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A novel immunoliposome mediated by CD123 antibody targeting to acute myeloid leukemia cells

Yin Wang[a,1], Furong Liu[a,1], Qiantao Wang[a], Honglin Xiang[a], Hui Jin[a,*], Hui Li[b,*], Shengjun Mao[a]

[a] Key Laboratory of Drug Targeting and Drug Delivery System, Ministry of Education & West China School of Pharmacy, Sichuan University, No.17, Section 3, Southern Renmin Road, Chengdu 610041, PR China
[b] Department of Hematology, Sichuan Academy of Medical Sciences & Sichuan Provincial People Hospital, Chengdu 610072, PR China

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ABSTRACT

The application of the tumor targeting antibody-mediated immunoliposomes (ILP) provides us a potential effective strategy for treating malignancies, such as acute myeloid leukemia (AML). CD123, which is specifically overexpressed on AML cells, plays an important role in cell cycling and enhances the cell resistance to the apoptotic stimuli. Given such a unique role of CD123 in AML cells, we aim to develop a novel drug targeting delivery system using CD123 monoclonal antibody (mAb) in this study. On the basis of the daunorubicin (DNR) loaded PEGylated liposomes (DNR-LP), a post-insertion method was applied to covalently attach the anti-CD123 mAb onto the surface of the liposomes to obtain the anti-CD123 mAb modified immunoliposomes (CD123-ILP). Immunoliposomes with different anti-CD123 mAb density (mAb/liposomal S_{100}PC, molar ratio, 0.06%, L-CD123-ILP and 0.14%, H-CD123-ILP) were prepared, respectively. The expressions of CD123 in KG-1a, Kasumi-1, HL-60, NB4 and THP-1 cells were determined by flow cytometry. The cell binding and uptake assays revealed that CD123-ILP was internalized into the $CD123^+$ AML cells, while the MTT assay indicated that CD123-ILP had stronger inhibitory effect on the growth of THP-1 and KG-1a cells, in which CD123 were highly expressed. Furthermore, *in vitro* drug release studies of DNR-LP and CD123-ILP showed a sustained release profile for both systems, which were further confirmed by *in vivo* pharmacokinetics study of liposomal DNR in rats. In this study, we reported the development of CD123-ILP for the first time by our best knowledge, which offered a promising drug targeting delivery system against $CD123^+$ AML cells.

1. Introduction

Acute myeloid leukemia (AML) is a clonal malignancy found in bone marrow (BM) and blood. It can be characterized by uncontrolled proliferation of biologically heterogeneous cells, and the accumulation of defective precursor myeloid cells (Busfield et al., 2014). The current standard treatment for AML is the so-called "7 + 3" regimen, i.e. cytarabine for 7 days and daunorubicin (DNR) for 3 days. Although the "7 + 3" regimen has been widely used in induction treatment of AML for over four decades, patients are still suffering from the drawbacks of such treatment, such as the high recurrence rates and the severe adverse side effects due to the lack of specificity of the drugs (Ehninger et al., 2014; Lee et al., 2015). It has been confirmed in many previous studies that the leukemia stem cells (LSCs) were responsible for the growth and recurrence of leukemia (Blair and Sutherland, 2000; Bonnet and Dick, 1997; Hope et al., 2004). Therefore, there are urgent needs to develop better therapies that can specifically targeting and eliminating both the AML cells and LSCs.

Monoclonal antibody (mAb) drugs have been a hot area in drug discovery, given their specificity and selectivity toward the disease cells, hence have direct impacts on enhancing the drug efficacy and reducing the drug toxicity (Majeti, 2011). Several mechanisms have been reported on how antibodies can effectively against cancers (Adams and Weiner, 2005; Braster et al., 2014; Rogers et al., 2014). Their superior specificity against the target cells making them perfect candidates in development of targeted therapies.

Although site-specific delivery of drugs to cancer cells using antibodies can potentially increase the therapeutic effect and reduce the side effect, cautions are remain to be consistently considered. For instance, gemtuzumab ozogamicin (GO) was a

* Corresponding authors.
E-mail addresses: jinhui_1974@126.com (H. Jin), lihui606606@163.com (H. Li).
[1] These authors contributed equally to this work.

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humanized anti-CD33 monoclonal antibody drug conjugated with calicheamicin. It received the accelerated approval by the FDA for the treatment of relapsed AML in May 2000 in the US, and was the first approved antibody-conjugate drug. However, it was voluntarily removed from the US market due to its potential toxicity and low treatment effect in post-approval clinical trials in June 2010 (Bross et al., 2001; Castaigne, 2013; O'Hear et al., 2013). Therefore, choosing an appropriate surface antigen of AML cells as therapeutic target is crucial to develop a truly effective and safe therapy for AML.

CD123, the transmembrane α chain of interleukin 3 receptor (IL-3R), is overexpressed on AML blasts, $CD34^+$ leukemic progenitors and AML-LSCs specifically, in comparison with the normal hematopoietic stem cells (HSCs). Thus, it is a potential therapeutic target for AML treatment, to eradicate both AML cells and the LSCs simultaneously. It has been reported that a neutralizing monoclonal antibody against CD123 (7G3) with both IL-3R-neutralizing and innate immunity activating properties have previously been shown to antagonize IL-3-mediated proliferation of AML cells, and to improve the survival of engrafted AML mice (Jin et al., 2009a, 2009b).

