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A poly(L-glutamic acid)-combretastatin A4 conjugate for solid tumor therapy: Markedly improved therapeutic efficiency through its low tissue penetration in solid tumor

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ABSTRACT

Combretastatin A4 (CA4) is a leading agent in vascular disrupting strategies for tumor therapy. Although many small-molecule prodrugs of CA4 have been developed to improve its solubility, the overall therapeutic efficiency is moderate. A key reason for this is the reversible effect that CA4 has on tubulin as well as its rapid clearance from plasma and tissues. In this study, we proposed a poly(L-glutamic acid)-CA4 conjugate (PLG-CA4) nanomedicine to fulfill the requirements for fully liberating the potential of CA4 on tumor therapy. Enhanced accumulation and retention of CA4 in tumor tissue, especially, high distribution and gradual release around tumor blood vessels resulted in prolonged vascular disruption and markedly enhanced therapeutic efficiency. We examined and compared the therapeutic effect of PLG-CA4 and commercial combretastatin-A4 phosphate (CA4P) in a murine colon C26 tumor. PLG-CA4 showed significantly prolonged retention in plasma and tumor tissue. Most importantly, the PLG-CA4 was mainly distributed around the tumor vessels because of its low tissue penetration in solid tumor. Pathology tests showed that PLG-CA4 treatment resulted in persistent vascular disruption and tumor damage 72 h after a single injection, this in contrast to CA4P treatment, which showed quick relapse at an equal dose. Tumor suppression tests showed that PLG-CA4 treatment resulted in a tumor suppression rate of 74%, which indicates a significant advantage when compared to tumor suppression rate of the CA4P group, which was 24%. This is the first time that an advantage of the polymeric CA4 nanomedicine with low tissue penetration for solid tumor therapy has been shown. Thus, the results presented in this study provide a new idea for enhancing the tumor therapeutic effect of vascular disrupting agents.

Statement of Significance

Nanomedicine usually has low tissue penetration in solid tumors, which limits the efficacy of nanomedicine in most cases. But herein, we demonstrate a nanosized vascular disruptive agent (VDA) PLG-CA4 has supper advantages over small molecular combretastatin-A4 phosphate (CA4P) because the PLG-CA4 was mainly distributed around the tumor vessels due to its low tissue penetration in solid tumor.

1. Introduction

The tumor vasculature is an attractive target for tumor therapy [1–3]. It has been reported that when tumors grow to a size larger than 2–3 mm^3, their continued proliferation strongly depends on angiogenesis [4–6]. As a result, various drugs targeting tumor-initiated angiogenic processes or destroying the established tumor vessel network (angiogenesis inhibitors (AIs) and vascular disrupting agents (VDAs) respectively), have been developed and are currently under clinical evaluation [7,8].

VDAs cause a rapid and selective vascular shutdown in tumors to produce extensive secondary neoplastic cell death due to ischemia [9–11]. CA4 is a lead agent in VDAs. As a microtubule depolymerizing agent, CA4 binds at or near the colchicine binding site of β-tubulin, which leads to cytoskeletal and morphological changes

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in endothelial cells [12,13]. These changes increase vascular permeability, disrupt tumor blood flow, and rapidly lead to widespread ischemic necrosis [2,14,15]. Studies have shown that CA4 has dramatic effects on the three-dimensional shape of newly formed endothelial cells, with little or no effect on quiescent endothelial cells [16]. In addition, CA4 has been demonstrated to disrupt cell-cell contacts between endothelial cells mediated by vascular endothelial (VE)-cadherin/β-catenin complexes and this effect could be inhibited in the presence of smooth muscle cells [17]. Since tumor blood vessels are characterized by recently formed endothelial cells and abnormal vessels that lack a full complement of smooth muscle or pericyte support, CA4 shows selective vascular damage in tumor tissues.

Because of its insolubility in water, CA4 cannot directly be intravenously administrated. In recent years, various small-molecule prodrug derivatives of CA4 have been developed [18,19]. Of these, CA4 phosphate (CA4P) is the leading agent [20]. Phosphate greatly improved the solubility of CA4, and is able to release CA4 in the presence of phosphoesterase [21]. Clinical studies of CA4P consist of 18 completed and ongoing clinical trials in oncology and ophthalmology [22]. Overall, CA4P monotherapy was well tolerated, with most adverse events being of mild to moderate intensity [23–25]. In a phase II clinical trial in which CA4P was used to treat anaplastic thyroid cancer (ATC), the median survival was 4.7 months of which 34% of patients were alive at 6 months and 23% of patients was alive at 12 months. Median duration of stable disease in seven patients was 12.3 months (4.4–37.9 months). However, there were no objective responses observed after single-agent CA4P administration in this trial and the primary endpoint of survival doubling was not observed [26]. As a result, phase III clinical trials of CA4P on ATC were conducted as a combination therapy using CA4P with carboplatin/paclitaxel.

