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Improved drug delivery and therapeutic efficacy of PEgylated liposomal doxorubicin by targeting anti-HER2 peptide in murine breast tumor model

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ABSTRACT

Targeted cancer therapy is a powerful therapeutic strategy to management of cancer. HER2 as an anticancer target has long been studied. Its overexpression plays an important role in the pathogenesis and progressiveness of breast and other cancers. To establish efficient and reliable drug delivery to HER2-overexpressing cells, the authors of this study have developed anti-HER2 (ErbB2) peptide-liposomal formulations of doxorubicin (DOX) by an engineered breast tumor-targeting peptide ligand, AHNP, Anti-HER2/neu peptide, (FCDGFYACYADV) with three glycine amino acids as spacer before its original sequencing. Towards this goal, PEGylated liposome doxorubicin (PLD) bearing different ligand densities of AHNP was prepared and characterized for their size, zeta potential and peptide conjugation. The AHNP functionalization and density effects on breast tumor cell uptake, selective cytotoxicity, prevention of tumor growth and the tissue biodistribution of encapsulated DOX were studied in mice bearing TUBO breast cancer tumor model. The findings demonstrated that increasing the ligand density of AHNP increases cytotoxicity and cell-uptake in SKBR3 and TUBO cells which overexpress HER2 but not in MDA-MB-231with low HER2 expression profile. The anticancer activity was also superior for targeted liposomal DOX with more AHNP densities. Overall, the results showed that optimum AHNP density functionalization of PLD can significantly improve selectivity and the therapeutic index of liposomal DOX in the treatment of HER2 positive breast cancer and merits further investigation.

1. Introduction

The most common chemotherapy regimens for treating cancer is based on the application of nonspecific cytotoxic substances which can induce toxic side effects; while inhibiting the quickly dividing cancer cells proliferation, it also damages quickly proliferating normal cells of the hair follicles and bone marrow (Chari, 1998; Malam et al., 2009; Schally and Nagy, 1999). Providing a therapeutic window to kill cancer cells while sparing healthy ones is currently the main purpose of cancer therapy. This procedure need to specifically target tumor cells at the malignancy location (Allen, 2002, Danhier et al., 2010; Sawyers, 2004). Ligand-targeted liposomal cancer chemotherapeutics compared to non-functionalized ones have been showed to improve the pharmacokinetics of drugs, including intracellular delivery, selective toxicity, and finally, in many cases, improved therapeutic efficacy and quality of life for cancer patients (Brannon-Peppas and Blanchette, 2012; Das et al., 2009; Heath et al., 1983; Noble et al., 2004; Pastorino et al., 2006; Sapra et al., 2005).

HER2 (also known as p185her2/neu and Neu), as oncogene product, is a member of the erbB family of tyrosine kinases. Overexpression of HER2 occurs in 20–30% of breast and ovarian cancers, usually due to gene amplification, and is linked with a poor prognosis in patients with these tumors (Bartlett et al., 2003, Berns et al., 1995; Slamon et al., 1987; Xu et al., 2002). HER2-overexpression also occurs frequently in other cancers. Moreover, HER2 in normal adult tissue is slightly expressed by particular cell types of the epithelium (Natali et al., 1990).

The activated erbB receptors have appeared as suitable selective tumor-targeting ligands. Receptor-guided therapy shows a powerful cancer management strategy. For targeting EGF receptors, numerous antibodies, recombinant proteins, peptide mimetics, and small molecules have been generated (Atalay et al., 2003; Cai et al., 2010; Master and Sen, 2012, Mendelsohn and Baselga, 2006; Scaltriti and Baselga, 2006).

Although antibodies are employed as tumor-specific ligands, utilization of tumor-targeting peptides is a more valuable procedure. The peptides with outstanding properties of the determinant tissue permeation, specific association and internalization into tumor cells are perfect

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candidates for the therapeutics delivery. In addition, they are almost concealed to the immune system which can result in minimal or without side effects (Shadidi and Sioud, 2003; Shahin et al., 2011; Wang et al., 2008).

