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An acid-seeking carrier-free drug achieves high antitumor activity via a "solution-particle" transition

Xiaoying Zhang[a,1], Cuifeng Wang[a,*,1], Jiamin Wu[a], Yajun Liu[a], Zeping Yang[a], Ye Zhang[a], Xiaofeng Sui[b], Min Li[a], Min Feng[a,*]

[a] School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510006, PR China
[b] Key Laboratory of Science & Technology of Eco-Textile (Ministry of Education), Donghua University, Shanghai 201620, PR China

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A B S T R A C T

Drug nanocarriers that have long been expected to revolutionize cancer therapy have yet to achieve the significant clinical success. Therefore, it remains controversial to pursue a complex drug nanocarrier that lacks clinical relevance. Herein, we developed an easily-synthesized anti-tumor drug that actively seeks the acidic tumor microenvironment while ignoring the normal tissue without the aid of additional carriers. This called "carrier-free" drug (CFD) is capable of switching its morphology from the unstructured solution to the spherical structure in response to tumor acidity. CFDs were the water-soluble zwitterionic unimers to prevent the nonspecific distribution in the circulation, whereas they spontaneously formed into the particles tending to accumulation in tumor. CFD overloading in tumor cells caused the lysosomal dysfunction and autophagy blockage, thereby triggered the cell death. All the in vitro and in vivo data demonstrated the tumor-acidity-selective cytotoxicity of CFD. This facile strategy to create a self-delivering anticancer drug may cast a new light on the development of cancer therapy.

1. Introduction

In the past decades, nanocarrier-based drug delivery systems have held great potential for progressing cancer chemotherapy due to their desirable pharmacokinetics and tissue distribution via passive targeting, known as the enhanced permeability and retention (EPR) effect [1–3]. Despite the compelling advantages over the free drugs, drug nanocarriers in the bloodstream are unavoidably taken up by mononuclear phagocyte system (MPS) organs, especially the liver, which greatly compromises the therapeutic efficacy of the drugs. To address this challenge, intensive studies are being conducted to produce diverse multifunctional nanocarrier-based platforms that actively target tumor cells via the ligand-receptor interaction. In addition, a pH variation between normal tissue (pH 7.2–7.4) and tumor (pH 6.0–6.8) represents an ideal target for cancer treatment because this distinctive feature can be exploited to artificially manipulate drug accumulation and toxicity [4,5]. Along with passive and active targeting strategies, a serial of tumor-acidity-responsive materials have been incorporated into nanocarriers to enhance tumor accumulation while sparing the normal tissue based on a pH difference [6–8]. Although there have been convincing preclinical outcomes for nanocarrier-based delivery systems, some of the systems are unfortunately too elaborate to translate into scale-up production, accompanied by the disadvantages such as complicated nanocarrier preparation, inefficient drug encapsulation, and nanocarrier-induced toxicity, immunogenicity or instability. Therefore, simplifying the drug delivery system may highlight an alternative avenue to accelerate clinical translation. Following the principle of "less is more", to design an ideal drug delivery system should minimize its architecture while maximizing the multifunction. "Drug self-delivery systems" (DSDSs) have been recently proposed to exclude the aid of additional nanocarriers [9–12], although, these revolutionized systems are still unlikely to escape the MPS recognition and clearance. DSDSs reserved the nanocarrier characteristics (e.g., particle size and surface properties [13]) which play a critical role in mediating serum protein adsorption and macrophage phagocytosis. It previously showed that nanomaterials modified with cysteine maintained a low-fouling zwitterionic surface to prevent the nonspecific adsorption of serum proteins. Therefore, zwitterionic modification has been developed as an alternative to traditional PEGylation to achieve the in vivo stability of the nanomaterials [14–16].

Herein, we demonstrate a proof-of-principle of a facile strategy in which acidotropic macromolecular therapeutics construct the drug-self-

* Corresponding authors.
E-mail addresses: wangcf6@mail.sysu.edu.cn (C. Wang), fengmin@mail.sysu.edu.cn (M. Feng).
[1] These authors contributed equally to this work.

