Journal of Pharmaceutical Sciences 105 (2016) 639–649

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutics, Drug Delivery and Pharmaceutical Technology

Preclinical Evaluation of an Epidermal Growth Factor Receptor–Targeted Doxorubicin–Peptide Conjugate: Toxicity, Biodistribution, and Efficacy in Mice

Fan Yang [1,*], Weilun Ai [2], Fagang Jiang [1], Xin Liu [2], Zebo Huang [2,3], Shibin Ai [2,*]

[1] Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430021, China
[2] School of Pharmaceutical Sciences, Wuhan University, Wuhan 430071, China
[3] Guangdong Province Key Laboratory for Biotechnology Drug Candidates, School of Biosciences and Biopharmaceutics, Guangdong Pharmaceutical University, Guangzhou 510006, China

ARTICLE INFO

Article history:
Received 9 July 2015
Revised 30 September 2015
Accepted 16 October 2015

Keywords:
receptor-mediated targeted delivery
epidermal growth factor receptor
doxorubicin
biodistribution
toxicity
antitumor activity

ABSTRACT

Doxorubicin (DOX) is known to induce apoptosis and necrosis in healthy tissue resulting in unwanted toxicities. To improve the ability of DOX to more specifically target tumors and minimize undesirable side effects, conjugation of DOX with epidermal growth factor receptor (EGFR)–binding peptide (DOX-EBP) has been developed to deliver DOX to EGFR-overexpressing neoplastic cells. Here, we investigated whether DOX-EBP was able to reduce toxicity and enhance anticancer efficacy *in vivo* through receptor-mediated targeted delivery system. Nude mice were treated with DOX or DOX-EBP to estimate general toxicity, normal tissue damage, biodistribution, and antitumor efficacy. In addition, the expression levels of EGFR in tumor tissues and normal organs were investigated by Western blotting, and their mRNA expression was analyzed by reverse transcription PCR. This study demonstrated that DOX-EBP was able to effectively decrease the distribution of DOX in normal tissues without EGFR overexpressing and reduce DOX-induced toxicity. On the other hand, the research also confirmed that DOX-EBP was able to preferentially accumulate DOX in EGFR-overexpressing tumor tissues and showed the enhanced anticancer efficacy over free DOX. DOX-EBP could be used for receptor-targeted chemotherapy with less toxicity and greater efficacy of tumor cells overexpressing EGFR. DOX-EBP conjugate is a good therapeutic agent for cancer treatment.

Introduction

The anthracycline antibiotic doxorubicin (DOX) is a frequently used therapeutic agent for the treatment of solid tumors, including breast cancer, lung cancer, ovarian cancer, and sarcomas, and is regarded as one of the most potent chemotherapeutic drugs approved by the Food and Drug Administration.[1] As DOX itself does not specifically target tumor cells, only an amount of the administered dose reaches the tumor site, whereas the rest is distributed nonspecifically throughout the whole body where it may damage normal tissues, resulting in toxic side effects such as bone marrow toxicity, gastrointestinal disorders, stomatitis, alopecia, acute and cumulative cardiotoxicity, and extravasation.[2,3] DOX may also induce potential liver and kidney lesions, causing hepatorenal toxicity. In the case of the excessive use of the drug, DOX may cause serious and irreversible cardiac toxicity.[4] Such high levels of nonspecific toxicity toward normal tissues and organs limit the clinical application of DOX because of its narrow therapeutic indices.[5,6]

In recent years, several approaches have been attempted to develop more specific drug delivery systems capable of limiting DOX toxicity while targeting tumor sites as much as possible.[7-10] Some of these drug delivery systems have been proved to enhance efficacy, selectivity, and the overall effect of DOX. For example, liposomes were proposed as a DOX delivery system that can improve accumulation within solid tumors compared to free DOX via the enhanced permeability and retention effect and reduce accumulation in healthy tissues.[11] Several DOX-encapsulating liposomes, such as Doxil®, Caelyx®, and Myocet®, have been

* *Correspondence to:* Shibin Ai and Fan Yang (Telephone: +86-27-68759869; Fax: +86-27-68759850).
E-mail addresses: uh_biotf@126.com (F. Yang), aishibin@tom.com (S. Ai).

http://dx.doi.org/10.1016/j.xphs.2015.10.020
0022-3549/

clinically employed.[12] Current efforts are focusing on developing the receptor-mediated active targeting liposomes that can promote more selective binding and internalization of the liposomes into target cells with enhanced specificity and improved drug bioavailability relative to enhanced permeability and retention–mediated passive targeting liposomes.[13,14]

It is well recognized that receptor-mediated DOX delivery, which selectively targets the receptors overexpressed on the surface of tumor cells, is less toxic and more effective than conventional DOX chemotherapy.[15–17] This strategy involves target-specific peptides, which are conjugated to DOX and have high specificity and affinity for the overexpressed receptor targets.[18] With this approach, DOX is accumulated at significantly lower levels in normal tissues and cells than in tumor cells, and thus, normal tissues suffer less collateral damage.[19,20] For example, AEZS-108 (AN-152), a conjugate of DOX and luteinizing hormone–releasing hormone via a hydrolysable spacer, is shown to be taken up via hormone receptor–mediated process and has been clinically used to provide targeted therapy for luteinizing hormone–releasing hormone receptor–positive human cancers such as ovarian and endometrial cancers, hormone-refractory prostatic tumors, and mammary neoplasms.[21,22] Similarly, in mouse models, a conjugate of DOX and cyclic Arg-Gly-Asp peptides has been shown to selectively target $\alpha_v\beta3$ and $\alpha_v\beta5$ integrins overexpressed in tumor cells during angiogenesis and can be used to treat angiogenesis or diagnose tumors.[23,24] The integrin family is an extensive group of structurally related receptors for extracellular matrix proteins and immunoglobulin superfamily molecules.[25] Accordingly, such treatment can improve the survival of mice bearing human breast carcinoma.

