BASIC SCIENCE

Nanomedicine: Nanotechnology, Biology, and Medicine
13 (2017) 713–722

nanomedjournal.com

Original Article

A drug-delivery strategy for overcoming drug resistance in breast cancer through targeting of oncofetal fibronectin

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Received 12 August 2016; accepted 6 October 2016

Abstract

A major problem with cancer chemotherapy begins when cells acquire resistance. Drug-resistant cancer cells typically upregulate multi-drug resistance proteins such as P-glycoprotein (P-gp). However, the lack of overexpressed surface biomarkers has limited the targeted therapy of drug-resistant cancers. Here we report a drug-delivery carrier decorated with a targeting ligand for a surface marker protein extra-domain B(EDB) specific to drug-resistant breast cancer cells as a new therapeutic option for the aggressive cancers. We constructed EDB-specific aptide (APT_{EDB})-conjugated liposome to simultaneously deliver siRNA(siMDR1) and Dox to drug-resistant breast cancer cells. APT_{EDB}-LS(Dox,siMDR1) led to enhanced delivery of payloads into MCF7/ADR cells and showed significantly higher accumulation and retention in the tumors. While either APT_{EDB}-LS(Dox) or APT_{EDB}-LS(siMDR1) did not lead to appreciable tumor retardation in MCF7/ADR orthotopic model, APT_{EDB}-LS(Dox,siMDR1) treatment resulted in significant reduction of the drug-resistant breast tumor. Taken together, this study provides a new strategy of drug delivery for drug-resistant cancer therapy.
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Key words: Aptide; Multi-drug resistance; Extra-domain B (EDB) of fibronectin; siRNA; Drug delivery; Liposomes

Multidrug resistance (MDR) is one of the biggest challenges in chemotherapy and a major impediment to current cancer therapy modalities.[1] A key characteristic of drug-resistant cancer cells is overexpression of P-glycoprotein (P-gp), a cellular membrane protein that actively pumps out a diverse class of drugs to the cell exterior, rendering treatment ineffective.[2] MDR-1 belongs to P-glycoprotein superfamily, in which overexpression of P-gp family members often results in active pump-out of drugs from the cytoplasm to cell exterior, a phenomenon known as drug resistance.[2,3] Drug resistance can also be acquired during the course of treatment, due to various adaptive responses of cells; *i.e.* activation of alternative compensatory signaling pathways for survival.[4] In addition to the presence of drug-resistance machinery, the lack of appropriate overexpressed surface biomarkers in these cells has limited the targeted drug delivery and treatment of drug-resistant cancers.[1] As proven by many others, nanoparticle-based drug delivery system could overcome drug resistance in cells using a Trojan horse approach, where drugs were carried into the cell cytoplasm, unrecognized by P-gp as a substrate.[5–13] In this regard, a drug-delivery carrier decorated with a targeting ligand for a surface marker protein specific to drug-resistant cancer cells could provide a new therapeutic option for hard-to-treat cancers.

Recently combinations of RNAi and chemotherapy have pre-clinically demonstrated synergistic effects in inhibiting tumor growth[7,9–11,14–18]. Generally, the purpose of the combination should determine the therapeutic regime. If the focus is to inhibit the expression of a Pgp protein to enhance drug uptake, a

Abbreviations: P-gp, P-glycoprotein; MDR, multidrug resistance; siRNA, small interfering RNA; siMDR1, siRNA specific to the MDR1 gene; EDB, extra-domain B of fibronectin; APT, aptide; Dox, doxorubicin; 9R, nona-arginine peptide; RT-PCR, reverse transcription-polymerase chain reaction; LS, liposome; APT_{EDB}-LS, EDB aptide-conjugated liposome.

Conflict of Interest: The author declares no conflict of interest.

This work was supported by a Global Research Laboratory grant (2015045887) through the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT & Future Planning and the David H. Koch-PCF Program in Cancer Nanotherapeutics.

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http://dx.doi.org/10.1016/j.nano.2016.10.005
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sequential treatment will be the best choice, *i.e.*, siRNA can be delivered to knock down the Pgp family protein and the drug delivered after cells are sensitized.[9,19,20] On the other hand, if the siRNA and drug are targeting different signaling pathways that are equally crucial to the proliferation or survival of cancer cells, it would be appropriate to deliver siRNA and drug simultaneously to produce a synergistic effect in halting tumor progression.[5,10,21] The combination of RNAi and chemotherapy with different mechanisms of action could down-regulate the expression of MDR-associated proteins and thus increase the chemosensitivity of cancer cells to anticancer drugs, raising therapeutic efficacy with minimal systemic toxicity. In the case of MDR1 siRNA and Dox co-delivery, several groups have reported synergistic effect of combination therapeutic efficacy as compared to monotherapy.[5,9] The synergistic effect of siMDR1 downregulating MDR1 protein increases Dox accumulation in cancer cell cytoplasm, which in turns increases cell cytotoxicity.

