Acta Biomaterialia 48 (2017) 131–143

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Engineering of cell microenvironment-responsive polypeptide nanovehicle co-encapsulating a synergistic combination of small molecules for effective chemotherapy in solid tumors

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ARTICLE INFO

Article history:
Received 3 August 2016
Received in revised form 21 October 2016
Accepted 25 October 2016
Available online 26 October 2016

Keywords:
Polypeptide
Doxorubicin
Quercetin
Nanovehicle
Combination

ABSTRACT

In this study, we report a facile method to construct a bioactive (poly(phenylalanine)-*b*-poly(L-histidine)-*b*-poly(ethylene glycol) polypeptide nanoconstruct to co-load doxorubicin (DOX) and quercetin (QUR) (DQ-NV). The smart pH-sensitive nanovehicle was fabricated with precisely tailored drug-to-carrier ratio that resulted in accelerated, sequential drug release. As a result of ratiometric loading, QUR could significantly enhance the cytotoxic potential of DOX, induced marked cell apoptosis; change cell cycle patterns, inhibit the migratory capacity of sensitive and resistant cancer cells. In particular, pro-oxidant QUR from DQ-NV remarkably reduced the GSH/GSSG ratio, indicating high oxidative stress and damage to cellular components. DQ-NV induced tumor shrinkage more effectively than the single drugs in mice carrying subcutaneous SCC-7 xenografts. DQ-NV consistently induced high expression of caspase-3 and PARP and low expression of Ki67 and CD31 immunomarkers. In summary, we demonstrate the development of a robust polypeptide-based intracellular nanovehicle for synergistic delivery of DOX/QUR in cancer chemotherapy.

Statement of Significance

In this study, we report a facile method to construct bioactive and biodegradable polypeptide nanovehicles as an advanced platform technology for application in cancer therapy. We designed a robust (poly(phenylalanine)-*b*-poly(L-histidine)-*b*-poly(ethylene glycol) nanoconstruct to co-load doxorubicin (DOX) and quercetin (QUR) (DQ-NV). The conformational changes of the histidine block at tumor pH resulted in accelerated, sequential drug release. QUR could significantly enhance the cytotoxic potential of DOX, induce marked cell apoptosis, change cell cycle patterns, and inhibit the migratory capacity of sensitive and resistant cancer cells. DQ-NV induced tumor shrinkage more effectively than the single drugs and the 2-drug cocktail in tumor xenografts. In summary, we demonstrate the development of an intracellular nanovehicle for synergistic delivery of DOX/QUR in cancer chemotherapy.

1. Introduction

Despite the tremendous increase in knowledge pertaining to tumor biology and pathogenesis, the success rate of current chemotherapies is often limited due to multiple factors including poor efficacy, drug-related side effects, heterogeneity of cancer cells, and drug resistance [1]. In this perspective, combination chemotherapy is a promising strategy to effectively treat cancer

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http://dx.doi.org/10.1016/j.actbio.2016.10.034
1742-7061/

in the clinic owing to its ability to enhance the therapeutic efficacy and minimize the adverse effects [2]. The use of combination drug therapy not only delays/suppresses cancer adaptation, mutation, and progression, but also decreases the dose of individual drugs required, reducing side effects [3,4].

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most effective anticancer agents used for treatment of a wide variety of solid and hematologic malignancies [5–7]. DOX-based therapy, however, is associated with severe side effects on healthy tissues including heart, liver, and kidney, which limits its therapeutic utility [8,9]. It is worth mentioning that the cellular pathways of DOX-mediated cytotoxicity differ between healthy and cancer cells. Cytotoxicity in cancer cells primarily occurs through intercalation of DNA and apoptosis, whereas, hepatotoxicity and cardiotoxicity result from the generation of free radicals leading to oxidative damage [10,11]. This prompted us to develop a new tumor-specific DOX-based combinatorial regimen that allows reducing the adverse effect, while potentiating the therapeutic efficacy of DOX and protecting the normal tissues (by countering oxidative damage). Quercetin (QUR), a bioflavonoid, has recently received much attention as a potential chemopreventive and pro-apoptotic antitumor agent, which acts exclusively on malignant cells [12–14]. Moreover, selective mitochondrial accumulation of QUR would promote apoptosis by cutting off the cellular "power supply" [15,16]. Few biological studies have demonstrated that the combination of QUR with DOX can overcome severe DOX-related toxicity, enhancing its therapeutic effect [17,18]. The nanoformulations, which allow encapsulation of multiple drugs while maintaining tunable and controlled synergistic therapeutic ratio are highly desired.

Advancements in nanomedicine have opened up unprecedented opportunities in combination drug delivery [19]. Particularly, self-assembled block copolymer-based nanocarriers have shown to possess great potential in cancer targeting, and offer numerous advantages including small size, long circulation time, systemic stability, and ability to surpass the reticuloendothelial system (RES), making them ideal carrier systems for anticancer drug delivery [20–22]. Recently, we prepared poly(acrylic acid)-based polymeric micelles and lipid-coated poly(aspartic acid)-based nanoparticles for dual-drug delivery to cancer tissues [23,24].

