Acta Biomaterialia xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Rational design of multimodal therapeutic nanosystems for effective inhibition of tumor growth and metastasis

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ARTICLE INFO

Article history:
Received 16 April 2018
Received in revised form 26 June 2018
Accepted 12 July 2018
Available online xxxx

Keywords:
Gold nanorods
Stimuli-responsive
Combined therapy
Tumor growth
Cancer metastasis

ABSTRACT

Simultaneous inhibition of both tumor growth and metastasis is the key to treating metastatic cancer, yet the development of effective drug delivery systems represents a great challenge since multimodal therapeutic agents must be rationally combined to overcome the biological mechanisms underpinning tumor cell proliferation and invasion. In this context, we report a hybrid therapeutic nanoscale platform that incorporates an anti-proliferative drug, doxorubicin (DOX), and an anti-NF-κB agent, p65-shRNA, for effective treatment of metastatic breast cancer. In our design, we first conjugated DOX via an acid-labile linker onto gold nanorods that were pre-modified with the tumor targeting peptide RGD and a positively charged, disulfide cross-linked short polyethylenimines (DSPEI), and then incorporated shRNA through electrostatic complexation with DSPEI. We show that this "all in one" nanotherapeutic system (RDG/shRNA@DOX) can be effectively internalized through RGD-mediated endocytosis, followed by stimuli-responsive intracellular co-release of DOX and shRNA. Our *in vitro* experiments suggest that this multimodal system can significantly inhibit cell proliferation, angiogenesis, and invasion of metastatic MDA-MB-435 cancer cells. Systemic administration of RDG/shRNA@DOX into a metastatic mouse model led to enhanced tumor accumulation, and, most importantly, significant inhibition of in situ tumor growth and almost complete suppression of tumor metastasis. We believe this hybrid multimodal nanotherapeutic system provides important insight into the rational design of therapeutic systems for the effective treatment of metastatic carcinoma.

Statement of Significance

The key to successfully treat metastatic cancer is the simultaneous inhibition of both tumor growth and metastasis. This represents a great challenge for the design of drug delivery systems since multimodal therapeutic agents must be rationally combined to overcome the respective biological mechanisms underpinning tumor cell proliferation and invasion. Toward this end, we developed a hybrid nanomedicine platform that incorporates an anti-proliferative drug, doxorubicin (DOX), and an anti-NF-κB agent, p65-shRNA, for effective treatment of metastatic breast cancer. We showed that this multimodal system (RDG/shRNA@DOX) enhanced tumor accumulation, led to prolonged circulation, and most importantly, significant inhibition of in situ tumor growth and almost complete suppression of tumor metastasis. We believe this hybrid multimodal nanotherapeutic system provides significant insight into the rational design of therapeutic systems for the effective treatment of metastatic cancer.

Abbreviations: DOX, doxorubicin; GNRs, gold nanorods; PEI, polyethylenimine; DSPEI, disulfide cross-linked short polyethylenimines; DG, DSPEI-GNR; RDG, RGD-PEG-DSPEI-GNR; RPG, RGD-PEG-PEI-25 kDa-GNR; RDG@DOX, DOX-tethered RDG; LA, α-lipoic acid; NF-κB, nuclear factor-kappa B; VEGF, vascular endothelial growth factor; MMPs, matrix metalloproteinases; RNAi, RNA interference; EB, ethidium bromide; GSH-OEt, glutathione reduced ethyl ester; GSH, L-glutathione; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; HMVEC, human microvascular endothelial cells; HRMS, high resolution mass spectra; CLSM, confocal laser scanning microscope; H&E, hematoxylin and eosin; IHC, immunohistochemical; EPR, enhanced permeability and retention; NSET, nanosurface energy transfer; PA, photoacoustic.

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https://doi.org/10.1016/j.actbio.2018.07.025
1742-7061/

1. Introduction

Malignant tumors are the main cause of the high mortality in cancer patients due to their uncontrolled growth and metastasis [1]. The disease can be treated at its early stage but becomes incurable and fatal once metastasized to other organs at advanced stages [2,3]. For all types of cancer, metastasis contributes to up to 90% of cancer-related deaths. Despite considerable progress in the development of new therapeutic agents, novel delivery systems, and targeted therapies, only 20% of metastatic cancer patients survive more than five years [4,5]. In contrast, there are only a few treatments that are reported to inhibit both the growth and metastasis of malignant tumors [6–8]. Consequently, it is highly beneficial to explore new strategies for metastatic cancer treatment.

The distinct biological mechanisms underpinning tumor proliferation and invasion necessitates the combined use of multimodal agents in the drug delivery system. Nuclear factor-kappa B (NF-κB) protein has been reported to play a key role in the development and metastasis of human tumors [9]. Activated NF-κB can induce the expression of various genes that stimulate cell proliferation, facilitate angiogenesis, regulate apoptosis and promote metastasis [10]. Specifically, the inhibition of NF-κB can markedly down-regulate the expression of vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), thereby suppressing angiogenesis and cell invasion [11,12]. Therefore, targeting this signaling pathway using RNA interference (RNAi) has great potential to prevent tumor metastasis. However, an anti-NF-κB agent alone may not be adequate to induce complete suppression of in situ tumor growth [13]. It is thus requisite to combine the anti-NF-κB agent with a chemotherapeutic agent to achieve optimal anti-cancer effect. Doxorubicin (DOX) is one of the broad spectrum anticancer agents, which is extensively used and can efficiently kill cancer cells [14]. Additionally, the inhibition of NF-κB can down-regulate the expression of surviving and Bcl-2, further increasing the sensitivity of cancer cells to DOX [9].

