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Regulating cancer associated fibroblasts with losartan-loaded injectable peptide hydrogel to potentiate chemotherapy in inhibiting growth and lung metastasis of triple negative breast cancer

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ABSTRACT

Preoperative chemotherapy is effective in improving the prognosis of patients, but its efficacy is impeded by cancer associated fibroblasts (CAFs) that enhance the survival, growth, and metastasis of cancer cells. To inhibit the activity of CAFs, prolonged and localized drug exposure is necessary. Here, we report on the rational design, screening, and evaluation of an injectable peptide hydrogel as a local losartan depot aiming to inhibit CAFs and potentiate chemotherapy. We synthesized a set of peptide derivatives and found that C_{16}-GNNQQNYKD-OH (C_{16}-N) surpassed the others in hydrogel formation and drug encapsulation, due to its flexible hydrocarbon tail and interpeptide hydrogen bonding that allowed supramolecular self-assembly into long filaments with hydrophobic cores. C_{16}-N co-assembled with losartan to form hydrogel from which losartan was sustainably released over 9 days. After intratumoral injection, the hydrogel could be retained in the tumor for more than 9 days, significantly inhibited the CAFs and collagen synthesis in orthotopic 4T1 tumors, and enhanced the efficacy of PEGylated doxorubicin-loaded liposomes (Dox-L) in inhibiting the tumor growth (64% vs. Dox-L alone) and lung metastasis (80% vs. Dox-L alone). These results provide important guiding principles for the rational design of injectable peptide hydrogels aiming to regulate CAFs and improve chemotherapy.

1. Introduction

Preoperative chemotherapy can improve and help predict the prognosis of cancer patients receiving adjuvant therapy [1–3]. It is particularly useful in treating triple negative breast cancer (TNBC), an aggressive subtype of breast cancer that responds to chemotherapy but not hormonal and anti-human epidermal growth factor receptor-2 therapies [4]. Its utility derives from its ability to facilitate lumpectomy and help identify adjuvant chemotherapies that can induce a complete pathological response [3,5]. However, the efficacy of preoperative chemotherapy is still not satisfactory, even with Abraxane® and Myocet® [6,7].

The presence of tumor stroma is one major hurdle for the successful chemotherapy of breast cancer [8]. In patients with TNBC, poor prognosis always associates with a high percentage of tumor stromal cells mainly composed of cancer-associated fibroblasts (CAFs) [9–11]. This is because (1) CAFs secrete various growth factors and cytokines that aid in the progression and metastasis of tumors [12,13]; (2) CAFs produce extracellular matrix (ECM) components that provide physical support for cancer cells and growth factors and greatly hinder the accumulation and penetration of nanotherapeutics [14–16]; and (3) CAFs promote the epithelial-to-mesenchymal transition (EMT) and the survival of metastasized cancer cells during and after dissemination [17,18]. Transforming growth factor-β (TGF-β) is a key promoter of CAF activity, inducing

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the secretion of ECM components such as collagen I [19]. Collagen I can down-regulate E-cadherin gene expression and stabilize the epithelial-to-mesenchymal transition [20], and also forms a network that facilitates the invasion of cancer cells [21], both of which functions can promote tumor metastasis. The TGF-β signaling pathway can be inhibited by angiotensin II receptor antagonist losartan, partially through down-regulation of the TGF-β activator thrombospondin-1, but only when the losartan is dosed >40 mg kg^{-1} day^{-1} intraperitoneally for at least 7 days [14,19,22,23]. Though effective in inhibiting CAFs, losartan treatment alone cannot reduce the growth rate of the tumor and subsequent chemotherapy is necessary [14]. Deleterious effects on blood pressure may contraindicate losartan [19] and, therefore, materials enabling the localized and prolonged delivery of losartan is desired during preoperative chemotherapy of TNBC to maximize the efficacy and minimize side effects such as low blood pressure.

Polymers have been widely used to construct localized drug delivery systems for cancer therapy, including micro-needle patches, implantable devices, microparticles, hydrogels and so forth [24–28]. Recently, supramolecular polymeric hydrogels, particularly those composed of peptide derivatives, have attracted more interest because they can be easily functionalized by varying the peptide sequence, can be delivered via syringe injection, and can be completely degraded into natural amino acids afterward [29–33]. For instance, high-molecular-weight peptides such as elastin-like peptides and co-polypeptides have long been used as materials to create stimuli-responsive hydrogels [34–36], and the self-assembly of collagen in steps from peptide chain to triple helix to nanofibres and to a hydrogel can now be replicated by shorter collagen-mimetic peptides with around 40 residues [37,38]. Even tripeptides with specific sequences are able to form hydrogels through self-assembly [39]. Peptide hydrogels of this type have been used for the controlled delivery of proteins which are trapped within the hydrogel network [40–42]. Peptide derivatives with hydrophobic moieties such as fatty acids, aromatic molecules, and drugs are another class of hydrogelators under broad investigation, gelating through an entropy-driven mechanism [43–46]. The ordered molecular arrangement of small peptide amphiphile gelators enables the formation of hydrogels with high drug loading at low concentration (<3 wt%) [45–47], which is in contrast with block-copolymer-based hydrogels [33,48].

