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Oral delivery of shRNA based on amino acid modified chitosan for improved antitumor efficacy

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ABSTRACT

In this investigation, chitosan–histidine–cysteine (CHC) was engineered for oral delivery of Survivin short hairpin RNA (shRNA)-expressing plasmid DNA (shSur-pDNA) to promote hepatoma regression through integrating the advantages of histidine and cysteine to conquer serial cellular and systemic barriers. CHC could effectively encapsulate shSur-pDNA to form compact nanocomplexes (NC) at adequate weight ratios. Sequential modification with histidine and cysteine conferred CHC NC with the beneficial attributes for shRNA delivery including improved stability, facilitated internalization, promoted endosomal escape, increased nuclear localization, and GSH-responsive release, which contributed to their superior performance in terms of apoptosis promotion, proliferation inhibition, and Survivin down-regulation of tumor cells. More importantly, in hepatoma-bearing mice, orally delivered CHC NC overweighed chitosan counterparts with respect to suppressed Survivin expression, retarded tumor growth, and prolonged surviving time, owing to their above-mentioned merits in combination with enhanced intestinal permeation. Especially, rapid intracellular release of CHC NC with lower molecular weight of 30 kDa (CHC30 NC) might be responsible for the most satisfactory antitumor efficacy with tumor inhibition ratio (TIR) of 92.5%, which rendered CHC30 NC a promising vehicle for oral delivery of shRNA. This investigation would shed light on the deliberate design of oral shRNA delivery vehicles to mediate effective antitumor efficacy.

1. Introduction

Short hairpin RNA (shRNA) can be introduced into target cells to mediate transcription of small interfering RNA (siRNA) through a plasmid DNA (pDNA) vector, which offers persistent down-regulation of the aberrantly overexpressing genes, *i.e.* RNA interference (RNAi) [1,2]. RNAi has been demonstrated as a promising modality for the management of tumor, nevertheless, its satisfactory therapeutic efficacy is handicapped by the lack of efficient and safe delivery vehicles to surmount extracellular and intracellular obstacles including nuclease degradation, cell entry, endosomal entrapment, and nuclear localization [3,4]. Oral administration is always regarded as the holy grail for systemic gene delivery due to patient compliance and convenience [5,6]. However, it also proposed more challenges for delivery vehicles considering the harsh environment in the gastrointestinal (GI) tract and poor intestinal permeation. Consequently, the realization of orally delivered shRNA-based RNAi required deliberate design of safe delivery vectors to facilitate overcoming aforementioned cellular and systemic obstructions [7]. To our knowledge, few investigations concerning oral delivery of shRNA for cancer therapy have been reported.

Following oral delivery of shRNA, whether to maintain structural stability, dissociate from the vehicles in time, and effectively

Abbreviations: ANOVA, analysis of variance; CH, chitosan–histidine; CHC30, chitosan–histidine–cysteine with molecular weight of 30 kDa; CHC30 NC, CHC30/shSur-pDNA nanocomplexes; CHC100, chitosan–histidine–cysteine with molecular weight of 100 kDa; CHC100 NC, CHC100/shSur-pDNA nanocomplexes; CLSM, confocal laser scanning microscopy; CS30, chitosan with molecular weight of 30 kDa; CS30 NC, CS30/shSur-pDNA nanocomplexes; CS100, chitosan with molecular weight of 100 kDa; CS100 NC, CS100/shSur-pDNA nanocomplexes; DMEM, Dulbecco's modified Eagle's medium; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; EDTA, ethylene diamine tetraacetic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GI tract, gastrointestinal tract; GSH, glutathione; MTT, methyl tetrazolium; NC, nanocomplexes; NHS, *N*-hydroxysuccinimide; P_{app}, apparent permeability coefficient; PBS, phosphate buffered solution; pDNA, plasmid DNA; RNAi, RNA interference; SD, standard deviation; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; shRNA, short hairpin RNA; shSur-pDNA, Survivin shRNA-generating pDNA; TIR, tumor inhibition ratio.

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permeate across intestinal epithelia, were closely related to successful RNAi. In this respect, thiolated polymers displayed advantages through spontaneously interacting with shRNA-generating pDNA *via* electrostatic forces to form nanocomplexes (NC). The introduction of thiol groups could strengthen the extracellular stability, promote the intracellular dissociation, and facilitate the intestinal permeation of NC [4,8,9]. Additionally, endo-lysosomal entrapment and degradation before nuclear localization constituted as vital obstacles for effective shRNA-induced gene knockdown [10]. Molecules containing buffering domains could cause osmatic imbalance in the endosomal lumen, thereby facilitating the endosomal escape *via* proton-sponge mechanism [11,12].

Based on the above-mentioned understandings, in this investigation, we sequentially conjugated naturally occurring cysteine bearing thiol groups and histidine with pH-sensitivity on the backbone of chitosan (CS), a reputed biocompatible and biodegradable polysaccharide, to integrate their distinct predominance in conquering delivery barriers and correspondingly mediate improved therapeutic efficacy for hepatoma *via* shRNA-based silencing expression of Survivin after oral administration. The obtained CS derivatives with different molecular weight were spontaneously interacted with shRNA-generating pDNA *via* electrostatic forces to form NC. The virtues of NC with amino acid modification were evaluated with regard to structural stability in simulated environment of GI tract, cellular uptake, endosomal escape, release behavior, pDNA permeation across intestine *ex vivo*, apoptosis induction of tumor cells, proliferation inhibition of tumor cells, *in vitro* and *in vivo* Survivin expression knockdown, and the *in vivo* antitumor efficacy.