The overexpression of epidermal growth factor receptor (EGFR) on more than a third of human solid tumors, including bladder, breast, colon, ovarian, prostate, renal cell, and squamous cell (non-small cell lung and head and neck) carcinomas.[26,27] EGFR has emerged as a key therapeutic target for the treatment of these tumors.[28] Two different types of EGFR-targeted therapeutic agents have been developed: selected EGFR-targeted monoclonal antibodies such as cetuximab and panitumumab and selected EGFR-tyrosine kinase inhibitors such as gefitinib and erlotinib.[29,30] More recent trials have suggested that conjugate of a cytotoxic agent with a ligand or an antibody provides another EGFR-targeted approach.[31,32] The use of the ligand or antibody as a vector molecule for the EGFR-targeted delivery of cytotoxic antitumor drug can have the advantage of killing the cell after internalization. The recombinant EGFR-binding active fragments of the ligand, that is, EGFR-binding peptides (EBPs), appropriate as carriers of cytotoxic agents intended to target EGFR-overexpressed tumor cells.[33] This type of targeted chemotherapy can improve antitumor efficacy compared to conventional systemic chemotherapy and can also reduce side effects of free DOX.[34] Therefore, we first designed a new cytotoxic analog that links DOX to an EBP (NH_2-CMYIEALDKYAC-COOH) via an ester bond at DOX position 14 through a glutarate spacer and demonstrated that this conjugate had high affinity and specificity for EGFR-overexpressed tumor cells, resulting in enhanced antitumor efficacy.[35] In previous studies, we found the cellular accumulation, intracellular distribution, and *in vitro* cytotoxicity of DOX-EBP with significantly higher levels in EGFR-overexpressing tumor cells than in non–EGFR-overexpressing tumor cells, demonstrating DOX-EBP conjugate with targeting ability to EGFR-overexpressing tumor cells. In addition, EGFR inhibition assay conducted through competitive blockade of EGFR using an anti-EGFR monoclonal antibody C225 (Erbitux®) confirmed that cellular uptake of DOX-EBP conjugate was mediated by EGFR. In addition, we also found that the DOX-EBP conjugate was capable of bypassing ABC drug-efflux pumps through receptor-mediated endocytosis and overcoming DOX resistance. As a consequence, the conjugate had increased antitumor activity in both DOX-resistant and nonresistant tumors compared to free DOX.[36]

Lung cancer is the leading cause of cancer-related deaths worldwide, with an overall 5-year survival rate of 15%.[37] EGFR is overexpressed in >60% of human lung carcinomas. More importantly, the level of the expression of EGFR has been found to correlate with high metastatic rate, poor tumor differentiation, and poor prognosis in lung carcinoma patients.[38] Therefore, EGFR has become an important therapeutic target in lung cancer.[39] However, only a few studies on EGFR-mediated antiproliferative effects of the covalently linked DOX–peptide conjugate for treatment of lung cancer *in vivo* are reported. Previously, we studied the antitumor efficacy of DOX-EBP in human colon cancer cells, and our main purpose was to investigate DOX-EBP's ability to overcome drug resistance.[36] In the present study, we used human lung cancer cells to investigate the preclinical evaluation of DOX-EBP, which would let us know the DOX-EBP's antitumor efficacy on lung cancer and helps us develop the anticancer spectrum of DOX-EBP. This research aims to evaluate whether DOX-EBP can reduce DOX-induced toxicity, to change the tissue distribution of DOX, and to enhance its antitumor efficacy. At first, we compared the effects of free DOX and DOX-EBP conjugate on the structure and function of normal tissues and organs in a mouse model, including heart, liver, and kidney. Next, we analyzed the distribution profiles of free DOX and DOX-EBP conjugate in both tumor tissues and normal organs in mice bearing human lung cancer cells (A549 xenografts). To analyze the biodistribution of DOX-EBP, DOX-EBP conjugate's toxicity effects and its distribution in tissues were evaluated by receptor-mediated response to EGFR after the expression of EGFR in tumor tissues and normal organs was examined. Finally, the antitumor efficacy of free DOX and DOX-EBP was compared in BALB/c nude mice with A549 xenografts.

Materials and Methods

Chemicals

Doxorubicin hydrochloride (DOX HCl) was obtained from Sigma-Aldrich (St. Louis, MO). DOX-EBP conjugate was synthesized as previously described.[35]

Cell Lines and Animals

A549 human lung cancer cells, which overexpress EGFR, were purchased from the China Center for Type Culture Collection (Wuhan, China) and maintained as previously described.[35] BALB/c-nu/nu female mice (6-8 weeks old) were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The animals were housed in laminar airflow cabinets under pathogen-free conditions with a 12-h light and 12-h dark schedule and fed with autoclaved standard chow and water *ad libitum*. Animal experiments were performed according to the Guidelines for Animal Care and Use Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Evaluation of Toxicity

Tumor-free mice (6 per group) were used to test the general toxicity of free DOX and DOX-EBP based on survival and body weight change after a single-dose intravenous (IV) administration at 10-, 20-, 40-, and 80-mg DOX (or DOX equivalent) per kg body weight, with 0.9% sodium chloride as a control.[40] The survival and