Biomaterials 94 (2016) 1–8

Contents lists available at ScienceDirect

Biomaterials

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

Albumin-binding caspase-cleavable prodrug that is selectively activated in radiation exposed local tumor

Seung Woo Chung [a], Jeong uk Choi [a], Beom Seok Lee [b, c], Julia Byun [d], Ok-Cheol Jeon [e], Seong Who Kim [f], In-San Kim [c], Sang Yoon Kim [b, **], Youngro Byun [a, g, *]

[a] Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul, 08826, South Korea
[b] Department of Otolaryngology, Asan Medical Center, College of Medicine, University of Ulsan, Seoul, 05505, South Korea
[c] Biomedical Research Institute, Korea Institute of Science and Technology, Seoul, 02792, South Korea
[d] Franklin & Marshall College, Lancaster, PA, 17604-3003, USA
[e] Pharosgen Co., Seoul, 06034, South Korea
[f] Department of Biochemistry and Molecular Biology, Asan Medical Center, College of Medicine, University of Ulsan, Seoul, 05505, South Korea
[g] Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergent Science and Technology, Seoul National University, Seoul, 08826, South Korea

ARTICLE INFO

Article history:
Received 19 January 2016
Received in revised form
11 March 2016
Accepted 28 March 2016
Available online 31 March 2016

Keywords:
Albumin
Apoptosis
Caspase
Drug delivery
Prodrug
Radiotherapy

ABSTRACT

Existence of the genomically and epigenomically diverse subclones in a tumor severely limits the therapeutic efficacy of targeted agents. To overcome such a limitation, we prepared a novel targeted prodrug, EMC-DEVD-S-DOX, which comprises two important features: radiation-induced apoptosis targeting and albumin-binding properties. In particular, the prodrug binds circulating albumin after intravenous administration and then activated by caspase-3, which is upregulated from apoptotic cells that responded to radiotherapy. The prodrug was designed to bind circulating albumin to extend half-life and facilitate tumor accumulation in order to increase the possibility of contacting caspase-3, which is only transiently upregulated during apoptosis. Our results showed that EMC-DEVD-S-DOX had a prolonged half-life with enhanced tumor accumulation, which clearly benefited the therapeutic effect of the prodrug. Also, agreeing with the *in vitro* studies that showed ignorable cytotoxic effect in the absence of caspase-3, the prodrug was effective only when combined with radiotherapy without any noticeable systemic toxicity *in vivo*. Due to the highly selective action of EMC-DEVD-S-DOX independent to the complex genomic profiles of tumor, the prodrug would overcome the limitation of current targeted therapy and potentiate radiotherapy in the clinical oncology.

1. Introduction

The use of targeted agents are fundamentally challenged by recent findings that revealed significant genomic and epigenomic variations of tumor cells in a single tumor, known as intratumor heterogeneity [1]. This could in fact severely impair the reliability of the genomic analysis of tumor tissue in order to predict the feasibility of a targeted agent. Moreover, specific targeting of a single biomarker is likely to affect only a limited population of tumor cells, thereby eventually resulting in tumor relapse [2,3].

In attempt to overcome the limitations of targeted therapy caused by the intratumor heterogeneity, we previously suggested a radiation-induced apoptosis-targeted chemotherapy (RIATC) [4,5]. RIATC delivers cytotoxic agents to tumor by targeting apoptosis occurred in an irradiated tumor. In particular, caspase-3 was selected as a target molecule, which is upregulated during apoptosis [6,7]. The major advantages of the RIATC are as follows: (i) the target site could be controlled actively by exogenous stimuli (e.g. stereotactic radiotherapy); (ii) caspase-3, which is the target molecule, could be continuously upregulated during the therapy, since the delivered cytotoxic agents again induce tumor cell apoptosis. This strategy was embodied by designing a prodrug that could release free doxorubicin in the presence of caspase-3 by

* Corresponding author. Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergent Science and Technology, College of Pharmacy, Seoul National University, Seoul, 08826, South Korea.
** Corresponding author.
E-mail addresses: sykim2@amc.seoul.kr (S.Y. Kim), yrbyun@snu.ac.kr (Y. Byun).

http://dx.doi.org/10.1016/j.biomaterials.2016.03.043
0142-9612/

conjugating doxorubicin and a caspase-3 substrate peptide.