Tumor targeting with peptide ligands as an efficient tumor therapy and imaging tool has been greatly investigated (Aina et al., 2002; Brown, 2010; Jie et al., 2012; Temming et al., 2005). Targeted therapy mediated by peptide is obtained either by direct coupling of a chemotherapeutic to peptide ligands or by constructing peptide-directed drug carriers (Pastorino et al., 2006; Schneider et al., 2006; Xiong et al., 2005a; Zhang et al., 2012).

Carriers including liposomal agents with several tumor targeting peptides in comparison with conjugates of peptide-drug provide long circulation with minimal immunogenicity, highly-concentrated delivery of potent anticancer agents, ultimately minimizing the potential for cytotoxicity *versus* normal tissue (Allen, 2002, Wu and Chang, 2010).

Anti-HER2/Neu peptidomimetic (AHNP) from the 3D structure of the heavy chain CDR3 loop of trastuzumab was first designed by Park et al., 2000. It is HER2-specific with an increased affinity and demonstrates similar strength to the intact Trastuzumab (Berezov et al., 2001; Park et al., 2000). Since its introduction, AHNP has been used as a ligand of targeting to effectively transport therapeutic agents into HER2–overexpressing tumor cells (Afshar et al., 2009; Fantin et al., 2005; Guillemard et al., 2005; Tai et al., 2010; Tan et al., 2006). Although AHNP, compared with the parental mAb, has a lower affinity forp185her2/neu, it can similarly disable p185her2/neu receptor activity *in vitro* and *in vivo*. The function of AHNP has been confirmed in several other studies (Fantin et al., 2005; Park et al., 2000, Tan et al., 2006; Zhang et al., 2007).

The present study reports the synthesizing of a new formulation of PEGylated liposomal doxorubicin functionalized by different peptide densities of AHNP, hoping to combine the AHNP tumor targeting characteristics and PLDs drug delivery characteristics in the optimum ligand density. The PEGylated peptide-liposomal formulations are evaluated in terms of particle size and pharmacokinetics compared to the plain liposome. The receptor-mediated internalization of the novel formulation was also evaluated through cytotoxicity and cellular uptake by HER2-overexpressing cells. Moreover, the *in vivo* survival benefit of peptide-liposomes was assessed and compared with that of unmodified liposomes in a murine model of breast cancer.

2. Materials and methods

2.1. Materials

AHNP (FCDGFYACYADVGGG) with purity greater than 95% was obtained from ChinaPeptides Co. (Shanghai, China). Methoxy-polyethylene glycol (MW 2000)–distearoylphosphatidylcholine (mPEG2000–DSPE), Cholesterol (Chol), hydrogenated soy phosphatidylcholine (HSPC), and COOH-PEG2000-DSPE were purchased from Avanti Polar Lipids (Alabaster, AL). Commercially available Caelyx® was purchased from Tocris Bioscience (USA). Isopropanol, chloroform, and Tryptan blue were obtained from Merck (Darmstadt, Germany). MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide was purchased from Sigma (Aldrich, Germany). Acidified isopropyl alcohol (90% isopropanol/0.075 M HCl) was prepared by adding 7.5 ml HCl 1 M and 2.5 ml water to 90 ml isopropanol (Merck, Darmstadt, Germany). All other chemicals and solvents were of analytical grade.

