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Sigma receptor-mediated targeted delivery of anti-angiogenic multifunctional nanodrugs for combination tumor therapy

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ABSTRACT

The potential of low molecular weight heparin (LMWH) in anti-angiogenic therapy has been tempered by poor *in vivo* delivery to the tumor cell and potentially harmful side effects, such as the risk of bleeding due to heparin's anticoagulant activity. In order to overcome these limitations and further improve the therapeutic effect of LMWH, we designed a novel combination nanosystem of LMWH and ursolic acid (UA), which is also an angiogenesis inhibitor for tumor therapy. In this system, an amphiphilic LMWH-UA (LHU) conjugate was synthesized and self-assembled into core/shell nanodrugs with combined anti-angiogenic activity and significantly reduced anticoagulant activity. Furthermore, DSPE-PEG-AA-modified LHU nanodrugs (A-LHU) were developed to facilitate the delivery of nanodrugs to the tumor. The anti-angiogenic activity of A-LHU was investigated both *in vitro* and *in vivo*. It was found that A-LHU significantly inhibited the tubular formation of human umbilical vein endothelial cells (HUVECs) ($p < 0.01$) and the angiogenesis induced by basic fibroblast growth factor (bFGF) in a Matrigel plug assay ($p < 0.001$). More importantly, A-LHU displayed significant inhibition on the tumor growth in B16F10-bearing mice *in vivo*. The level of CD31 and p-VEGFR-2 expression has demonstrated that the excellent efficacy of antitumor was associated with a decrease in angiogenesis. In conclusion, A-LHU nanodrugs are a promising multifunctional antitumor drug delivery system.

1. Introduction

It is widely accepted that tumor growth and metastasis depend on angiogenesis, as these neovasculatures must provide the nutrition necessary for the dividing tumor cells [1,2]. Angiogenesis plays a fundamental role in physiological and pathological conditions such as cancer and chronic inflammation, which is regulated by a number of growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelet derived growth factor (PDGF). These growth factors bind to heparan sulfate proteoglycans (HSPGs) present endothelial cells (ECs), as well as in the extracellular matrix (ECM), and thereby trigger the proliferation and migration of ECs [3–7]. In many human tumors, the molecule heparanase is more highly expressed, and can partially digest these HSPGs, producing fragments which seem even more effective than the native heparin sulfate in potentiating the activity of bound growth factors. As compared to traditional cancer therapy, anti-angiogenic therapy can inhibit tumor growth by targeting blood vessels more effectively and stably by overcoming impediments such as drug resistance and inadequate drug delivery [8]. Therefore, antiangiogenesis has represented a potential target for cancer therapy.

Low molecular weight heparin (LMWH) is a non-cytotoxic, biodegradable, and water-soluble natural glycosaminoglycan. It has been reported that LMWH can reduce angiogenesis induced by bFGF and VEGF when administered systemically by competitively inhibiting the binding of growth factors to their endothelial receptors [3,9–11]. Collen et al. demonstrated that LMWH inhibited both bFGF and VEGF-induced proliferation of human microvascular endothelial cells (HUVECs) [12]. Moreover, heparin and some chemically modified heparins can inhibit tumor cell heparanase activity, which correlates with a lower metastatic potential. The effects of heparins on the outgrowth of primary tumors, angiogenesis, and metastasis have been studied in several animal models. Nathan et al. have found that heparin significantly inhibited the growth of transplanted rodent Murphy–Sturm lymphosarcoma by the 15th day of therapy [13]. However, a long-term administration of a high concentration of heparin was necessary to produce an effect on the primary tumor growth, which would unfortunately put patients at risk of hemorrhage [14]. Various chemically modified heparins have therefore been synthesized to minimize its anticoagulant activity, such as periodate-oxidized, N-acetylated, N-desulfated, O-desulfated or carboxyl-reduced heparin [15]. It has been described that periodate-treated, non-anticoagulant heparin carrying polystyrene exhibited

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stronger anti-angiogenic properties and much lower anti-coagulant activity than heparin itself [16]. In addition, LMWH chemically modified with hydrophobic segments also exhibited this reduced anticoagulant activity and enhanced tumor growth inhibition *via* anti-angiogenesis [15,17,18]. Park et al. have also developed the chemical conjugation LMWH-deoxycholic acid (LHD), which exhibits no anticoagulant activity and high inhibition of tumor growth *via* anti-angiogenic effect [15].