The prodrug was finally synthesized as an albumin-binding agent that could bind *in situ* to the circulating albumin after intravenous administration according to Kratz et al. [8–13]. Albumin is the most abundant protein in the plasma (35–50 mg/mL) [14], and about 70% of that has an accessible free thiol on Cys34, which is a very unique property among the plasma compartments [10]. In fact, the free thiol on albumin account for more than 80% of the total free thiols in the blood and is the most reactive among the thiols that exist in the plasma substances. Therefore, thiol-reactive molecules could selectively bind to the endogenous albumins when entered the blood. Using albumin as a drug carrier has several advantages. Albumin has a very long plasma half-life due to its large molecular size above the renal clearance threshold and neonatal Fc receptor-mediated recycling [15,16]. In fact, albumin has a half-life 19 days in human. Also, albumin could be accumulated in the tumor due to the high metabolic turnover and EPR (enhanced permeability and retention) effect, which is shown in macromolecules by the increased vascular permeability and lack of lymphatic drainage [17,18]. In addition, many different tumors overexpress SPARC protein, which is a high affinity albumin-binding protein and facilitate albumin accumulation in the tumor [19].

This study demonstrates about the prodrug that could effectively deliver cytotoxic agents to tumor by adopting radiation-induced apoptosis targeting and *in situ* albumin-binding mediated passive targeting together (Fig. 1A). The doxorubicin prodrug, EMC-DEVD-S-DOX, was prepared as a representative agent by conjugating the ε-maleimidocaproic acid (EMC) to doxorubicin via DEVD peptide spacer (Fig. 1B). The maleimide group in the EMC moiety could selectively react with thiol in physiological pH [20], allowing the prodrug to covalently bind to the circulating albumin.

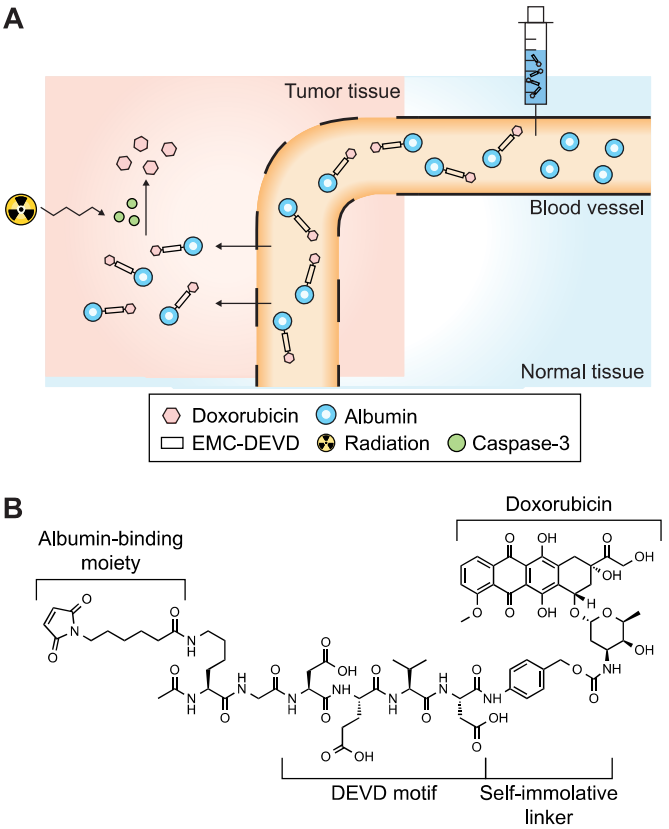

Fig. 1. (A) Schematic diagram of EMC-DEVD-S-DOX therapeutic strategy. (B) Chemical structure of EMC-DEVD-S-DOX.

