International Journal of Pharmaceutics 515 (2016) 449–459

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Polysialic acid-modifying liposomes for efficient delivery of epirubicin, *in-vitro* characterization and *in-vivo* evaluation

Ting Zhang, Songlei Zhou, Ling Hu, Bo Peng, Yang Liu, Xiang Luo, Yanzhi Song, Xinrong Liu, Yihui Deng*

College of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, PR China

ARTICLE INFO

Article history:
Received 15 May 2016
Received in revised form 6 October 2016
Accepted 23 October 2016
Available online 24 October 2016

Keywords:
Polysialic acid
Long circulation time
Tumor shed
Antitumor efficacy

ABSTRACT

Polysialic acid (PSA) serves as a hydrophilic polymer and affords conjugated biologically active molecules a longer circulation time *in vivo*. Furthermore, PSA could potentially target tumor tissues and help achieve better curative effects. In this study, PSA was conjugated with octadecyl dimethyl betaine (BS18) to yield a PSA-BS18 conjugate. The PSA-BS18 modified liposomal epirubicin (EPI-SL), had a particle size of 133.63 ± 0.92 nm, a *zeta* potential of -26.23 ± 1.50 mV and an encapsulation efficiency (%EE) of $96.23 \pm 1.16\%$. *In vitro* release studies showed that PSA-BS18 could delay EPI release from the modified liposomes. The MTT assay suggested that EPI-SL led to stronger cytotoxic activity than that exhibited by common and PEGylated liposomes. The pharmacokinetic study showed that EPI-SL prolonged the residence time of the EPI in the blood compared with that observed from common liposomes. Biodistribution results obtained from tumor-bearing mice clearly demonstrated that PSA-BS18 increased the accumulation of modified liposomes in tumors compared with that of common liposomes. In the antitumor efficacy study, EPI-SL showed the best antitumor and life-prolonging effects among all of the tested formulations. These findings strongly indicate EPI-SL might have great potential as an effective approach for anticancer therapy.

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1. Introduction

Poly (ethylene glycol) (PEG) increases the hydrophilicity of liposomal surfaces and provides a steric barrier (Klibanov et al., 1990; Mori et al., 1991), which markedly reduces the recognition of liposome by the mononuclear phagocyte system (MPS) and prolongs the circulation time of intravenously administered liposomes (Allen et al., 1991; Nichols and Bae, 2012). Consequently, the long-circulating liposomes show selective accumulation in tumors and inflammation areas through the enhanced permeability and retention (EPR) effect (Maeda et al., 2000). Regrettably, PEGylation significantly reduces the cellular uptake and interferes with the antitumor efficacy of liposome-based drug delivery systems (Hatakeyama et al., 2011; Hong et al., 2013). Another disadvantage of PEGylation liposomes is skin toxicity generally known as “hand-foot syndrome”, which results in skin eruptions/ulcers on the palms of the hands and soles of the feet (Cao et al., 2010). Most importantly, recent reports have revealed that the intravenous injection of PEGylation liposomes significantly alters the pharmacokinetics and biodistribution of the second dose after an interval of several days, leading to the loss of their long circulation time and extensive accumulation in the liver and spleen. This phenomenon is referred to as the “accelerated blood clearance (ABC) phenomenon” (Abu Lila et al., 2013; Dams et al., 2000; Laverman et al., 2000). In recent years, much attention has been paid to this irregular pharmacokinetic behavior. Many studies have been performed to identify approaches that will avoid the ABC phenomenon, including altering the injection regimens, lipid composition, size, and surface charge of liposomes (Abu Lila et al., 2013; Ishida et al., 2004; Wang et al., 2005). However, practically none of these methods can eliminate the ABC phenomenon and these methods are accompanied by attenuation of the therapeutic efficacy of the PEGylation liposomes. Therefore, a pre-requisite for