On the other hand, liposomal formulations have also been widely studied as a versatile drug vehicle since they are nontoxic, non-immunogenic and highly efficient for drug encapsulation (Schuster et al., 2015). Liposomes can also decrease the distribution volume of the loaded drugs, reduce their toxicities and hence dramatically improve the therapeutic efficacy (Yang et al., 2007). Stealth liposomes with polyethylene glycol (PEG) modification can avoid phagocytosis by reticular endothelial system (RES), hence greatly prolong the circulation time *in vivo* (Torchilin and Trubetskoy, 1995; Yoo et al., 2010; Zhang et al., 2012). Comparing with solid tumors, hematologic diseases, including AML, have limited permeability and retention (EPR) effects. Therefore, common passive liposomal delivery systems may have limited function against such diseases. Hence, it is of great interest to develop an active-targeting drug delivery system (Liu et al., 2014). Immunoliposomes modified with different ligands (e.g., antibody, peptide, transferrin, and folate) provide a promising approach for such active-targeting abilities.

The efficacy of the immunoliposome depends firstly on the target specificity of the vector, and secondly on cellular uptake and intracellular release of the loaded drugs. The mAb modified immunoliposome have the important advantages of both high drug loading and cell targeting abilities. Therefore, by connecting relatively few mAb molecules to immunoliposomes, the therapeutic efficacy can be further improved comparing to the non-targeting liposomes (Sapra and Shor, 2013).

Based on those knowledges, we aim to construct a DNR-loaded PEGylated immunoliposome mediated by CD123 antibody (CD123-ILP) that targeting the CD123 antigen on AML cells. Firstly, in order to explore the relationship between the CD123 antigen density on AML cells and the targeting ability of the immunoliposomes, we use multicolor flow cytometry to quantify the CD123 expression level on different AML subtypes, defined by French-American-British (FAB) classification. Secondly, a post-insertion method is applied to prepare the CD123-ILP. We then evaluate the targeting capacity of CD123-ILP against different AML cells, and consequently determine the best formulation for efficient inhibition of AML cells. It should be noted that CD123-ILP is constructed for the first time by us to our best knowledge. We hope this work can shed light on the development of new ideas and approaches for the treatment of AML.

2. Materials and methods

2.1. Materials

Daunorubicin was purchased from Baili Biotech (Shanghai, China) with greater than 97% purity. Soybean phosphatidylcholine (S_{100}PC, purity >98%) was purchased from the Lipoid (Ludwigshafen, Germany). Cholesterol (Chol), 1,2-distearoyl-sn-*glycero*-3-phosphoethanolamine [methoxy (polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) and Maleimide derivatized PEG_{2000}-DSPE (Mal-PEG_{2000}-DSPE) were bought from Avanti Polar Lipids (Alabaster, AL, USA). 2-iminothiolane (Traut's reagent), 5,5′-Dithiobis (2-nitrobenzoic acid) (Ellman's reagent), [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl] tetrazolium bromide (MTT), 4, 6-diamidino-2-phenylindole (DAPI) and dialysis bag (MWCO, 7000) were obtained from Sigma-Aldrich ((St. Louis, Mo, USA). Sepharose CL–4B was obtained from Yuanye Biotech (Shanghai, China) and Sephadex G-50 from Amersham Pharmacia Biotech (Stockholm, Sweden). Bicinchoninic acid (BCA) protein assay kit was obtained from KeyGen Biotech (Nanjing, China). RPMI-1640 medium, penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Gibco Invitrogen (USA). Plastic cell culture dishes and plates were purchased from Corning Incorporation (USA). Purified mouse anti-Human CD123 monoclonal antibody (clone 7G3), mouse anti-human CD123-APC and mouse IgG2a-APC were purchased from Becton Dickinson (USA) and anti-mouse IgG (H + L) Alexa fluor 647 conjugate as secondary antibodies were purchased from Cell Signaling Technology (USA). All other reagents were of analytical grade and were used without further purification.

2.2. Cell lines and animals

KG-1a (acute myelogenous leukemia cell line, FAB-M0), Kasumi-1 (acute myeloblastic leukemia cell line, FAB-M1), HL-60 (acute myeloblastic leukemia with maturation cell line, FAB-M2), NB4 (acute promyelocytic leukemia cell line, FAB-M3) and THP-1 (acute monocytic leukemia cell line, FAB-M5), which are representative subtypes of AML cells, were obtained from ATCC (Manassas, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified 37 °C incubator with 5% CO_2. Only cells in the exponential phase of growth were used for further experiments.

Male Sprague-Dawley (SD) rats with body weights ranging from 200 to 250 g were kindly provided by Laboratory Animal Center of Sichuan University (Sichuan, China) and housed in the SPF room of the Experimental Animals Center. All animal experiments were performed under a protocol approved by the University Ethics Committee for the Use of Laboratory Animals and in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3. Preparation of PEGylated liposomes

The daunorubicin-loaded PEGylated liposomes (DNR-LP) of S_{100}PC/Chol/$mPEG_{2000}$-DSPE at 2.85:1:0.15 molar ratio were prepared by the thin film hydration method. DNR was loaded into liposomes using the ammonium sulfate gradient method (Haran et al., 1993). Briefly, S_{100}PC, Chol and $mPEG_{2000}$-DSPE were dissolved in chloroform/methanol (2:1, v/v), and a thin uniform lipid film was generated by evaporation during vortexing at 37 °C under vacuum for 15 min. Subsequently the lipid film was put in a vacuum drying oven for at least 4 h at room temperature to get rid of organic solvents. The formed lipid film was then hydrated with 0.25 M ammonium sulfate solution at 60 °C for 30 min. The final lipid concentration was 9 mM. The obtained large unilamellar liposomes were probe sonicated for 75 s in ice-water bath and dialyzed using a dialysis bag (MWCO, 7000) against 10% sucrose solution for 12 h, and the solution was exchanged after 4 h. The resultant liposomes were mixed with daunorubicin sucrose solution (DNR:LP = 1:15, w/w) and incubated at 60 °C for 2 h. Free