Ursolic acid (UA) is a pentacyclic triterpenoid derived from berries, leaves, flowers, and fruits of medicinal plants [19]. It has been described that hydrophobic UA possesses pleiotropic biological effects such as antibacterial, hepatoprotective, antitumor, anti-inflammatory and anti-angiogenic activities [20–23]. The antitumor effect of UA is related to its ability to influence the activity of several enzymes, which modulate the process of tumor growth. For example, the MAPK/ERK and PI3K/AKT/mTOR signaling cascades play critical roles in the transmission of signal from growth factor receptors to regulate gene expression, which are responsible for anti-apoptotic and drug resistance effect in cells [24]. UA has the ability to suppress communication through these routes to inhibit the tumor growth. In addition, forkhead box (FOX) M1 is a kind of transcription factors that is able to cross-talk with other molecules in cancer development such as NF-κB, COX-2, ERK and MMPs [25,26]. The study has reported that UA inhibited the Forkhead box M1 expression on MCF-7 human breast cancer cells [26]. UA has demonstrated the capability to inhibit key steps of angiogenesis *in vitro*, including endothelial cell proliferation, migration, and differentiation [27]. It was found that UA inhibited angiogenesis in a dose-dependent manner, with the dose required for half-maximal inhibition in a chick embryo chorioallantoic membrane being a low dose of 5 μg [23]. The potential of UA to inhibit angiogenesis *in vivo* was also investigated. Kanjoormana et al. found that UA inhibited tumor-associated capillary formation in B16F10 melanoma grown in C57BL/6 mice. Meanwhile, as compared to the control, the VEGF, NO, and pro-inflammatory cytokines were highly reduced and serum TIMP-I (tissue inhibitor of metalloproteinase-I) and IL-2 (interleunkin-2) levels were significantly increased in UA-treated mice [19]. The anti-angiogenic abilities of UA are usually attributed to the inhibition of the downregulation of matrix metalloproteinase (MMP) activity, which are group of the enzymes involved in degradation of extracellular matrix. The studies reported that UA inhibited the activity of MMP-9 and MMP-2 [28,29].

Taking the anti-angiogenic advantages of both LMWH and UA, we have successfully prepared a LMWH-UA (LHU) conjugate as a polymeric drug by covalently binding UA to LMWH *via* an amide linker for combination cancer therapy. In this way, an additive inhibition of tumor angiogenesis could be achieved over the single anti-angiogenic effect of LMWH or UA alone. More importantly, compared to common chemical drugs, the amphiphilic LHU conjugate is able to form nano-sized particles in aqueous condition, thereby possessing the excellent properties of polymeric nanoparticles, including good stability, improved pharmacokinetic and distribution characteristics (*e.g.* EPR effect-based passive tumor targeting), and reduced side effects [30,31]. Moreover, the water solubility of UA can also be increased by binding to the hydrophilic LMWH, which facilitates the intravenous administration of insoluble UA.

