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Research Paper

Targeted delivery of epirubicin to tumor-associated macrophages by sialic acid-cholesterol conjugate modified liposomes with improved antitumor activity

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ABSTRACT

With the knowledge that the receptors of sialic acid are overexpressed on the surface of tumor-associated macrophages (TAMs), which play a crucial role in the tumor's progression and metastasis, a sialic acid-cholesterol conjugate (SA-CH) was synthesized and modified on the surface of epirubicin (EPI)-loaded liposomes (EPI-SAL) to improve the delivery of EPI to the TAMs. The liposomes were developed using remote loading technology via a pH gradient. The liposomes were evaluated for particle size, encapsulation efficiency, *in vitro* release, stability, *in vitro* cytotoxicity and pharmacokinetics. And the *in vitro and in vivo* cellular uptake studies demonstrated EPI-SAL achieved enhanced accumulation of EPI into TAMs. The antitumor studies indicated that EPI-SAL provided the strongest antitumor activity compared with the other formulations (EPI-S, EPI-CL and EPI-PL represent EPI solution, conventional liposomal EPI, PEGylated liposomal EPI, respectively), and the survival percent of tumor-bearing mice was 83.3%. The superior antitumor efficacy was probably attributed to the killing of TAMs by EPI-SAL, and modulating the tumor microenvironment with the depletion of TAMs. These findings suggested that SA-CH decorated EPI-loaded liposomes may present an effective strategy to eradicate TAMs, which may be a promising approach for cancer therapy.

1. Introduction

Over the past 60 years, conventional chemotherapy has been designed to target the tumor cell itself, but few types of cancers have been cured (Blansfield et al., 2008). In recent years, much attention has been paid to the strategy designed to target the tumor microenvironment (Huang et al., 2016b; Zhao et al., 2015). The tumor microenvironment is rather heterogeneous, which consists of the extracellular matrix (ECM) and various stromal cells such as vascular endothelial cells, fibroblasts, lymphatic endothelial cells and inflammatory cells (mainly macrophages and lymphocytes) (Hida and Ishii, 2016; Trédan et al., 2007). Stromal cells and ECM interact with each other, or with tumor cells in a complex manner and the constant cross-talk between cells plays a pivotal role in tumor growth, invasion, angiogenesis, and metastatic spread (Diakos et al., 2014). There is increasing evidence that tumors are able to create an immunosuppressive microenvironment and favor their growth and progression (Song et al., 2016a). Macrophages are the major factors contributing to immunosuppressive microenvironment and tumor progression (Kumar et al., 2016). Macrophages are the most abundant inflammatory cells of the tumor microenvironment, amounting to 50% of the tumor mass, and are present at all stages of tumor progression and are key players in the tumor microenvironment (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Muenst et al., 2016). These prominent populations are termed as tumor-associated macrophages (TAMs). TAMs tend to promote tumor growth, proliferation, and survival, and are active in all stages of tumor metastasis and invasion, the specific protumoral mechanisms involved in tuning inflammatory responses and adaptive immunity, scavenging debris, and promoting angiogenesis, tissue remodeling and repair (Dirkx et al., 2006; Sica et al., 2006).

Abbreviations: EPI, ,epirubicin; SA, sialic acid; TAMs, tumor-associated macrophages; SA-CH, sialic acid-cholesterol conjugate; EPI-S, EPI solution; EPI-CL, conventional liposomal EPI; EPI-PL, PEGylated liposomal EPI; EPI-SAL, SA-CH modified liposomal EPI; EPR effect, enhanced permeation and retention effect; SA-ODA, sialic acid-octadecylamine conjugate; CH, cholesterol; EE, encapsulation efficiency; $AUC_{(0-t)}$, area under the drug concentration-time curve values; C_{max}, maximum concentration.

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Accordingly, in various tumors in humans, the presence of extensive TAMs infiltration is correlated with worse clinical outcome and frequent recurrence (Mantovani et al., 2006). These evidence support the idea that TAM targeting is a promising strategy for treating neoplastic diseases (Huang et al., 2016a; Locke et al., 2012; Piaggio et al., 2016; Song et al., 2016a); several therapies have already shown great prospect in improving therapeutic responses to chemotherapy (Joseph and Isaacs, 1998; Solinas et al., 2009).

It is well-known that enhancing the cellular uptake by decorating a targeting ligand on the surface of nanocarriers is a classical method. Sialic acid (SA) is the name for a family in excess of 50 structurally diverse monosaccharides with a nine-carbon backbone, and is also a generic term for the N- or O-substituted derivatives of neuraminic acid (Angata and Varki, 2002). Its binding receptor, Sialoadhesin (Sn), called Siglec-1, is a prototypic member of the SA binding family of lectins and highly expressed on TAMs (Nath et al., 1999). Studies have shown that Siglecs are endocytic receptors, which makes them candidates for targeted therapies that involve the delivery of cytotoxic agents into the target cells (Macauley et al., 2014). Therefore, SA has the potential to be used as a ligand or targeting moiety to enhance the distribution of anticancer drugs to TAMs.