To create a peptide-based hydrogel for localized drug delivery, the gelators should first self-assemble into one-dimensional (1D) filaments with hydrophobic cores for drug encapsulation and further entangle with each other to form a hydrogel. It is well-documented that 1D supramolecular self-assembly is mainly guided by hydrogen bonding and π-π stacking between gelators [49,50], a process that is assisted by hydrophobic interactions between molecules via an entropy-driven mechanism [51]. Although some hydrophobic drugs can be covalently linked to a peptide for self-delivery, many therapeutics are not suitable for conjugation. Thus, peptide amphiphile-based filaments with hydrophobic cores for drug entrapment are desirable. The drug loading capacity of the hydrophobic core is mainly determined by the hydrophobic moiety of the peptide amphiphile, but may also be influenced by the peptide segment [52]. It is thus necessary to optimize the molecular design to obtain hydrogels with suitable properties for localized drug delivery. Herein, we report on the design and synthesis of three short peptide derivatives with similar hydrophilic segments and different hydrophobic tails, all of which can self-assemble into 1D nanostructures. We investigated the influence of molecular design on the molecular packing, self-assembly, hydrogel formation, and losartan encapsulation capability. The optimal losartan-loaded hydrogel was then evaluated for its efficacy in inhibiting CAFs and enhancing chemotherapy. Our findings highlight the importance of molecular design in the preparation of injectable hydrogels and demonstrate that the losartan-loaded peptide hydrogel could improve the effect exhibited by chemotherapy in the inhibition of growth and lung metastasis of TNBC through regulation of CAFs.

2. Materials and methods

2.1. Materials and animals

All Fmoc-protected amino acids and Fmoc-Asp(OtBu)-Wang resin were purchased from GL Biochem (Shanghai, China). *O*-benzotriazole-*N*,*N*,*N*,*N*-tetramethyluronium hexafluorophosphate (HBTU), *N*,*N*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from J&K (Shanghai, China). Losartan potassium was purchased from Tokyo Chemical Industry (Japan). DiR was obtained from Life Technology (USA). Unless noted otherwise, all other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd and used as received.

4T1 mice-derived triple-negative breast cancer cells were obtained from the Cell Bank of Shanghai, Chinese Academy of Sciences (Shanghai, China). 4T1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% antibiotics. Cells were maintained at 37 °C in a humidified incubator containing 5% CO_2.

Female Balb/c nude mice (16–18 g) and Balb/c mice were purchased from the Shanghai Experimental Animal Center (Shanghai). All animal procedures were performed under guidelines approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

2.2. Peptides synthesis

Peptides were synthesized manually using fluorenylmethoxycarbony (Fmoc) solid-phase peptide synthesis protocols as previously described [53], employing 20% 4-methylpiperidine in DMF (*v*/*v*) for Fmoc removal and amino acid (or palmitic acid)/HBTU/DIEA (4:4:6 M ratio relative to the resin) in DMF for couplings (with 2 min activation time and 2 h reaction time). The molecules were then cleaved from the resin with a cocktail of TFA/TIS/H_2O (95:2.5:2.5 *v*/*v*) for 2.5 h and precipitated in cold ethyl ether to obtain the crude products. The molecules were further purified using RP-HPLC. The purity and molecular mass of the molecules were confirmed by analytical HPLC and mass spectrometry, respectively.

2.3. Critical micelle concentration (CMC) measurement

The CMC values of the synthesized molecules were determined using a Nile Red-based method [54]. Samples of various concentrations were prepared in DPBS and added to vials containing dried Nile Red, such that the final dye concentration was 1 μM. The mixture was then sonicated and incubated overnight to equilibrate. Each sample was excited at 560 nm and the fluorescence emission at 655 nm was monitored using an F4600 fluorescence spectrophotometer (Hitachi, Japan). The fluorescence intensity was then plotted against the sample concentration (on a log scale).

2.4. Transmission electron microscopy (TEM)

Seven microliters of each sample was deposited onto a copper grid covered with a carbon film. After removing the excess solution to leave a thin layer, the sample was stained using uranyl acetate (2 wt %). The specimens were then air dried at room temperature