2.2. Liposome and peptide-liposome preparation

Non-targeted sterically stabilized liposomes (SL) were prepared with HSPC:CHOL:mPEG2000-DSPE at a 56.2:38.3:5 molar ratios. Liposomes were prepared using the hydration of thin lipid films as previously reported and downsized by sonication and extrusion to a mean size of 100 ± 10 nm (de Menezes et al., 1998). Doxorubicin was loaded into liposomes by the ammonium sulfate gradient technique (Bolotin et al., 1994; Haran et al., 1993). Targeted liposomes containing Caelyx® as plain liposome were prepared using the post-insertion method(Ishida et al., 1999). Peptide-coupled micelles for the post-insertion method were prepared by coupling AHNP to distearoyl-N-(3-carboxypropionoyl poly (ethylene glycol)succinyl)phosphatidylethanolamine (DSPE–PEG–COOH) micelles through amide bonds as previously described (Ishida et al., 2001). Briefly, 1 ml of PEG-micelles (4 μmol lipids) in Mes buffer (pH 5.5) was activated by mixing it with EDC 400 mM in water and NHS 200 mM for 15 min at room temperature. Following the increase of pH to 7.5, the desired amount of peptide (0.4 μmol) was added and the whole was then incubated for 3 h at room temperature with gentle stirring. The coupled micelles with different ligand densities were then incubated with preformed liposomes, based on earlier estimates that a 100-nm liposome approximately contains 80,000 phospholipids (Allen, 2002, Amin et al., 2013; Schiffelers et al., 2003) for one hour at 60 °C. After incorporating, the mixture of liposome and micelle was dialyzed against His/dextrose buffer (pH 6.5) to separate the peptide-liposomes from micelles and free peptide.

2.3. Liposome characterization

The average diameter, polydispersity index (PDI), and zeta potential of liposomes was determined in triplicate by the Dynamic Light Scattering Instrument (Nano-ZS; Malvern, UK). All data were given as means ± standard deviation (n = 3). The Bartlett's method was used to measure phospholipid concentration of liposomes (Bartlett, 1959).

2.4. Determination of peptide coupling efficacy

To avoid peptide destruction and affecting its binding efficacy, three glycine amino acids were considered as spacer before original amino acid sequencing of peptide. AHNP covalently linked to COOH-PEG2000-DSPE *via* binding between the carboxyl group of lipids and the amine group of glycine residue of spacers. Conjugation efficacy of AHNP was indirectly determined by measuring of free peptide levels with HPLC (KNAUER smartline). The Nucleosil C18, 5 μm, 150 × 4.6 mm, 100 A° reversed phase column (KENAUER), and a UV detector (KENAUER S2600) set at 220 nm were used for analytical HPLC. The mobile phases used for gradient elution consisted of A (water + 0.1% TFA) and B (acetonitrile + 0.1% TFA), starting with 100% A and enhancing to 25% B in 2 min, 55% B in 15 min, with the flow rate of 1 ml/min. Furthermore, the formation of AHNP-PEG2000-DSPE was qualitatively investigated by tricine-sodium dodecylsulfate-polyacrylamide (tricine-SDS-PAGE) electrophoresis (16% SDS-PAGE) (Schägger, 2006).

2.5. Cell lines

HER-2/neu-positive cell clone TUBO, was kindly supplied by Dr. Pier-Luigi Lollini (Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy). It was successfully cultured in DMEM (Dulbecco's Modified Eagle's Medium) + 20% FBS. **S**K-BR-3 and MDA-MB-231 cell lines were obtained from Pasteur Institute (Tehran, Iran) and RPMI-1640 medium containing 10% FBS was used for culturing.

2.6. In vitro cell uptake study

Different cell lines (SK-BR-3, TUBO, and MDA-MB-231) were seeded into 24-well plates at densities of 2×10^5 cells/well and treated overnight at 37 °C. Free DOX, unmodified liposomes, liposomes with 25, 50, 100, and 200 AHNP ligand densities at 100 nmol phospholipid/mL concentration were added and treated with cells at either 37 °C or 4 °C for 3 h and 6 h. Cells were then washed 3 times with cold PBS and separated via 100 μL of trypsin–EDTA solution (Gibco). To extract the cellular uptake of DOX, 0.9 mL acidified isopropanol per well was added for an overnight at 4 °C. Cellular debris was deposited and supernatants were then analyzed spectrofluorimetrically to determine