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assembly nanoparticles via a "solution-particle" transition upon encountering the acidic tumor microenvironment. This pH-induced morphological switch guarantees that soluble zwitterionic unimers escape MPS capture and prolong blood circulation, whereas micellar aggregates contribute to the enhanced cellular uptake by tumor cells. Hence, this called the "carrier-free" drug (CFD) system processes the tissue-selective toxicity.

2. Materials and methods

2.1. Chemicals, plasmids and antibodies

Branched polyethylenimine (PEI 25 KDa), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), azobisisobutyronitrile (AIBN) and low melting point agar were obtained from Sigma-Aldrich (USA). D_2O and DCl were purchased from ASFIRST Science (China). Allyl glycidyl ether, S-Allyl-L-cysteine, L-cysteine, 2,2′-Azobis (2-methylpropionitrile), Pyrene and Tris (hydroxymethyl) aminomethane were purchased from Aladdin Chemistry (China). DMEM medium, fetal bovine serum (FBS) and trypsin were obtained from Gibco (Canada). Lipofectamine 2000 was purchased from Invitrogen (USA). Cy5 mono NHS ester was obtained from GE Healthcare (UK). Chloroquine was purchased from Ark Pharm (USA). LysoTracker Red DND-99 and Lysosensor Green DND-189 were obtained from Life Technologies Corporation Eugene. Oregon (USA). Annexin V-FITC apoptosis detection kit was purchased from BestBio Biology (Shanghai, China). The plasmids DNA GFP-LC3, mRFP-GFP-LC3 were kind gifts from Dr. M. Li (Department of Pharmcology, Sun Yat-sen University) [17]. Anti-rabbit LC3 and anti-mouse GAPDH were purchased from Sigma-Aldrich (USA) and ABclonal (USA), respectively. HRP conjugated goat anti-rabbit or anti-mouse antibodies were purchased from Thermo Scientific (USA). ECL western blotting detection kit was purchased from GE Healthcare Bioscience (UK). Calcein-AM/PI kit was purchased from Shanghai YEASEN Biotechnology (China).

2.2. CFD synthesis and characterization

The synthesis of the carrier free drug (CFD) was illustrated in Fig. S1 [18,19]. Briefly, allyl glycidyl ether (AGE, 1.33 g, 0.0115 mol) was dissolved in a 7.5 mL methanol/aqueous (3/4, v/v) mixed solvent. Next, PEI 25 KDa (0.5 g, 0.02 mmol) dissolved in 2.5 mL deionized water was added dropwise into the AGE solution for a 3 h reaction at 25 °C. The product PEI-AGE was purified using dialysis (molecular weight cutoff, 3.5 KDa) against Tris-HCl buffer (15 mM, pH 8.0) for 48 h. Cysteine (3.38 g, 0.028 mol) was dissolved in 60 mL Tris-HCl buffer (15 mM, pH 8.0) in 250 mL round-bottom flask. Subsequently, the purified PEI-AGE was slowly added into cysteine solution in the present of the catalyst azobisisobutyronitrile (AIBN, 18 mg, 0.1 mmol). After reaction at 65 °C for 24 h under N_2 atmosphere, the mixture was transferred to a dialysis tube (molecular weight cutoff, 3.5 KDa) and then dialyzed against deionized water for 3 days. CFD was finally obtained using freeze-drying under vacuum.

Molecular weight of CFD was determined by gel permeation chromatography (GPC). Proton Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded in D_2O/DCl solution (pH 7.4 and pH 6.5) with a Bruker Avance III 400 MHz NMR spectrometer and 100 MHz NMR spectrometer, respectively. ^{1}H NMR (D_2O) δ 5.92 (d, J = 6.0 Hz, 1H), 5.27 (dd, J = 26.7, 13.6 Hz, 2H), 4.78–4.32 (m, 14H), 4.00 (d, J = 31.5 Hz, 2H), 3.89 (s, 2H), 3.64–3.22 (m, 6H), 3.02 (d, J = 75.1 Hz, 5H), 2.84–2.31 ppm (m, 14H). ^{13}C NMR (D_2O) δ 174.939 (1C), 133.693 (1CH), 118.034 (1CH_2), 69.686 (1CH_2), 67.747 (1CH_2), 57.045 (1CH_2), 56.073 (1CH_2), 53.734 (1CH_2), 50.734 (1CH_2), 45.086 (2CH_2), 39.061 (1CH_2), 33.423 (1CH_2), 28.152 ppm (1CH_2) [20].

