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Dual-functional bio-derived nanoparticulates for apoptotic antitumor therapy

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ABSTRACT

The application of bio-derived nanoparticulates has gained a remarkable degree of interest as a promising sustained-release, site-targeted and completely biodegradable delivery system for chemotherapeutics. We hereby introduce a dual-functionalized biomimetic nanovector, cell-penetrating peptide (CPP)-anchored recombinant high density lipoproteins (cp-rHDL), which affords high payload and improved targeting of gambogic acid (GA), a therapeutic agent for apoptotic antitumor therapy. GA-loaded cp-rHDL nanoparticles (cp-rHDL/GA) consisted of hydrophobic core modulating GA, apolipoprotein A-I (apo A-I) for attractive integrating and tumor-homing, and lipophilic anchored R6H4 (RRRRRRHHHH, a pH-responsive CPP) offering a pH-controlled penetrating potential. Upon stepwise incubation with apo A-I and R6H4, cp-rHDL/GA presented several merits, including desirable physicochemical properties, superior biostability, and favorable buffering capacity resulting in proton sponge effect. Synergistic intracellular mechanism for scavenger receptor class B type I (SR-BI)-mediated direct transmembrane delivery, and pH-responsive R6H4 associated endocytotic pathway with rapid endolysosomal escape was also observed. This tailored cp-rHDL/GA displayed remarkable cytotoxicity and apoptotic effect via triggering p53 pathway, and provided approximately 5-fold increase in IC_{50} compared to free GA. Moreover, this rational biomimetic therapeutic strategy attained superior tumor accumulation and significant inhibition of tumor growth in HepG2 xenograft tumor animal models without measurable adverse effect. Results of this study demonstrated that bio-derived cp-rHDL/GA presents pH-responsive penetrating potential and efficient cellular internalization. This dual-functionalization model will open an avenue for exploration of multi-functional bio-derived drug delivery, thereby rendering potential broad applications in apoptotic anticancer therapy.

1. Introduction

Natural particulates—ranging from mammalian cells to endogenous proteins are emerging as a class of therapeutic carriers for diseases as they are highly optimized for specific functions *in vivo* and possess several desirable features in drug delivery. Recent clinical results suggest that natural particulates-derived therapeutics can result in enhanced efficacy. Owing to properties such as targeted accumulation in tumors and attractive cellular internalization, it can simultaneously help to reduce side effects [1]. Despite the fact that most of the drug delivery vehicles presently in use for clinical applications are chemically synthesized carrier forms and actively developed due to their small size and prolonged circulation half-life [2,3], success of these synthetic nanocarriers relies largely on the selection of appropriate design parameters to address the physicochemical limitations of free drugs (solubility and stability). Overcoming biological hurdles (immune clearance, cell entry and off-target deposition) in reaching the target is also of significance [4]. With synthetic carriers struggling to meet clinical expectations, natural particulates-derived drug delivery carriers such as biomimetic vehicles are therefore worth exploring. Researchers have started to exploit more potential natural particulates for multiple applications. By mimicking endogenous shape and structure of native high density lipoprotein (HDL), a

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successfully developed biomimetic nanovector reconstituted HDL (rHDL), can evade the reticuloendothelial cells in the body's defenses, remain in circulation for an extended period of time, and bind to cancer cells expressing high levels of receptors known as scavenger receptor class B type I (SR-BI) [5,6]. Inasmuch as rHDL mediates therapeutic payloads delivery to cancer cells, it is likely to become a promising and attractive natural cell-specific strategy for cancer treatment.

Stimulus-responsive carriers provide additional opportunities to increase the local concentration of the drugs within solid tumors by triggering changes in certain material properties [7]. The stimulus may be intrinsic to the tumor environment, such as its low extracellular pH [8,9], upregulated protease expression [10] or the presence of reactive oxygen species [11]. Considering these intrinsic triggers, cell-penetrating peptides (CPPs) with cationic or amphipathic in nature, facilitates efficient translocation across cell membrane [12,13] leading to increased tumor-localized cellular uptake. However, application of CPPs in most cases is hampered by extensive penetration *in vivo* with improper selectivity and requires suitable strategies to mask its permeability before reaching target sites. To address this dilemma posed by conventional CPPs, pH-induced CPPs have been developed to unveil or unmask CPPs only within tumor tissue as a means to endow specificity of CPPs [14]. These 'protected' or 'masked' CPPs with pH triggered exposure mechanism, allow for controlled effect at tumor microenvironment. Owing to pH gradient between tumor milieu (pH 6.4) and physiological environment (pH 7.4), CPPs preferentially enhance intracellular uptake of drugs by tumor cells. Among them, R6H4 (RRRRRRHHHH) has been reported as a tumor pH-sensitive CPP, possessing optimal penetrating potential at pH 6.4 when a series of synthetic CPPs which are known to be rich in arginines and histidines were screened. As investigated, arginine provides R6H4 with a strong capability of cell penetration whereas histidine offers R6H4 with pH-responsive cellular uptake and a potential proton sponge effect on endo-lysosomal escape. The balance between arginine and histidine in R6H4 can be utilized as an important step in developing tumor-specific stimulus-sensitive drug delivery systems for improved anti-cancer therapy [15].

