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Dual pH-responsive multifunctional nanoparticles for targeted treatment of breast cancer by combining immunotherapy and chemotherapy

Yuanyuan Liu, Linan Qiao, Sipei Zhang, Guoyun Wan, Bowei Chen, Ping Zhou, Ning Zhang *, Yinsong Wang *

Tianjin Cancer Institute and Hospital, Laboratory of Breast Cancer Prevention and Therapy, Ministry of Education, Research Center of Basic Medical Science, School of Basic Medical Sciences, School of Pharmacy, Tianjin Key Laboratory on Technologies Enabling Development of Clinical Therapeutics and Diagnostics (Theranostics), Tianjin Medical University, Tianjin 300070, China

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ABSTRACT

In the present study, a dual pH-responsive multifunctional nanoparticle system was designed for combining immunotherapy and chemotherapy to treat breast cancer through targeting immune cells and cancer cells. A proven anti-tumor immune regulator, R848, was encapsulated with poly(L-histidine) (PHIS) to form PHIS/R848 nanocores. Doxorubicin (DOX) was conjugated to hyaluronic acid (HA) through an acid-cleavable hydrazone bond linkage to synthesize polymeric prodrug HA-DOX, which was subsequently coated outside PHIS/R848 nanocores to form HA-DOX/PHIS/R848 nanoparticles. Ionization of PHIS around pH 6.5 (a pH value close to that of tumor microenvironment) switched the nature of this material from hydrophobic to hydrophilic, and thus triggered the release of R848 to exert immunoregulatory action. The rupture of hydrazone bond in HA-DOX at about pH 5.5 (pH of endo/lysosomes) accelerated the release of DOX to exert cytotoxic effects. In immune cells, PHIS/R848 nanocores exhibited strong immunoregulatory activities similar to those induced by free R848. In breast cancer cells overexpressing CD44, HA-DOX was specially internalized by CD44-mediated endocytosis and significantly inhibited the cell growth. In 4T1 tumor-bearing mice, HA-DOX/PHIS/R848 nanoparticles showed excellent tumor-targeting ability and remarkably inhibited the tumor growth by regulating tumor immunity and killing tumor cells. In summary, this multifunctional nanoparticle system could deliver R848 and DOX respectively to tumor microenvironment and breast cancer cells to achieve synergistic effects of immunotherapy and chemotherapy against breast cancer.

Statement of Significance

Combination of immunotherapy and chemotherapy is becoming a promising new treatment for cancer. The major challenge is to target cancer and immune cells simultaneously and specifically. In this study, a dual pH-responsive multifunctional nanoparticle system based on poly(L-histidine) and hyaluronic acid was designed for co-loading R848 (immune-regulator) and doxorubicin (chemotherapeutic drug) through different encapsulation modes. By responding to the acidic pHs of tumor microenvironment and intracellular organelles, this multifunctional nanoparticle system could release R848 extracellularly and deliver DOX targetedly to breast cancer cells, thus achieving synergistic effects of immunotherapy and chemotherapy against breast cancer.

1. Introduction

Breast cancer is the most common cancer and ranks the second-highest female mortality rate. In 2016, approximately 249,260 new breast cancer cases and 40,890 breast cancer deaths (40,450 women, 440 men) are estimated throughout the world [1]. Recent

* Corresponding author.
E-mail addresses: zhangning@tmu.edu.cn (N. Zhang), wangyinsong@tmu.edu.cn (Y. Wang).

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genetic studies have revealed that cancer is a highly heterogeneous disease where each tumor mass consists of cancer cells with different genetic backgrounds, which exhibit different malignancies and drug responsiveness [2]. Therefore, conventional chemotherapy with one single drug, although effective immediately, often result in deteriorated relapse in prolonged period of treatments. It has been proposed that blocking multiple targets simultaneously by combination therapy is an optimal strategy to combat cancer heterogeneity [3]. Some approaches have been tested in the laboratory and showed promising outcomes in clinical trials [4].

Nanotechnology, which allows delivery of multiple drugs in a controlled manner, shows unique advantages in combination therapy and needs further development. Different drugs can be co-loaded into a single nanocarrier system and co-delivered efficiently to the tumor through the enhanced permeability and retention (EPR) effect, and/or the active tumor-targeting effect; thus, this delivery mode is very favorable for exerting synergistic effects of these drugs [5,6]. Moreover, stimuli-sensitive or smart nanocarriers can effectively control drug release at the target site in a desirable fashion by responding to the inherent stimuli (e.g., pH, temperature, redox, enzymes, and reactive oxygen species) or the external stimuli (e.g., light, ultrasound, or magnetic field), thus alleviating toxic and side effects of the drugs on normal tissues [7]. To date, many tumor-targeted and stimuli-sensitive nanocarriers have been developed for targeted co-delivery and controlled release of antitumor drugs with different mechanisms, and some of them have shown significant synergistic effects [8,9].