The selective binding of EMC-DEVD-S-DOX on albumin was confirmed on commercially available human serum albumin (HSA), mouse plasma, and human plasma. DEVD peptide is a well-known substrate of caspase-3. The caspase-3 recognizes the DEVD sequence and enzymatically hydrolyzes the amide bond following the Asp residue [21]. Consequently, doxorubicin could be released from the bound albumin when it reaches the irradiated tumor, thus potentiating the therapeutic outcome of radiotherapy. The release of doxorubicin in the presence of caspase-3 was confirmed by various *in vitro* studies. Pharmacokinetic and tumor regression studies were also carried out in rodent models.

2. Materials and methods

2.1. Synthesis

Firstly, Ac-Lys-Gly-Asp-Glu-Val-Asp-PABC-DOX (named as DEVD-S-DOX; 20 mg, 0.013 mmol) was synthesized as described previously [4]. DEVD-S-DOX (20 mg, 13.4 μmol) and *N*-(ε-maleimidocaproyloxy)succinimide ester (EMCS; 8.26 mg, 26.8 μmol, 2 eq; Pierce, Rockford, IL) were then dissolved in anhydrous DMF (1.5 ml) followed by addition of Et_3N (4.64 μl, 2.5 eq; Sigma-Aldrich, St. Louis, MO). The reaction mixture was stirred at room temperature for 2 h under inert atmosphere. The product was purified by semi-preparative HPLC (Shimadzu, Kyoto, Japan) with an ODS-A 5 μm reverse phase semi-preparative column (150 mm × 20 mm; YMC, Dinslaken, Germany) in a gradient system (water and CH_3CN with 0.05% TFA as an additive, CH_3CN 20–50% over 50 min, 8 mL/min). The collected fraction was lyophilized to obtain Ac-Lys(EMC)-Gly-Asp-Glu-Val-Asp-PABC-DOX (named as EMC-DEVD-S-DOX) as a red amorphous powder (16.7 mg, 73.6% yield). The final product was analyzed by analytical HPLC (Agilent 1300 series, Agilent Technologies, Santa Clara, CA) using an ODS-A analytical column (150 × 3 mm, 5 μm; YMC) in a gradient system (water and CH_3CN with 0.1% TFA as an additive, CH_3CN 5–95%/5–30 min, 1 mL/min) and the purity was determined to be ≥ 95% (UV 214 nm). HRMS (ESI-QTOF): calculated for $C_{73}H_{90}N_{10}O_{29}$, 1570.5875; found 1570.5891 (Δ = 0.98 ppm).

2.2. Albumin binding study using HPLC analysis

HSA was purchased from Sigma-Aldrich. Human and mouse serum was obtained from a healthy volunteer and C3H/HeN mice (Orient Bio, Seongnam, South Korea), respectively. To a solution of HSA (700 μM in PBS, pH 7.4) or serum, EMC-DEVD-S-DOX (100 μM) was added and incubated at room temperature for 3 min and 60 min. For blocking study, the HSA solution and serum were pre-incubated with an excess amount of γ-maleimidobutyric acid (Santa Cruz Biotechnology, Dallas, TX) for an hour before adding EMC-DEVD-S-DOX. The samples were analyzed by an analytical HPLC using an ODS-A analytical column (150 × 3 mm, 5 μm; YMC) in a gradient system (water and CH_3CN with 0.1% TFA, CH_3CN 30–50%/5–25 min, 1 mL/min) under a fluorescence detector (470/580 nm).

2.3. Albumin binding study using SDS-PAGE analysis

To a solution of HSA (70 μM in PBS, pH 7.4), EMC-DEVD-S-DOX (100 μM) or DEVD-S-DOX (100 μM) was added and incubated for an hour at room temperature. To serum, EMC-DEVD-S-DOX was added at final concentrations of 0.2, 0.5, and 1 mM, and incubated for an hour at room temperature. The samples were analyzed with a native SDS-PAGE (12% polyacrylamide gel). The gels were observed by trans-UV under the ImageQuant LAS 4000 imaging system (GE Healthcare Life Sciences, Uppsala, Sweden) and then stained with