2. Materials and methods

2.1. Materials and animals

Chitosan (deacetylation degree of 85% and molecular weight of 30 and 100 kDa) was obtained from Golden-shell Biochemical Co., Ltd. (Zhejiang, China). L-cysteine, Boc-histidine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), fluorescein isothiocyanate (FITC), and Hoechst 33258 were purchased from Sigma (St. Louis, MO, USA). DNase I was obtained from Worthington (Lakewood, NJ, USA). pGL3-control vector without shRNA cassette and shSur-pDNA (target sequence of GAATTAACCCTTGGTGAAT) were amplified in *Escherichia coli* and purified using EndoFree Maxi Plasmid Kit (TianGen, Beijing, China).

Human liver cancer cell line QGY7703 was provided by Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, NY, USA) containing 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Hepatoma H-22 ascites were supplied by Shanghai Institute of Materia Medica (Shanghai, China).

Kunming mice (6 weeks, 18–22 g) were obtained from Slaccas Experimental Animals Co., Ltd. (Shanghai, China) and the investigation protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Fudan University, China.

2.2. Synthesis and characterization of chitosan–histidine–cysteine (CHC)

CHC conjugates were synthesized by sequential modification of chitosan with histidine and cysteine. Briefly, carboxyl groups of Boc-histidine were activated by EDC/NHS in HCl solution (pH 4.8) for 2 h, and then reacted with the amine groups of CS under stirring for 24 h. Chitosan–histidine (CH) was obtained through the detachment of Boc group with HCl, purification by dialysis, and lyophilization sequentially. Cysteine was then conjugated onto CH to achieve CHC according to the methods previously reported by Yin et al. [13]. CHC was purified by dialysis in HCl (pH 4.8) for three days and then subjected to lyophilization. CS and CHC polymers were characterized by H NMR in 1% DCl and D_2O, respectively, on an AVANCE DMX 500 NMR spectrometer (Bruker, Germany). The grafting ratios of histidine and cysteine were determined by ninhydrin test and Ellman's method, respectively, as previously described [13,14]. CS with molecular weight of 30 and 100 kDa were as denoted as CS30 and CS100, respectively. Correspondingly, CHC synthesized from CS30 and CS100 were denoted as CHC30 and CHC100, respectively.

2.3. Buffering capacity of polymers

The buffering capacities of polymers were assessed by acid–base titration method [15]. In brief, polymers were dissolved in water at the concentration of 0.2 mg/mL and adjusted to around pH 4.0 with HCl solution (0.1 M). NaOH solution (0.1 M) was used to titrate polymer solutions and pH values were recorded by a pH meter.

2.4. Preparation and characterization of NC

Certain volume of Survivin shRNA-generating pDNA (shSur-pDNA) aqueous solution (0.2 mg/mL) was mixed with various volumes of polymer aqueous solution (2 mg/mL) under vortex to achieve NC with different weight ratios. CS30/shSur-pDNA NC (CS30 NC), CS100/shSur-pDNA NC (CS100 NC), CHC30/shSur-pDNA NC (CHC30 NC), and CHC100/shSur-pDNA NC (CHC100 NC) were all incubated at 37 °C for 30 min before the application. The determination of particle sizes and Zeta potentials was performed on a Zetasizer Nano (Malvern, Worcestershire, UK). To evaluate the condensation efficiencies of polymers towards shSur-pDNA at various weight ratios, gel retardation assay was conducted on 1% agarose gel at 130 V for 30 min.

2.5. NC stability

NC with polymer/shSur-pDNA weight ratio of 20 (*i.e.* N/P ratio of 40 and 34 for CS NC and CHC NC, respectively) were incubated with DNase I (1 mg/mL) at 37 °C for 30 min before inactivating the enzyme by incubating at 80 °C for 5 min and the addition of 2.5 μL of ethylene diamine tetraacetic acid (EDTA) (200 mM). shSur-pDNA was then dissociated by heparin (5 mg/mL) from NC and its bands were observed by electrophoresis at 130 V for 30 min.

The stabilities of NC against dilution, ion challenges, and pH changes were evaluated in terms of particle sizes and Zeta potentials. Briefly, NC with polymer/shSur-pDNA weight ratio of 20 (*i.e.* N/P ratio of 40 and 34 for CS NC and CHC NC, respectively) were diluted with FBS-free DMEM by 50 folds and phosphate buffered solution (PBS, 0.2 M, pH 7.4) by 250 folds, respectively, to simulate *in vitro* gene silencing environment and *in vivo* physiological conditions. As for the influence of pH alteration, NC suspension was modulated to pH 1.2, 6.5, and 7.4 by HCl solution (1 M) or NaOH solution (1 M) before the measurement of particle sizes and Zeta potentials.

2.6. shSur-pDNA permeation across the ileum ex vivo

Fluorescein isothiocyanate (FITC)-shSur-pDNA was synthesized as previously described [16]. The experiment of shSur-pDNA permeation across the rat ileum *ex vivo* was performed according to the method described in a previous study [17]. Briefly, following anesthesia and sacrifice of rats, the ileum tissues were collected and