Nanoparticle-based delivery systems represent a logical choice to effectively combine gene and chemotherapeutics, given their capacity for co-loading gene/anticancer drugs and co-releasing them in response to specific biological stimuli [15–19]. These co-delivery systems could avoid multiple dosing and the construction of respective delivery systems for each agent while enabling precise control of their stoichiometric ratio [20–22]. Recently, gold nanorods (GNRs) have been used as delivery platforms for both *in vivo* and *in vitro* applications due to their unique properties. The plasmon resonance of GNRs can be tuned from the visible to NIR regions that depend on the nanorod's aspect ratio [23]. With suitable aspect ratios, GNRs can absorb and scatter strongly in the NIR region, which can be used for enhanced optical imaging, biosensing and photothermal therapy [24,25]. More importantly, GNRs can be easily functionalized with polymers and drugs through the Au-thiol bond [26,27]. Fine-tuning the surface chemistry of GNRs is pivotal for improving their circulation, biocompatibility, and targeted therapeutics delivery. Due to their distinct optical properties, facile chemistry and biocompatibility, GNRs have been considered as a promising delivery platform for small molecule drug and macromolecular agents including DNA and RNA [26–29]. Consequently, we designed an intracellular-cleavable disulfide bond cross-linked short PEI (DSPEI) as GSH-responsive gene delivering vector, with the cyclic RGD peptide chosen for tumor targeting and selective purposes [30–32]. The RGD peptide was incorporated into the DSPEI via a PEG spacer. Subsequent binding of the cationic RGD-PEG-DSPEI (denoted as RD) to the GNRs surface then produced a tumor-targeting and intracellular stimuli responsive systems (RDG, Fig. S1A). Built upon this RDG platform, we established an "all in one" nanotherapeutic system (RDG/shRNA@DOX) that incorporates the anti-proliferative DOX and anti-NF-κB agent, p65-shRNA, thus allowing the combination of active targeting, optical imaging, bioresponsive gene transport and chemotherapy. As shown in Scheme 1, this multimodal therapeutic platform, RDG/shRNA@DOX, was prepared by conjugating DOX to RDG *via* an acid-labile hydrazone linker, followed by complexing cationic DSPEI with negatively charged p65shRNA. Our results reveal that the cell apoptosis, anti-metastasis and anti-angiogenesis effects on metastatic cancer could concurrently occur as a result of the combined chemotherapy and RNA silencing. We believe that this multimodal nanotherapeutic system holds great promise for effective suppression of primary tumor growth and its distant metastasis.

2. Materials and methods

2.1. Materials

DG (DSPEI-GNR), RDG (RGD-PEG-DSPEI-GNR) and RPG (RGD-PEG-PEI-25 kDa-GNR) (Please find detailed chemical structure in Fig. S1) were produced in our lab [29]. Doxorubicin hydrochloride (DOX·HCl), α-lipoic acid (LA), acetyl chloride, hydrazine hydrate, crystal violet were obtained from Aladdin Industrial Inc. Polyethylenimine branched (PEI-25 kDa), trypan blue, Hochest 33342, ethidium bromide (EB), glutathione reduced ethyl ester (GSH-OEt) and L-glutathione (GSH) were purchased from Sigma Co., Ltd. Lyso Traker Red and YOYO-1 were purchased from Invitrogen Molecular Probes. The fetal bovine serum (FBS), penicillin–streptomycin, Dulbecco's modified Eagle medium (DMEM), and 0.25% trypsin were obtained from Gibco BRL.

The pGPU6/Neo-p65shRNA that targets GCCCTATCCCTTTACGTCA and pGPU6/GFP/Neo-p65shRNA expressing pDNA (p65 shRNA and encoding for green fluorescent protein) were purchased from Gene Pharm Co. Ltd.

2.2. Cell culture

MDA-MB-435 cancer cell line, human microvascular endothelial cells (HMVEC) and mouse embryonic fibroblasts NIH3T3 were generously donated by Shanghai Institute of Materia Medica. MDA-MB-435 cells and NIH3T3 cells were grown in DMEM and HMVEC cells were maintained in MCDB131 medium, respectively. Cells were grown in 5% CO_2 incubator at 37 °C and subcultured every other day.

2.3. Synthesis of LA-Hyd-DOX

LA-Hyd-DOX was prepared through three-step reaction. Firstly, to a solution of 0.35 mL of acetyl chloride in MeOH (50 mL) was added 412.6 mg (2 mmol) of α-lipoic acid (LA). The mixture was refluxed over 8 h and the solvent was removed in vacuum giving a yellow solid residue of LA-oet. In the second step, LA-oet (330 mg, 1.5 mmol) in 30 mL of ethanol was reacted with 10 mL of 85% hydrazine monohydrate under reflux for 6 h, thereafter, the reaction misture was poured onto ice and affording a yellow solid of LA-Hyd. In the third step, LA-Hyd (220 mg, 1 mmol) and doxorubicin hydrochloride (580 mg, 1 mmol) were reacted in 3 mL of DMSO at the presence of a drop of trifluoroacetic acid at 60 °C overnight. Then, acetonitrile was added to precipitate the desired product. LA-Hyd-DOX was collected by centrifugation and dried at high vacuum. ^{1}H NMR spectra and high resolution mass spectra (HRMS) were obtained to confirm the structure and molecular weight of the compounds.

Please cite this article in press as: F. Wang et al., Rational design of multimodal therapeutic nanosystems for effective inhibition of tumor growth and metastasis, Acta Biomater. (2018), https://doi.org/10.1016/j.actbio.2018.07.025