On the basis of encouraging results, we contemplated the design of a robust nanoconstruct (poly(phenylalanine)-*b*-poly(L-histidine)-*b*-poly(ethylene glycol) (pPhe-pHis-PEG), loaded with DOX and QUR, considering their chemical structure, the optimal dose ratio, and the drug-loading capacity of the carrier (Fig. 1). The construct was designed to allow endolysosomal escape for intracellular delivery of the drugs. The pPhe block provides a stable hydrophobic core, while pHis is a pH-responsive polypeptide with a lone electron pair on the unsaturated nitrogen, which enables the protonation-deprotonation process in the intracellular environment, promoting endosomal escape [24,25]. In this study, we defined a ratiometric approach in which DOX is released faster than QUR, which would enhance the therapeutic efficacy of DOX, while circumventing oxidative damage to normal tissues. The combined cytotoxic effects of DOX and QUR were tested in MCF-7, SCC-7, and MDA-MB-231 resistant cancer cell lines. The synergistic effect of DOX/QUR co-loaded polypeptide nanovehicle (DQ-NV) was characterized at *in vitro* level and investigated in SCC-7 xenograft tumors and immunohistochemical analysis of caspase-3, PARP, Ki-67, and CD-31. We hypothesized that polypeptide-based nanovehicle would enhance the therapeutic effect of combined DOX and QUR in solid tumors. To the best of our knowledge, this is first report of the application of a polypeptide nanovehicle as a carrier to enhance the therapeutic efficiency of DOX in the presence of a mitochondrial targeting drug, QUR.

2. Materials and methods

2.1. Synthesis of pPhe-b-pHis-b-PEG block copolymer

$mPEG_{113}$-NH_2 (0.06 mM) was dissolved in 20 mL of anhydrous dimethylformamide (DMF) and stirred at 40 °C under constant nitrogen atmosphere. Bz-His-NCA (1.875 mM) was separately dissolved in DMF (5 mL) and added dropwise to the mPEG organic solution and stirred for 24–36 h. The Phe-NCA was then added in a dropwise manner and the mixture was stirred for an additional 24 h at 40 °C. The final product (pPhe-*b*-pHis-*b*-PEG) was precipitated by the addition of excess diethyl ether (2–3 times) and dried in vacuum. The benzyl group of His was removed in the presence of metallic sodium. Proton nuclear magnetic resonance (^{1}H NMR) spectra and GPC experiment was performed.

2.2. Preparation of DOX/QUR-loaded polypeptide nanovehicle

The DOX/QUR-loaded polypeptide-based nanovehicle (DQ-NV) was prepared by dialysis. First, DOX hydrochloride was converted to DOX base by the addition of trimethylamine (TEA). DOX and QUR were dissolved in DMSO and stirred for 10 min. The pPhe-*b*-pHis-*b*-PEG was dissolved in DMSO and subsequently, the drug mixture was added and stirred for 4 h. The mixture was transferred to a pre-swollen dialysis membrane (MWCO = 3500 Da) and dialyzed against distilled water for 24 h. The DQ-NV was collected and freeze-dried for further analysis.

2.3. Physicochemical analysis

The diameter and zeta potential of micelles were determined using the dynamic light scattering method with a Zetasizer Nano ZS (Malvern, UK) at 25 °C. The morphology of the micelles was observed by TEM (CM 200 UT; Philips, Andover, MA, USA) with an accelerating voltage of 100 kV. For AFM, a Multimode NanoScope IV system (Veeco, Santa Barbara, CA, USA) was used in tapping mode.

2.4. In vitro cytotoxicity assay and synergism analysis

The *in vitro* cytotoxic potential of individual formulations was assessed by MTT assay. The cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Approximately 1×10^4 cells were seeded in a 96-well plate and incubated for 24 h. Then, the medium was replaced with fresh medium containing the indicated formulations (free DOX, free QUR, free DOX/QUR, DOX-NV, and DQ-NV) at different concentrations, and the cells were incubated for another 24 h. After incubation, the cells were washed twice with PBS, 20 μL of MTT solution (5 mg/mL) was added, and the cells were incubated for 3 h at 37 °C. The absorbance at 570 nm was measured using a microplate reader (Multiskan EX, Thermo Scientific, USA). The IC50 value (the concentration that inhibits cell growth by 50%) was calculated using SPSS software 17.0 (Chicago, IL, USA). The synergism was expressed in terms of the CI using the CalcuSyn software (Biosoft, Cambridge, UK).

2.5. Western blotting

Equal amounts of protein were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel and immediately transferred to a nitrocellulose membrane (Bio-Rad) using a TE22 Transfer Tank (Hoefer Inc., MA, USA) at 210 kV for 1.5 h. The transfer buffer contained 25 mM Tris HCl, 200 mM glycine, and 10% methanol. The membrane was blocked for 1 h in either 5% milk or 5% BSA on a horizontal shaker. The membranes were incubated overnight with primary