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Fabrication of dual responsive co-delivery system based on three-armed peptides for tumor therapy

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ABSTRACT

Introducing drugs into gene delivery systems to fabricate co-delivery systems for synergy therapy has become a promising strategy for tumor therapy. In this study, a dual responsive co-delivery system RHD/p53 was fabricated to enhance the antitumor efficacy with a low dose of doxorubicin (DOX). The reducible branched cationic polypeptide (RBCP), which was cross-linked *via* the thiol groups of two three-armed cationic peptides $(CRR)_2KRRC$ and $(CHH)_2KHHC$, was designated as RH. Then, DOX was immobilized on RH *via* pH-sensitive hydrazone bonds to obtain RHD. The positively charged RHD could compress p53 plasmid to form RHD/p53 complexes. After RHD/p53 complexes accumulated in tumor sites, the ability of cell penetrating by cationic peptide $(CRR)_2KRRC$ would facilitate the cellular internalization of complexes. Then, the complexes would be trapped in endosome, and the cleavage of hydrazone bonds in the intracellular acidic endosome could lead to pH-induced release of DOX. Additionally, the ability of protonation by $(CHH)_2KHHC$ could promote the escape of complexes from endosome to cytoplasm. Due to the cleavage of disulfide bonds triggered by the high-content GSH in cytoplasm, the complexes would be degraded and released p53 for co-therapy to improve antitumor efficacy. Both *in vitro* and *in vivo* studies indicated that dual responsive co-delivery system RHD/p53 could enhance antitumor efficacy, which provides a useful strategy for co-delivery of different therapeutic agents in tumor treatment.

1. Introduction

Although many researchers have spared no effort to seek effective ways for tumor therapy, tumor is still a serious threat to human health [1]. Gene therapy, as an attractive method to cure tumors, has seen some success in the past decades. However, the application of gene therapy was still limited by many barriers, such as inefficient gene packaging [2], suboptimal cell uptake [3], endosome barrier [4], vector-induced toxicity [5] and gene unpacking [6]. These barriers were finally led to the limited transfection efficiency and unsatisfactory antitumor efficacy. To overcome these barriers, various functions were introduced to the gene delivery system, such as the ability of effective gene packaging, the capacity to reduce vector-induced toxicity, cell penetrating ability, endosomal escape capability and the capacity of stimuli-responsive gene unpacking *etc*.

Because of the water solubility, good biocompatibility and immune-compatibility, the reducible cationic polypeptides (RCPs) with high molecular weight (HMW) after connecting low molecular weight (LMW) cationic peptides through the cleavable disulfide bonds, have been proposed as promising gene delivery vehicles [7]. Compared with traditional cationic polypeptides, RCPs could degrade in cytoplasm under the stimulation of GSH to efficaciously reduce cytotoxicity and rapidly liberate gene [8]. Moreover, the various functions of peptides such as cell penetrating ability and endosomal escape capability could be easily introduced to RCPs to realize the multi-functionality, and the multi-functionality of RCPs could further improve the gene transfection efficiency. Furthermore, RCPs with highly branched three-dimensional architecture could realize multivalency for effective gene binding and conformational flexibility for tight gene compacting simultaneously, which were beneficial for cellular entry [9]. Therefore, the reducible branched cationic polypeptides (RBCPs) could be considered as potential vectors for gene delivery.

The tumor suppressor gene p53, a therapeutic gene, can not only induce cell senescence and cell apoptosis, but also inhibit the cell proliferation [10,11]. Successful transfection of p53 gene in tumor

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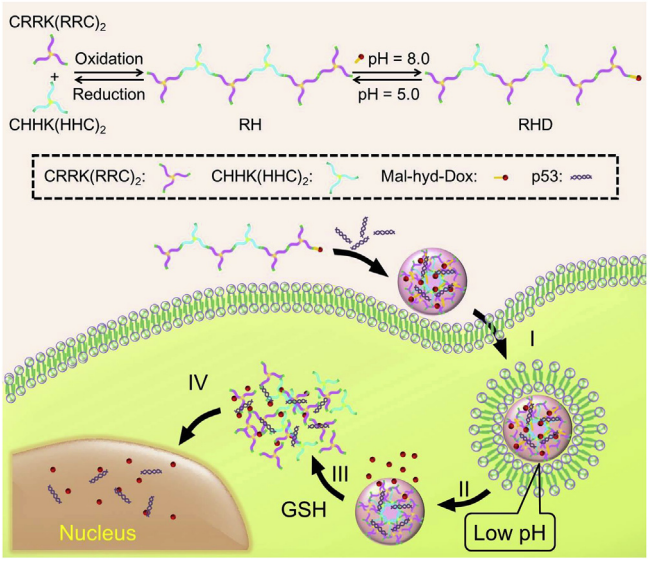
cells could activate the apoptosis program and result in 'commit suicide' of tumor cells [12]. The p53-based gene therapy has achieved great success in tumor therapy with the development of gene delivery system [13,14]. However, arising from the pathological complexity of tumor, treatment of therapeutic gene alone could not achieve the desirable therapeutic effect [15]. To further enhance the therapeutic effects of gene therapy, chemotherapy drug could be introduced to improve the antitumor activity by co-administration of gene and drug [16,17]. It is known that encapsulation is one of the most commonly strategies to load drugs in co-delivery systems [18,19]. Nevertheless, the non-covalent physical interaction may result in premature release of drugs in blood circulation [20]. Therefore, susceptible linkage between the drug and vector which could be cleaved by environment stimuli was developed to retard drug release [21,22]. With the help of the acid-labile linkages such as acetal and hydrazone bonds, the drugs could be intactly locked on the vector under the neutral physiological condition. After the co-delivery system accumulated in tumor site or trapped in endosome, the extracellular or intracellular acidic environment could lead to the cleavage of the acid-labile bonds and result in drug release [23,24].