The limited therapeutic efficacy of small-molecule prodrug derivatives of CA4 lies in the reversible effects CA4 has on tubulin as well as its rapid clearance from plasma and tissues. Unlike changes induced by colchicines and vinblastine the changes in endothelial cell shape induced by CA4 can be reversed after drug removal [16]. CA4 rapidly binds to tubulin and dissociates from tubulin over 100 times faster than colchicines. At 37 °C, CA4 half-life is 3.6 min compared to 405 min for colchines [27]. Moreover, these small-molecule drugs have a relatively short half-life *in vivo*. In phase I clinical trials, the distribution half-life time ($t_{1/2\alpha}$) of CA4P is 0.103 h and the elimination half-life time ($t_{1/2\beta}$) is 0.489 h [25]. As a result, the tentatively closed tumor vascular can recover and continuously provide oxygen and nutrients and nutrients for tumor growth. Therefore, single administration of small-molecule prodrug derivatives of CA4 may not significantly affect primary tumor growth [28,29].

A key point in improving the therapeutic efficacy of CA4 is to keep a constant concentration around the endothelial cells and enhance the action time on tubulin. Nanocarrier-based drug delivery systems provide an ideal medium to realize this. Nanocarrier-enwrapped or -conjugated drugs have been extensively examined for prolonging the retention time of small-molecule drugs *in vivo*, and increasing drug accumulation in the tumor tissue by virtue of the "enhanced permeability and retention" (EPR) effect [30–35]. This system allows for gradual or temporary release of free active drugs from the nanocarriers in a controlled fashion, and allows for a constant drug concentration in the tumor tissue [36–38]. More importantly, because of the high interstitial pressure and low diffusion efficiency inside solid tumors, nanocarrier-loaded drugs are frequently observed around blood vessels in solid tumors because of the low tissue penetration [39–43]. We consider these characteristics are especially important to improve the efficiency of CA4 treatment in tumor therapy, since the action target of CA4 are endothelial cells of tumor blood vessels. Therefore, a nanocarrier-loaded CA4 prodrug is expected to enhance CA4 accumulation and retention in the tumor tissue, high distribution and gradual release CA4 around tumor blood vessels, resulting in prolonged vascular disruption and markedly enhanced tumor therapeutic efficacy.

In this study, we proposed to derive a poly(L-glutamic acid)-CA4 (PLG-CA4) conjugate nanomedicine to examine the effect of polymeric design on CA4 efficacy. The following studies were conducted: 1) Preparation and characterization of PLG-CA4, 2) pharmacokinetics of PLG-CA4, 3) multispectral optoacoustic tomography (MSOT) and immunofluorescence analysis of PLG-CA4 distribution inside tumors, 4) pathological response of murine C26 tumors to PLG-CA4 and CA4P treatment, 5) tumor therapy tests. In summary, we found that nanosized polymeric CA4 prodrugs have advantages over small-molecule CA4 prodrugs.

2. Experimental

2.1. Materials

γ-Benzyl-L-glutamate-N-carboxyanhydride (BLG-NCA) was purchased from Chengdu Enlai Biological Technology CO., Ltd., China. mPEG5k and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Sigma-Aldrich, USA. Combretastatin A4 (CA4) and combretastatin-A4 phosphate (CA4P) were purchased from Hangzhou Great Forest Biomedical Ltd., China. 2,4,6-trichlorobenzoyl chloride, 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) and N-hydroxysuccinimide (NHS) were supplied by Aladdin Reagent Co. Ltd, China. Rhodamine B-NH_2 (RhoB-NH_2) was a kind gift from Dr. Chunsheng Xiao, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. Anti-mouse, human and pig CD31 antibody (ab28364) was purchased from Abcam, USA. All other reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd, China.

2.2. Preparation and characterization of PLG-CA4

Poly(L-glutamic acid)-*graft*-methoxy poly(ethylene glycol) copolymer (PLG-*g*-mPEG) was prepared as described previously [43–45]. PLG-*g*-mPEG has an average of 160 L-glutamic acid repeating units and an average of 8.3 mPEG5k chains. The number-average molecular weight (M_n) and molecular weight distribution (PDI) of the PLG-*g*-mPEG (Determined by GPC using PEG as standards and phosphate buffer pH 7.4 as eluent) were 37.3×10^3 g mol^{-1} and 1.91, respectively [44]. CA4 was grafted to PLG-*g*-mPEG by the Yamaguchi reaction. Briefly, PLG-*g*-mPEG (585 mg) was dissolved in 10 mL anhydrous N,N-dimethylformamide (DMF) in a glass reactor, then CA4 (1.0 mmol, 316 mg), 2,4,6-trichlorobenzoyl chloride (1.1 mmol, 268 mg), DMAP (1.1 mmol, 135 mg) and triethylamine (1.1 mmol, 111 mg) were dissolved in 5 mL anhydrous DMF and added to the above mixture. The reaction was allowed to proceed at room temperature for 2 h. After that, the reaction mixture was precipitated into excess diethyl ether, re-dissolved in DMF, and dialyzed against distilled water (MWCO 3500). The final product poly(L-glutamic acid)-*graft*-methoxy poly(ethylene glycol)/combretastatin A4 (PLG-CA4) was obtained after lyophilization.

Chemical structure of the synthesized PLG-CA4 was confirmed by ^{1}H NMR (D_2O, Bruker AV 400 NMR spectrometer). M_n (57.3×10^3 g mol^{-1}) and PDI (1.69) of the PLG-CA4 were determined by gel permeation chromatography (GPC, Waters 515 pump, 2414 detector, DMF containing 0.01 M LiBr as the eluent and using polystyrene as standard samples). Free CA4 contents were measured using a high-performance liquid chromatography