2.3. Particle size and zeta potential

CFD stock solution (10 mg/mL in dH_2O) was dispersed in 10 mM Hepes buffer either at pH 7.4 or pH 6.5 at a concentration of 10 μg/mL, and then it was allowed to incubate at room temperature for 10 min prior to measurements. Particle size and zeta potential of each sample were measured using dynamic light scattering (DLS) and electrophoretic light scattering on a Zetasizer NS90 (Malvern Instruments, UK), respectively. Three independent DLS measurements of each sample were repeated using a 4 Mw He-Ne laser at a wavelength of 633 nm and a scattering angle of 90° at 25 °C. Real-time dynamic CFD visualization was carried out by nanoparticle tracking analysis (NTA) using a NanoSight LM10 instrument (Malvern Instruments, UK). A movie was captured and stored at a rate of 30 frames/s, and further was analyzed with NTA software (version 2.2, Nanosight, UK).

2.4. Morphology analysis

CFD solution was prepared in 10 mM Hepes buffer (10 μg/mL) either at pH 7.4 or at pH 6.5 for morphological observation using transmission electron microscope (TEM) and atomic force microscopy (AFM). For the TEM imaging, 10 μL of CFD solution was dropped onto a copper grid and then stained with 2.5% phosphotungstic acid. Next, the grids were allowed to air dry before being observed using a JET1400 transmission electron microscope at a voltage of 120 kV. In addition, 15 μL of CFD solution (at pH 7.4 or pH 6.5) was immediately dropped onto the surface of freshly prepared mica and allowed to air dry. AFM imaging was performed using a Digital Instruments Nanoscope IIa (Digital Instruments, Santa Barbara).

2.5. Determination of critical micelle concentration (CMC)

The CMC value of CFD at pH 6.5 was measured by using pyrene as a fluorescence probe. A volume of 25 μL pyrene solution in acetone (1.8×10^{-6} M) was added to a separate test tube. Once the acetone was evaporated, aqueous solution of CFD with various concentrations at pH 6.5 was added to each test tube. The solution was mixed well and allowed to incubate in the dark for 24 h at 25 °C to achieve the equilibrium. Pyrene was excited at 336 nm and its emission was recorded at 373 nm and 384 nm with a fluorescence spectrophotometer (PTI QuantaMaster QM4CW, USA). The fluorescent intensity ratios of I_{373}/I_{384} were calculated and plotted against the logarithm of CFD concentrations. The CMC was obtained from the intersection of the two tangent plots.

2.6. Cell culture

Human colorectal LoVo cells, human lung cancer A549 cells, and human lung fibroblasts HLF cells (American Type Culture Collection, ATCC) were cultured in DMEM medium containing 10% fetal bovine serum in a humidified incubator at 37 °C with 5% CO_2.

2.7. In vitro cytotoxicity

LoVo cells, A549 cells, or HLF cells grown in 96-well plates at a seeding density of 5×10^3 cells per well overnight prior to MTT assay. When reaching to 70%–80% confluency, the cells were exposed to varying concentrations of CFD solution suspended in pH-adjusted medium for 24 h, followed by the addition of 20 μL of MTT solution (5 mg/mL). After incubation for 4 h, the MTT containing medium was aspirated, and 150 μL of dimethyl sulphoxide (DMSO) was immediately added to solubilize MTT formazan produced in viable cells. The absorbance of formazan at 490 nm was measured with a background correction using a Bio-Tek ELX800 ELISA reader. The cell viability was expressed as a percentage of the viable cells in the treated group to the untreated control group.

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