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Targeting the leptin receptor: To evaluate therapeutic efficacy and anti-tumor effects of Doxil, in vitro and in vivo in mice bearing C26 colon carcinoma tumor

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ABSTRACT

Leptin is an appetite regulatory hormone that is secreted into the blood circulation by the adipose tissue and it functions via its over expressed receptors (Ob-R) in a wide variety of cancers. In the present study, the function of a leptin-derived peptide (LP16, 91–110 of Leptin) was investigated as a targeting ligand to decorate PEGylated liposomal doxorubicin (PLD, Doxil®) surface and the anti-tumor activity and therapeutic efficacy of Doxil in C26 (Colon Carcinoma) tumor model were also evaluated. As a result of this, Doxil with different LP16 peptide density (25, 50, 100 and 200 peptide on the surface of each liposome) was successfully prepared and characterized. In vitro results showed significant enhanced cytotoxicity and cellular binding and uptake of LP16-targeted Doxil formulations (LP16-Doxil) in C26 cells as compared to Doxil. In BALB/c mice bearing C26 murine carcinoma, at a dose of 15 mg/kg, LP16-Doxil groups (100 ligand) significantly suppressed the growth of the tumor and showed higher inclination to tumor as compared to non-targeted Doxil. This study revealed that the potential of LP16 peptide targeting increased the therapeutic efficacy of Doxil and highlighted the importance of optimizing the ligand density to maximize the targeting ability of the nanocarriers and merits further investigations.

1. Introduction

Cancer being one of the major causes of death worldwide is a serious threat to human life. Chemotherapy is a major therapeutic approach to treat cancer and it may be used alone or in combination with other forms of therapy. However, conventional therapy suffers from lack of selectivity and this has led to rapid damage in proliferating normal cells. Recent advances in tumor drug delivery systems suggested that nanocarriers can improve the clinical treatment and tumor delivery of anticancer agents, because of Enhanced Permeability and Retention (EPR) effect, which is a kind of passive targeting [1,2]. Amid the different nanocarriers, PEGylated Liposomal doxorubicin (Doxil®), as the first approved nanomedicine, is more effective than free doxorubicin in drug delivery to tumors, especially in tumors with hypervascular characteristics including Kaposi sarcoma and ovarian cancers [3,4]. Nevertheless, most of these agents have failed to show enough therapeutic effects in vivo, because of insufficient EPR effect as a result of certain characteristics of their cancer microenvironment, which include hypovascularity and thick fibrosis [5]. Recent studies have shown that the clinical effectiveness of liposomes can also be improved by decorating the liposome surface with cancer targeting ligands, called active targeting [6,7].

Targeting ligands have special affinity selectively for a receptor expressed by the tumor or tumor vasculature cells (and not

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expressed by normal cells) and accumulated preferentially in these cells. This process has led to the direct killing of the targeted cancer cells without damaging normal cells [8,9]. Leptin is an adipocyte–derived hormone that operates as a major body mass regulator and its deficiency or resistance can result in obesity, diabetes, and infertility in humans [10]. Leptin effects through its receptors (Ob-R) belong to the class I cytokine receptor superfamily and is expressed both centrally and peripherally [11]. Leptin receptors are expressed in almost all major organs, such as lung, liver, heart, ovary and the major components of the central nerve system (CNS) [12]. In the CNS, these specific receptors Ob-R on the luminal side of the brain capillary are responsible for transcytosis of leptin to the brain to regulate energy balance [13]. In peripheral tissues, leptin exerts other physiological responses which include its effects on the immune response, angiogenesis, reproduction, and an intracellular cross talk with signaling pathways of growth hormones, such as insulin and lipid metabolism pathways [14,15].

In addition to leptin receptor expression in normal tissues, functional leptin receptors are over-expressed in a variety of cancers including adipocyte, adrenal, breast, bladder, endometrial, liver, leukemia, ovarian, pituitary, and prostate cancers [16–24]. Leptin mediated signaling pathways play an important role in cancer cell proliferation, invasion, and metastasis [25]. Thus, leptin as a targeting ligand could be desirable for drug delivery to tumors that over-expressed Ob-R and helpful vehicle in cancer chemotherapy. Recent studies suggest that high serum Ob-R levels are independent risk factor for colonic cancer [26]. Also, there are reports about the role of leptin receptor as a prognostic biomarker in colorectal cancers [27,28]. These evidences predict that targeting leptin receptor as novel therapeutic agent could be helpful in more effective drug delivery in colorectal cancers.

Very recent studies showed that different leptin fragments, overlapping different Ob-R-binding site, are taken up by the brain that over expressed Ob-R receptor to an extent as compared to wild-type leptin [29,30]. Moreover, in a recent study, peptide consisting of amino acid residues 91–110 of leptin (LP16) with recombinant leptin antisera was successfully used for leptin assay by using sandwich assay method. The amino acid sequence 91–110 was shown to be at least a part of an epitope for bovine leptin that cross-reacted with mouse and human leptins and this would be useful for antigen determination [31]. LP16 peptide synthesis was based on the relative hydrophilicity and flexibility of the region analyzed by a computer program [32].