Recent breakthroughs have revealed that immunotherapy is a new potent approach for cancer treatment [10,11]. Immunotherapy can modulate patient's own immunity to combat cancer, and therefore showing much lower toxicity and longer efficacy than the conventional treatment methods. Activation of dendritic cells (DCs), mediated by Toll-like receptors (TLR), plays a key role in anti-tumor immunity [12]. TLR agonists have been successfully employed to treat cancer in rodent tumor models [13,14]. Small-molecule agonists for Toll-like receptor (TLR) 7 and 8 have sparked a vivid interest in cancer research owing to their profound antitumor activity [15]. Resiquimod (R848) is a TLR7/8 agonist that can promote the maturation of DCs and enhance their functions through the myeloid differentiation factor (MyD88)-dependent pathway [16]. Compared to imiquimod, a TLR7/8 agonist approved by the US Food and Drug Administration (FDA) for topical administration in cancer therapy [14], R848 can induce more pronounced cytokine secretion and macrophage activation. Combination of immunotherapy with chemotherapy shows promising outcomes for patients, and recently, some carrier systems have been developed for co-delivery of immunotherapeutic and chemotherapeutic drugs. A thermosensitive hydrogel system based on poly(ethylene glycol)-poly(γ-ethyl-l-glutamate) diblock copolymers was prepared for co-delivery of interleukin-15 and cisplatin, and significant synergistic effects were achieved against melanoma [17]. Lim et al. designed a stable dispersion system containing both paclitaxel and imiquimod using poly (γ-glutamic acid) as the matrix. In DCs, this dispersion system remarkably enhanced the secretion of pro-inflammatory and Th1 cytokines [18]. However, none of these co-delivery systems addressed the differentiated targeting of immune cells versus tumor cells.

In the present study, a dual pH-responsive multifunctional nanoparticle system based on poly(L-histidine) (PHIS) and hyaluronic acid (HA) was designed for combining immunotherapy and chemotherapy to treat breast cancer through targeting immune cells and cancer cells simultaneously and specifically. PHIS is a peptide containing branched imidazole group that has lone pairs of electrons on the unsaturated nitrogen, which endows this peptide with pH-dependent amphoteric property [19]. The ionization of PHIS at around pH 6.5, a pH value close to that of tumor microenvironment [20], can switch the nature of this material from hydrophobic to hydrophilic [21]. HA is an acidic polysaccharide that consists of alternating units of β-1,4-D-glucuronic acid-β-1,3-N-acetyl-D-glucosamine [22,23]. As a natural ligand for CD44 that is often overexpressed by breast cancer cells, HA has been widely used in active targeting treatment of breast cancer [24–26]. The preparation of this multifunctional nanoparticle system is illustrated in Scheme 1A. R848-loaded PHIS (PHIS/R848) nanocores are first prepared in a weak alkaline solution. DOX is conjugated to HA through an acid-cleavable hydrazone bond linkage to synthesize polymeric prodrug HA-DOX. Next, HA-DOX is coated outside PHIS/R848 nanocores to form HA-DOX/PHIS/R848 nanoparticles, thus realize the co-loading of R848 and DOX. Scheme 1B illustrates the function mechanisms of HA-DOX/PHIS/R848 nanoparticles. After intravenous injection, HA-DOX/PHIS/R848 nanoparticles are delivered to and accumulated in the tumor site through the EPR and active targeting effects. In weakly acidic tumor microenvironment, the hydrophobic/hydrophilic transformation of PHIS triggers the disintegration of HA-DOX/PHIS/R848 nanoparticles, which further mediates the release of R848 to exert its activation effects targeting immune cells. On the other hand, HA-DOX is specifically internalized into breast cancer cells through CD44-mediated endocytosis and the rupture of the hydrazone bond then occurs in response to the acidic endo/lysosomal pH. Hence, DOX is released in breast cancer cells to exert its cytotoxic effects. In this study, we also investigated the *in vitro* and in vivo effects of this multifunctional nanoparticle system to evaluate its potential for combination treatment on breast cancer.

2. Materials and methods

2.1. Materials

HA with molecular mass of 47 kDa was purchased from Bloomage Freda Biopharm (Jinan, China). DOX·HCl was obtained from Meilun Biology Technology (Dalian, China). Succinic dihydrazide, D-Luciferin, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 3,3′-dioctadecyloxacarbocyanine perchlorate (Dio), and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, USA). Cyanine 5.5 (Cy5.5) was obtained from Fanbo BioChemicals (Beijing, China). Cell counting kit-8 (CCK-8) was purchased from Dojindo (Japan). All other chemical reagents were analytical grade and obtained from various commercial sources.

2.2. Cells and animals

Human breast cancer cell lines (MCF-7 and MDA-MB-231) and mouse breast cancer cell lines (4T1 and luciferase-labeled 4T1 (4T1-Luc)) were obtained from American Type Culture Collection (ATCC) and cultured in DMEM medium (Gibco, Life Technologies, USA) containing 10% v/v fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin. Dentritic cell (DC) lines including murine DC2.4 and human DC-like CAL-1 were gifted by Dr. De Yang from Frederick National Laboratory for Cancer Research (Frederick, Maryland, USA). Murine macrophage RAW264.7 cell line was obtained from ATCC. These cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) FBS.

Sprague-Dawley rats and female Balb/c normal and nude mice were bought from the Laboratory Animal Center (Tianjin Medical University, China) and housed in a specific pathogen-free environ-