With above considerations in mind, a reduction and pH dual responsive co-delivery system RHD/p53 was fabricated to improve the antitumor efficacy. In detail, the highly branched polypeptide RH was obtained by cross-linking two three-armed peptides of $(CRR)_2KRRC$ (a kind of arginine-riched peptides with cell penetrating ability) and $(CHH)_2KHHC$ (a kind of histidine-riched peptides with endosomal escape capability). Then, RHD was synthesized by conjugating antitumor DOX *via* pH-sensitive hydrazone bonds to highly branched polypeptide RH. Ascribed to the united multivalency and the conformational flexibility, RHD could bind p53 to form RHD/p53 complexes. As shown in Scheme 1, once RHD/p53 complexes accumulated in tumor tissue, the moiety of $(CRR)_2KRRC$ would improve the cell internalization ability of complexes [25]. After the encapsulation of complexes in endosome, the hydrazone bonds would be cleaved in the intracellular acidic environment, resulting in the liberation of DOX. Meanwhile, the protonation of $(CHH)_2KHHC$ could induce endosomal escape to some extent and allow migration of complexes to the cytoplasm. Then the complexes were degraded under the stimulation of GSH in cytoplasm to release p53 plasmid for co-therapy [26]. The characteristics of RHD/p53 complexes, such as particle size, zeta potential, drug release behavior, cell uptake, gene transfection, toxicity and antitumor efficacy were evaluated. Both *in vitro* and *in vivo* studies confirmed that the highly branched dual responsive co-delivery system RHD/p53 exhibited a good synergistic effect for tumor treatments.



Scheme 1. Transport p53 and DOX to cell by RHD/p53 complex for synergistic therapy. I: Endocytosis of RHD/p53 complex; II: Acid-mediated DOX release and endosomal escape; III: GSH-triggered complex degradation and p53 unpacking; IV: Nuclear import of p53 and DOX for synergistic therapy.

2. Materials and methods

2.1. Materials

2-Chlorotrityl chloride resin (100–200 mesh, loading: 1.20 mmol/g), *N*-fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids (Fmoc-His(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Lys(-Boc)-OH and Fmoc-Cys(Trt)-OH), 1-hydroxybenzotriazole (HOBt) and *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU) were purchased from GL Biochem. Ltd. (Shanghai, China). Trifluoroacetic acid (TFA), diisopropylethylamine (DIEA) and *N*,*N*-dimethylformamide (DMF) were obtained from Shanghai Chemical Co. (Shanghai, China) and purified before use. Dichloromethane (DCM), methanol, piperidine, phenol, thioanisole, ethanedithiol (EDT), anhydrous ether, dimethylsulfoxide (DMSO) and glutathione (GSH) were provided by Shanghai Chemical Reagent Co. (Shanghai, China). Doxorubicin hydrochloride (Dox·HCl) was obtained from Hisun Pharmaceutical Co. Ltd. (Zhejiang, China). All other solvents and reagents were analytical grade and used as received.

QIAfilter™ plasmid purification Giga Kit was acquired from Qiagen (Hilden, Germany). GelRed™ was acquired from Biotium (California, USA). Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS), phosphate buffered saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and molecular probes (Hoechst 33342, LysoTracker Red DND-99, YOYO-1 iodide) were purchased from Lonza Group Ltd. (Basel, Switzerland). Micro BCA protein assay kit was acquired from Thermo Fisher Scientific Inc. (Rockford, USA). Polyethyleneimine (PEI, Mw 25 kDa) was purchased from Sigma-Aldrich Co. LLC (Missouri, USA).

2.2. Synthesis of peptides

Three-armed peptides $(CHH)_2KHHC$ and $(CRR)_2KRRC$ were synthesized on 2-chlorotrityl chloride resin using standard Fmoc-based solid-phase synthesis technique [27,28]. Peptides were cleaved from resin with 15 ml cleavage cocktail for 100 min. The collected solutions were concentrated and then precipitated with ether. The precipitates were dried under vacuum. The molecular weights of $(CHH)_2KHHC$ and $(CRR)_2KRRC$ were assayed by electrospray ionization mass spectrometry (ESI-MS). $(CHH)_2KHHC$: calculated 1277.5, found 1278.3 $(M+H)^+$ and 639.8 $(M+2H)^{2+}$ (Fig. S1, see supplementary data). $(CRR)_2KRRC$: calculated 1391.7, found 696.0 $(M+2H)^{2+}$ (Fig. S2). The purity of peptides was evaluated by analytical high performance liquid chromatography (HPLC), and the purity of both peptides was proved to be above 90%.

2.3. Synthesis of (6-maleimidocaproyl) hydrazone of doxorubicin (Mal-hyd-Dox)

Mal-hyd-Dox was synthesized base on the literature reported by Li et al. [29]. Briefly, 4.5 g of DOX·HCl and 8.0 g of 6-maleimidocapro-hydrazide trifluoroacetate salt were dissolved in