A strong conservation degree of multiple sequences of the leptin protein has been observed in distinct mammals, particularly in evolutionarily close species. To determine the extent of Ob gene conservation between human and mice, Zhang et al. isolated and sequenced cDNA clones hybridizing to Ob from the human adipose tissue cDNA library. The results showed that the nucleic sequences from human and mice were highly homologous in the predicted coding sequence and it showed 84% overall identity between human and mouse amino acid sequences [33]. With reference to leptin receptors, there are 5 isoforms of Ob-R in mice and 6 isoforms of Ob-R in humans. Takaya et al. reported that amino acid sequences of mouse Ob-Rb isoform to be 75% homologous to that of humans. Similar situations also apply to other isoforms of leptin receptors [33,34].

To the best of our knowledge, to date, all studies have investigated the role of leptin derived peptides as carrier molecules for brain therapeutics. In this study, for the first time, Doxil was decorated with LP16 peptide to evaluate the anti-tumor activity and therapeutic efficacy of Doxil in colon carcinoma tumor model.

MTT assay and flow cytometry (FCM) were used to investigate the cytotoxicity and cellular binding and uptake of LP16-Doxil. In vivo studies were conducted on BALB/c mice bearing C26 colon carcinoma. After a single dose injection of Doxil, LP-Doxil formulations and doxorubicin at a dose of 15 mg/kg therapeutic efficacies of LP16-Doxil as compared to non-targeted liposomal formulation and free drug were evaluated. In addition, the best ligand density on each liposome was optimized to achieve maximum targeting ability and therapeutic efficacy of LP16-Doxil formulations. Although, LP16-Doxil liposomes still need stability studies and further experimental proof of validity in different human tumor models that over-expressed Ob-R in nude mice along with safety studies in future studies to generalize the findings on human tumor models.

2. Materials and methods

2.1. Materials

LP16 peptide, *SRNVIQISNDLENLRDLLHVGGGC*-Ac, >95% pure by HPLC and mass spectrographic analyses was purchased from China Peptides Co. (Shanghai, China). Maleimide-PEG2000 distearoylphosphatidylethanolamine (Mal-PEG2000-DSPE) was purchased from Avantipolar lipids (Alabaster, AL). Doxorubicin hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). 3-(4, 5-Dimethylthiazol-2-yl) – 2 [MTT], diphenyltetrazolium bromide, was purchased from Sigma-Aldrich. Acidified isopropyl alcohol (90% isopropanol/0.075 M HCl) was prepared by addition of 7.5 ml HCl 1 M and 2.5 ml water to 90 ml isopropanol (Merck, Darmstadt, Germany). C26 cells were purchased from Cell Lines Service (Eppelheim, Germany). Commercially available Doxil® was purchased from BehestanDarou Company (Tehran, Iran). All other solvents and reagents were used as chemical grade.

2.2. Modification of doxil with LP16 peptide

LP16-Doxil liposomes were prepared by using post-insertion method. Briefly, LP16-DSPE-PEG2000-Mal conjugate was synthesized via thioether bond between the thiol group of cysteine residue of peptide and the pyrrole group of maleimide [35]. Peptide and DSPE- PEG2000-Mal were dissolved in dimethyl sulfoxide (DMSO) and chloroform at 1:1 (v/v), respectively. The molar ratio of peptide: DSPE-PEG2000-Mal was 1.2:1. After LP16-DSPE preparation, the organic solvents (chloroform and DMSO) were removed using rotary vacuum evaporation and freeze drier, respectively. Lipid film was hydrated with injectable water to form micelles. Then, LP16-DSPE micelles and Doxil liposomes were co-incubated at 60 °C for 4 h with gentle shaking [36] and purified using dialysis against Histidin buffer in 10% sucrose (Histidin 10 mM, pH 6.5) in dialysis cassettes (Spectrum) with 30 kDa molecular weight cut off (MWCO).

LP16-Doxil formulations were prepared by directing 25, 50, 100, and 200 LP16 peptides on the surface of each liposome. The following parameters were used to calculate the number of peptide molecules per liposomes [37]: (a) phospholipids concentration of Doxil: 13.279 mM; (b) liposome average size: 100 nm in diameter; (c) lipid molecules per liposome with average size of 100 nm: 8×104; (d) number of liposomes per each ml: 1×1014; (e) total peptide content: 0.4 mM; (f) number of peptide molecules per each ml aliquots of peptide-micelles: 2.4×1014; (g) the number of peptide per each liposome (25, 50, 100, and 200 ligand).

2.3. Characterization of liposomes

LP16-DSPE conjugation efficiency was determined by thin layer chromatography (TLC) method using chloroform: methanol at 15:85 (v/v) with iodine vapors (silica gel 60 F254, Merck, USA) and verified using SDS-PAGE. Particle size and polydispersity index and zeta potential of post-inserted liposomes were determined by using dynamic light scattering (Nano-ZS; Malvern, UK). Doxorubicin concentration of formulations before and after post-insertion