Abbreviations: $AUC_{(0-t)}$, area under the drug concentration-time curve values; C_{max}, maximum concentration; CLz, total clearance; DiR, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indotricarbocyanine iodide; DiR-CL, DiR-labeled common liposomes; DiR-SL, DiR-labeled PSA-BS18 modified liposomes; DiR-PL, DiR-labeled mPEG2000-DSPE modified liposomes; EE, encapsulation efficiency; EPI, epirubicin; EPI-S, EPI solution; EPI-CL, common liposomal EPI; EPI-SL, PSA-BS18 modified liposomal EPI; EPI-PL, mPEG2000-DSPE modified liposomal EPI; FALT, fixed aqueous layer thickness; FGFs, fibroblast growth factors; $MRT_{(0-t)}$, mean residence time; MPS, mononuclear phagocyte system; TEM, transmission electron microscopy.

* Corresponding author.
E-mail address: pharmdeng@gmail.com (Y. Deng).

http://dx.doi.org/10.1016/j.ijpharm.2016.10.051
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resolving this issue is to find a suitable and biodegradable material to improve the circulatory stability of liposomes without increasing immunogenicity.

Polysialic acid (PSA) is a highly hydrophilic polysaccharide primarily composed of α-2,8-linked 5-N-glycolylneuraminic acid (Neu5AC) subunits, with the characteristics of inherent biodegradability and non-immunogenicity (Zhang et al., 2016). PSA has been found on the surface of both mammalian cells and bacteria. In mammalian tissue, PSA is a post-translational modification of the neural cell adhesion molecule (NCAM), and the large, negatively charged chains are thought to be a steric mechanism for promoting neural plasticity by reducing cell–cell interactions of NCAM (Kiss and Rougon, 1997). Bacterial PSA has chemical and immunological properties similar to those of mammalian PSA (Mühlenhoff et al., 1998; Troy and Frederic, 1995. Therefore, bacterial PSA could mask the antigenicity from the host immune system. In summary, PSA acts in nature to reduce undesirable cellular interactions and prevents recognition by the MPS, which could be utilized for an effective drug delivery system. Gregoriadis et al. (1993) first showed that the half-life of PSA conjugate with a fluorescent tag approached 40 h following intravenous administration in mice. The circulation time of asparaginase, insulin, GCSF, and IFN-a2b in animal models significantly increased *via* polysialylation (Fernandes and Gregoriadis, 2001; Gregoriadis, 1999; Gregoriadis et al., 2000; Jain et al., 2003). Although the underlying mechanisms that are responsible for the extension of drug half-life remain unclear, it is believed that the fixed aqueous layer produced by PSA plays a critical role. (Wilson et al. (2014) reported the fixed aqueous layer thickness (FALT) of PSA–poly-caprolactone micelles and stated that it was comparable to that obtained from traditional PEGylation liposomes. PSA is considered as to be surrounded by a protective envelope of water, referred to as a "watery cloud", which mainly owed to its high hydrophilicity and chain flexibility. Consequently, PSA can protect the modified drug/carrier from interacting with plasma proteins or macrophages, resulting in low uptake by the MPS and a prolonged circulation half-life (Fernandes and Gregoriadis, 1997, 1997, 2001; Zhang et al., 2014).