Sigma receptors are well known membrane-bound proteins which are over-expressed on many types of cancer cells, such as melanoma, non-small cell lung carcinoma, breast tumors of neural origin, and prostate tumors [32–37]. The PEG-lipid containing anisamide (DSPE-PEG-AA) exhibits a high affinity to sigma receptors over-expressed on the tumor cells [37]. Therefore, the DSPE-PEG-AA was used to modify LHU nanodrugs in this study to facilitate the targeted delivery of nanodrugs to the tumor. Based on sigma receptor-mediated endocytosis and the tumor's EPR effect, DSPE-PEG-AA-modified LHU (A-LHU) nanodrugs should show increased accumulation at the tumor site. PEG, a biocompatible hydrophilic polymer, is known to contribute to the long circulation time of nanoparticles, and it was added to A-LHU nanodrugs to benefit its targeted delivery *in vivo* [38]. A-LHU nanodrugs are therefore multifunctional ternary antitumor drug delivery systems used for synergistic angiogenetic inhibition *via* LMWH and UA, as shown in Fig. 1. In this study, the capacity of A-LHU nanodrugs to inhibit bFGF-induced angiogenesis was evaluated *in vitro* and *in vivo*. *In vitro* cellular uptake of A-LHU nanodrugs was monitored on two different cell lines: B16F10 cells (high level of sigma receptor) and HUVECs (low level of sigma receptor). Antitumor efficacy of A-LHU nanodrugs was also investigated.

2. Materials and methods

2.1. Materials

LMWH (100 IU/mg), average molecular weight near 4500 Da, was obtained from Nanjing University. UA was purchased from Wuhan Yuan Cheng Co-created Technology Co. Ltd. (Wuhan, China). DSPE–PEG–anisamide (DSPE–PEG–AA) was synthesized in our lab as described [39].DSPE-PEG-OCH_3 was purchased from Xiamen Sinopeg Biotech Co. Ltd. (Xiamen, China). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) were purchased from Aladdin Industrial Corporation (Shanghai, China). N, N-dicyclohexylcarbodiimide (DCC) and pyrene were from Sinopharm Chemical Reagent Co. Ltd. (Nanjing, China). Anhydrous dimethylformamide (DMF) and anhydrous formamide were from Shanghai Lingfeng Chemical Reagent Co. Ltd. (Shanghai, China). Acetone was purchased from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China). All other chemicals were of analytical grade and were used without further purification.

2.2. Synthesis and characterization of LHU conjugate

The LHU conjugate was obtained by coupling aminated LMWH with UA, as shown in Fig. 2A. Firstly, LMWH dissolved in phosphate-buffered solution (PBS) (0.01 M, pH 7.4) was reacted with EDC and NHS (molar ratio 1:3:3) for 4 h at room temperature, followed by adding ethylenediamine as the linker. The reaction of activated LMWH and ethylenediamine was for 24 h at room temperature. After reaction with ethylenediamine, the mixture was dialyzed against deionized water for 48 h using a dialysis membrane (MWCO 3500). The pure LMWH-NH_2 was obtained followed by lyophilization. The degree of substitution (DS) of ethylene diamine on LMWH was 17.4% according to the ^{1}H NMR assay. Secondly, UA dissolved in THF was reacted with NHS and DCC (molar ratio 1:1.5:1.2) for 24 h at room temperature. The precipitated side product dicyclohexylurea (DCU) was removed by filtration. The activated UA was precipitated in *n*-hexane and then filtered, followed by vacuum drying at room temperature. Finally, the activated UA dissolved in DMF was added into the mixture of LMWH-NH_2 dissolved in formamide and EDC (molar ratio 4∶1∶4), and then reacted for 24 h at room temperature. The mixture was then precipitated in excess cold acetone and the precipitate was carefully washed three times with cold acetone. The dried LHU conjugate was suspended in water and dialyzed against deionized water for 24 h using a dialysis membrane (MWCO 3500), followed by lyophilization.

The chemical structure of LHU conjugate was characterized by ^{1}H NMR spectra. The hydroxyl of UA can be easily dehydrated with the presence of sulfuric acid resulting in the formation of the chromophores in the visible range. Therefore, the DS of UA covalently attached to LMWH was determined spectrophotometrically after a reaction with sulfuric acid [15,40]. The particle size and zeta potential of LHU nanodrugs were determined by dynamic light scattering (DLS) measurements (BI-200SM, Brookhaven Instruments Corp., USA). The morphology of LHU nanodrugs was observed by transmission electron microscopy (H-600, Hitachi, Japan). The critical micelle concentration (CMC) of the LHU conjugate was investigated by fluorescence spectroscopy, using pyrene as a probe as described previously [41]. Briefly, 1 mL