Epirubicin (EPI) is a 4′-epimer of doxorubicin, belonging to the anthracyclines group. It has an important role in chemotherapy, and has been available for various types of cancer in clinical settings (Di-Wen et al., 2016; Tian et al., 2010). The mechanism of action of EPI occurs through intercalating into DNA strands and suppressing DNA and RNA synthesis (Tariq et al., 2015). Nevertheless, the long-term use of EPI in clinical settings is greatly restricted because of the lack of tumor selectivity and serious non-specific toxicity to normal tissue (Nasr et al., 2014). Meanwhile, the rapid clearance by the mononuclear phagocyte system (MPS) reduces the extravasation of EPI into the tumor site and weakens its efficacy accordingly (Šimůnek et al., 2009). Taken together, these revealed the need for a desirable delivery system that selectively accumulates in the tumor area, is less toxic, and improves therapeutic efficacy.

Liposomes are closed spherical nanoparticles composed of phospholipid bilayers (Sauvage et al., 2016). Liposomes as carriers for the targeted delivery of anticancer drugs have been extensively studied and have achieved significant success compared to other nanomedicines (Harrington et al., 2000; Shah et al., 2014). For instance, Doxil® and Myocet® encapsulated doxorubicin and were approved for the treatment of Kaposi sarcoma and late stage ovarian cancer (Cho et al., 2008; Kibria et al., 2016; Taurin et al., 2012). Thus, liposome encapsulation is a strategy to prolong the blood circulation time of EPI and subsequently enhance the tumor accumulation of EPI via the enhanced permeation and retention (EPR) effect.

In our previous study, a sialic acid-octadecylamine conjugate (SA-ODA) was synthesized. The SA-ODA modified liposomes encapsulating pixantrone (Pix) reduced the systemic side effects of Pix and improved its anticancer effects, which was supposed to the killing effect of TAMs (She et al., 2014). Therefore, the aim of this research was to demonstrate the hypothesis that the improved antitumor activity was attributed to the TAMs eradicating effect by EPI-SAL. On the other hand, the following acute toxicity study suggested that SA-ODA has a certain toxicity (Song et al., 2016b), which may hinder its application in drug delivery system. Cholesterol (CH), an essential structural component of animal cell membranes, regulates fluidity and the phase behavior of membranes, and is often used to decrease membrane fluidity and improve drug retention properties in liposomal formulations (Ercole et al., 2015). Thus, CH is better suited to serve as the liposome anchor (Qin et al., 2011).

To our knowledge, there have been no reported studies that explored whether SA modification improved the targeting efficiency of EPI to TAMs. On the other hand, there are only a few reports on SA-based nanocarriers, especially *in vivo* antitumor effects. In the current work, with the aim to target TAMs and improve the therapeutic effect of EPI, a sialic acid-cholesterol conjugate (SA-CH) was synthesized and decorated onto the surface of the EPI-loaded liposomes. The various parameters and antitumor studies of the liposomes were evaluated *in vivo* and *in vitro*.

2. Materials and methods

2.1. Materials

Sialic acid (SA) was purchased from Changxing Pharmaceutical Co. Ltd (Huzhou, China). Epirubicin·HCl (EPI, purity 99.0% by high-performance liquid chromatography) was purchased from Olympic Star Pharmaceutical Co., Ltd. (Shenzhen, China). Sialic acid-octadecylamine conjugate (SA-ODA) was prepared by our laboratory (She et al., 2014). Cholesterol (CH) was provided by Shanghai Advanced Vehicle Technology Pharmaceutical, Ltd. (Shanghai, China). Acryloyl chloride (AC) and 2-aminoethanethiol (AE) were obtained from J2000-DSPE) was purchased from Genzyme Corporation (Cambridge, MA, USA). 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich Chemical Co., Ltd. (St. Louis, MO). ZB-1 and ZB-2 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 S180 murine sarcoma and RAW264.7 murine macrophage cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Wistar rats (aged 7–8 weeks; weighing 180–220 g) and male Kunming mice (aged 6–7 weeks; weighing 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 SA-CH

2.3.1. SA-CH was synthesized by the following three steps

2.3.1.1. AC-CH. AC-CH was synthesized according to a modified procedure (Oh et al., 2013). Briefly, CH (386 mg, 1 mmol) was dissolved in 15 mL anhydrous dichloromethane (DCM) containing TEA (417 μL) in an 100 mL eggplant-type flask with a stir bar. AC (245 μL, 3 mmol) was added dropwise to the flask at 0 °C under nitrogen. The reaction mixture was stirred at 0 °C for 1 h and stirred for another 24 h at room temperature. The solvent was removed in vacuo and the residual mixture was washed with cold ethanol and recrystallized to yield a pale yellow solid.

2.3.1.2. AE-AC-CH. AC-CH (220 mg, 0.5 mmol) was dissolved in 15 mL ethanol. Then, AE (154 mg, 2 mmol) was added to the mixture under nitrogen. After the reaction progressed for 10 h, the organic solvent was removed in vacuo. The white crude product was washed with deionized water to yield the purified AE-AC-CH.

2.3.1.3. SA-CH. SA-CH was synthesized by conjugation of the carboxylic group of SA with the amine group of the AE-AC-CH,