a solution of HCl 10% was added to the above solution and the mixture was well stirred for 2 h. The organic phase was then separated and was dried by anhydrous sodium sulfate. In the next step, the organic solvent was removed under the vacuum and the structure of final resulting conjugated PGG polymer was confirmed using Fourier Transform Infrared Spectroscopy (FTIR) by a Nicolet Magna IR-550, USA spectrophotometer.

2.2.3. Synthesis and purification of folate ethylenediamine

To prepare folate ethylenediamine, the carboxyl group of folate structure was conjugated to ethylenediamine. As a result, NH_2 group on folic acid became ready for further reactions with the PGG carboxylic groups.

Folate carboxylic groups were activated by adding EDC and NHS (1:10:9 molar ratios) in the presence of dried methanol as a solvent. The mixture was stirred at 50 °C for 24 h under light protection and N_2 atmosphere. The final reaction was stirred continuously for another 24 h in dark condition and in the presence