In an overlap of these two areas, bioinspired-targeted and stimulus-responsive biomimetics provide an interesting example of this class of materials incorporating both natural particulates and cell-interactive peptides. Up to date, this class of dual-functional biomimetics have experienced attractive applications in various fields such as biological and membrane science as well as intelligent stimulus-responsive macromolecule and controlled drug delivery [16,17]. Depending on their natural biological safety, favorable diseased site accumulation and accurate dynamic response to specific triggers, stimulus-responsive biomimetic materials have been leveraged in cancer treatments to enhance intratumoral drug distribution, promote cellular internalization, and increase tumor drug concentration. In addition, this combination has been employed to help minimize harmful effects, thereby providing more effective and better-tolerated cancer therapies. Studies have been reported for successful preparation of CPP-fused HDL particles by genetic engineering method [18,19]. However, biosynthetic strategy of this HDL species is still limited with regard to controllability and gene mutation. In this study, we sought to design a pH-responsive CPP-functionalized reconstituted high density lipoproteins (cp-rHDL) via a stepwise incubation method, which can maximize the delivery of hydrophobic drugs into cancer cells by means of active cell targeting and stimulus-responsive penetration. This will contribute to the development of an applicable approach for further manufacturing and control assay *in vitro*. As to the model drug, gambogic acid (GA) has been reported to activate impaired apoptosis pathways in cancerous cells (*e.g.*, lung carcinoma, and hepatoma) through a complex mechanism involving the participation of multi-molecular targets [20,21]. GA is shown to suppress angiogenesis by acting on human umbilical vein endothelial cell receptor [22], following triggered mitochondrial dependent- and independent-apoptosis pathways by combination with transferrin as its receptor [20,23]. It has also been evidenced as a potent p53 inducer to trigger cancer cell apoptosis and inhibit the anti-apoptotic Bcl-2 family proteins [24,25].

Herein, dual-functional biomimetic cp-rHDL was introduced by combining HDL-derived natural tumor targeting for SR-BI-mediated direct transmembrane delivery, and pH-sensitive CPP-triggered stimulus-responsive property to the mildly acidic environment of tumors. The research project is aimed at controlling permeability and rapid endo-lysosomal escape, while providing a safe and effective carrier platform for GA-induced apoptotic antitumor therapy (Scheme 1). As shown, GA-loaded lipid nanoparticle (LNP/GA) was first prepared as a lipid core by nano-precipitation/solvent diffusion method and apo A-I and stearyl-R6H4 (STR-R6H4) were inserted onto the surface of the LNP successively to formulate GA-encapsulated rHDL (rHDL/GA) and CPP-anchored rHDL/GA (cp-rHDL/GA). After intravenous injection (*i.v.*), cp-rHDL is expected to exhibit a considerable accumulation at tumor site due to retention (EPR) effect; further improved by the affinity of rHDL with its binding receptors and pH-responsive permeability, subsequently results in intracellular delivery and intracellular GA release via designed synergistic mechanism. For proof of our idea, boistability, buffering capacity and release profile of cp-rHDL/GA were thoroughly investigated. In addition, the cellular uptake and mechanism of coumarin 6-loaded cp-rHDL (cp-rHDL/C6), *in vitro* cytotoxicity and apoptosis of cp-rHDL/GA were estimated on HepG2 cells. The *in vivo* biodistribution and antitumor activity were examined with HepG2 xenograft model in nude mice as well.

2. Materials and methods

2.1. Materials

GA was extracted and isolated from the resin of gamboges with a purity of 99.7%. All other reagents were of analytical grade; HPLC-grade was used without further purification. Apolipoprotein A-I (apo A-I) was isolated and highly purified from albumin by-product in our laboratory according to an established protocol [26]. Soybean phospholipids (PC, purity 90%) were obtained from Evonik Degussa China Co., Ltd. (Shanghai, China). Cholesterol (Chol) and cholesteryl esters (CE) were purchased from Huixing Biochemical Reagent Co., Ltd. (Shanghai, China). Glycerol trioleate was obtained from Tokyo Kasei Kogyo Co. Ltd. (Japan). Stearyl R6H4 (STR-R6H4) was purchased from the GL Biochem Co., Ltd. (Shanghai, China). Coumarin 6 (C6) was purchased from Sigma–Aldrich Co. (Shanghai, China). Hoechst 33258 was purchased from Sigma–Aldrich (St. Louis, USA). LysoTracker Red was purchased from Invitrogen Corporation (Carlsbad, USA). Primary antibodies and IRDye™ 800-conjugated second antibody were obtained from Sunshine Biotechnology Co., Ltd. (Nanjing, China).

2.2. Formulation and characterization of cp-rHDL/GA

The preparation process consisted of the construction of LNP/GA and subsequent stepwise incubation with apo A-I and Stearyl pH-responsive CPP (STR-R6H4) for cp-rHDL/GA formation. Firstly, LNP/GA was prepared by nano-precipitation/solvent evaporation method [27]. Soybean phospholipids, glycerol trioleate, cholesterol, cholesteryl esters and gambogic acid were dissolved in organic solvent (acetone: absolute ethanol = 4: 1, v/v) with an optimized mass ratio of 50:15:5:1:5. The solution was sonicated (DL-720,