The extracellular matrix in most tumors becomes substantially remodeled in various ways as cancer progresses. One such modification is insertion of extra-domain B (EDB) into fibronectin through alternative splicing.[22,23] EDB is specifically over-expressed around new blood vessels in tumors as well as in tumor-associated extracellular matrix, and is thus considered a promising cancer marker.[24,25] It has also been shown that EDB expression is linked to cancer aggressiveness and malignancy.[26] Very recently, we reported that EDB is a putative biomarker of a breast cancer stem cell or tumor-initiating cell; thus, knockdown of EDB could significantly inhibit breast tumor growth.[27] An additional characteristic of cancer stem cells is that they possess intrinsic/acquired drug-resistance properties, which could hamper effective chemotherapeutic intervention.[28–31] Drawing on our previous findings and those of others, we thus hypothesized that drug-resistant breast cancer cells might overexpress the EDB domain as drug-resistant cells are more malignant and aggressive.[32,33] True to our expectation, we observed that EDB was minimally expressed in MCF7 breast cancer cells yet highly expressed when MCF7 cells attain drug resistance. We speculate that targeting EDB domain of fibronectin could specifically direct nanomedicine to dox-resistant MCF7/ADR cells, resulting in an effective modality in treating resistant cancers. Drawing from our experience in developing anionic liposomal system for ODN and drug delivery respectively,[34–36] herein, we developed an EDB-targeting liposomes that simultaneously encapsulate doxorubicin (Dox) and siRNA targeting MDR1 (siMDR1) for use as therapeutic modality for treatment of drug-resistant breast cancer. An aptide specific to EDB (APT_{EDB}) was used as a targeting peptide ligand, and MCF7 and MCF7/ADR cells were chosen as standard and Dox-resistant human breast cancer cell variants, respectively. APT_{EDB} has been extensively studied and verified for its target specificity and targeting ability by our group.[37,38] We have previously reported APT_{EDB} conjugated liposomal system for various cancer treatment modalities, including imaging, drug delivery and theranostic application.[34–36,39] In this regard, we anticipate that co-delivery of siMDR1 and Dox would exert synergistic effects by inhibiting P-gp–mediated efflux of the internalized drug.[15,40–43] In this report, we prepared EDB-targeting liposomes simultaneously encapsulating Dox and siMDR1, termed APT_{EDB}-LS(Dox, siMDR1), and evaluated their efficacy in the treatment of Dox-resistant breast cancer *in vitro* and *in vivo* xenograft and orthotropic mouse models.

Methods

Materials

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (POPG), polyethylene glycol(2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (ammonium salt) (PEG_{2000}-DSPE), N-maleimide-PEG_{2000}-DSPE (ammonium salt) (Mal-PEG_{2000}-DSPE), plant cholesterol (Chol), and a mini handheld extruder set were purchased from Avanti Polar Lipids (Alabaster, AL, USA). An aptide specific for the EDB domain of fibronectin (APT_{EDB}) containing an additional cysteine in the β-hairpin constant loop region, *N*′-CSSPIQGSWTWENGK(**C**)WTWGIIRLEQ-*C*′, was custom-synthesized by Anygen Corp (Gwangju, South Korea). MDR1 gene-specific siRNA (siMDR1) was purchased from Bioneer (Daejeon, South Korea). The hydrophilic anticancer drug, doxorubicin hydrochloride (Dox), was purchased from Boryung Pharmaceuticals Co. Ltd. (Seoul, South Korea). Mounting solution was purchased from Dako (Glostrup, Denmark). All antibodies were purchased from Abcam (MA, USA) unless otherwise stated. All other chemicals were of reagent grade and were used as received. All animal experiments were performed according to the rules and regulations of animal care and handling procedures of Korea Advanced Institute of Science and Technology (KAIST).

Methods

Cell culture

MCF7 and MCF7/ADR cells were kindly provided by Prof. Keon Wook Kang (Jeon Nam National University, Korea). All cells were maintained at in cell incubation chamber at 37 °C in a humidified 5% CO_2 environment. Cells were cultured in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. For MCF7/ADR cells, cultures were further supplemented with 10 μg/mL insulin.

EDB and MDR1 expression in MCF7 and MCF7/ADR cells

Conventional and real-time quantitative RT-PCR

Cells were collected and RNA was isolated with RiboEx using an RNA isolation kit (Geneall, South Korea). cDNA was synthesized by reverse transcription (RT) using 1 μg of total RNA from each sample. Polymerase chain reactions (PCR) were carried out using a BioRad thermocycler (Biorad®; Hercules, *CA*, USA). The primers used for specifically detecting the EDB domain of fibronectin were 5′-AAC TCA CTG ACC TAA GCT TT-3′ (forward) and 5′-CGT TTG TTG TGT CAG TGT AG-3′ (reverse), yielding a 263-bp fragment. The primers used for detecting MDR1 were 5′-GCT CAT CGT TTG TCT ACA GTT CGT-3′ (forward and 5′-ACA ATG ACT CCA TCA TCG AAA CC-3′ (reverse), yielding a 159-bp fragment. The PCR protocol consisted of an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing), 72 °C for 2 min (extension), and a final extension at 72 °C for 7 min.[44] PCR products were analyzed by agarose gel electrophoresis. For quantitative real-time RT-PCR (RT-qPCR), 1 μg of cDNA was added to 4 μL of ultra-pure water and 5 μL of SYBR Green real-time mixture (Takara, Tokyo, Japan) and quantification

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