Although PSA is not present on NCAM in most adult tissues, the re-expression of PSA-NCAM has been observed in some malignant tumors (Komminoth et al., 1991; Livingston et al., 1988; Roth et al., 1988; Suzuki et al., 2005; Tanaka et al., 2000). PSA-positive tumor cells can detach from the primary tumor by attenuating the adhesive property of NCAM, and PSA also contributes to the metastatic potential by escaping detection from the immune system (Tanaka et al., 2000). The impact of PSA on the biological system processes described above has been attributed primarily to its ability to function as an anti-adhesive molecule, either directly or by modulating NCAM interactions with signaling receptors (Colley et al., 2014; Kiss and Rougon, 1997). However, recent results from the Sato's group revealed that PSA could directly bind and regulate the function of a number of biologically active molecules, such as neurotrophins, growth factors, and neurotransmitters (Sato and Kitajima, 2013). Fibroblast growth factors (FGFs) that signal through FGF receptors (FGFRs) regulate fundamental developmental pathways, which are responsible for many functions, including cell proliferation, survival and migration. To signal, FGFs are released from the extracellular matrix by heparinases, proteases of specific FGF-binding proteins, and the liberated FGFs subsequently bind to the cell surface heparin sulphate proteoglycans (Turner and Grose, 2010). Now therapeutic targeting of FGFs and their receptors is a major area of drug development research (Ori et al., 2008). FGF2 is a prototypical membrane of the FGF family that stimulates the growth of various cell types, from fibroblasts to tumor cells (Itoh, 2007; Sato and Kitajima, 2013). It has been shown that NCAM stimulates FGFR signaling(Cavallaro et al., 2001), while PSA-NCAM and FGF2 overlap in location and have similar deficiency (Graham and Richardson, 2011). One et al. demonstrated that PSA specifically binds to FGF2 and inhibits the cell growth facilitated by signaling through a ternary complex of FGF2, FGFR, and heparan sulfate (HS) (Ono et al., 2012). As mentioned above, FGF2 which liked to the cell surface heparin heparin sulphate proteoglycans sulphate proteoglycans might be responsible for binding of PSA-modified liposome encapsulated cytotoxic drugs to the cancer cell. These findings suggest that PSA-modified liposome encapsulated cytotoxic drugs may target tumor tissues to provide a desirable curative effect.

In the present study, we synthesized a PSA derivative conjugated with the long hydrophobic aliphatic chain octadecyl dimethyl betaine (BS18). We attached the resulting compound PSA-BS18 to the liposomes and studied their pharmacokinetic behavior in Wistar rats. Moreover, the anticancer effects of EPI-SL were evaluated. We assumed that PSA could prolong the circulation time of the modified liposomes, and serve as a binding molecule to the tumor tissue, which would facilitate tumor-site accumulation and therefore enhance the antitumor activity of the liposome-based drug delivery system.

2. Materials and methods

2.1. Materials

Poly[2,8-(*N*-acetylneuraminic acid sodium salt)] (PSA, isolated from *E. coli*, average Mw 30 kDa, about 100 sialic acid (SA) units) was purchased from Carbosynth China, Ltd. (Shanghai, China). Octadecyl dimethyl betaine (BS18) was purchased from Xingguang Auxiliaries Co., Ltd. (Tianjin, China). Epirubicin·HCl (EPI, purity 99.0% by high-performance liquid chromatography) was purchased from Olympic Star Pharmaceutical Co., Ltd. (Shenzhen, China). 1,1'-Dioctadecyl-3,3,3',3'-tetramethyl indotricarbocyanine Iodide (DiR) was purchased from American ATT Bioquest, Inc (California, USA). Hydrogenated soy phosphatidylcholine (HSPC) and 1,2-dioctadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (DSPG) were purchased from Lucas Meyer (Düsseldorf, Germany). *N*-(Carbonyl-methoxy polyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (mPEG2000-DSPE) was purchased from Genzyme Corporation (Cambridge, MA, USA). *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide HCl (EDC) and *N*-hydroxysuccinimide (NHS) were supplied by China National Medicines Corporation, Ltd. (Shenyang, China). Cholesterol (CH) was obtained from Shanghai Advanced Vehicle Technology Pharmaceutical, Ltd. (Shanghai, China). ZB-1 exchange fibers were obtained from Guilin Zhenghan Technology Development Co., Ltd. (Guilin, China). All other chemicals used in this study were of analytical or HPLC grade.

2.2. Cells and animals

The murine sarcoma S180 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Male Wistar rats (age, 7–8 weeks; weight, 180–220 g) and male kunming mice (age, 6–7 weeks; weight, 18–22 g) were purchased from the Laboratory Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All animals had free access to food and water. The animal care and experiments were performed in accordance with the guidelines of the local Animal Welfare Committee and Guide for the Care and Use of Laboratory Animals (Care et al., 1985).

2.3. Synthesis of PSA-BS18

PSA-BS18 was synthesized by direct conjugation of the hydroxyl groups of PSA with the carboxylic group of BS18 by using EDC/NHS as a catalyst, the synthetic scheme is shown in Fig. 1. Briefly, BS18

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