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Co-delivery of multiple drug resistance inhibitors by polymer/inorganic hybrid nanoparticles to effectively reverse cancer drug resistance

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ABSTRACT

To effectively reverse multiple drug resistance (MDR) in tumor treatments, a functional nano-sized drug delivery system with active targeting function and pH sensitivity was prepared for the co-delivery of multiple drug resistance inhibitors. Buthionine sulfoximine (BSO) to inhibit GSH synthesis and celecoxib (CXB) to down-regulate P-gp expression were co-loaded in polymer/inorganic hybrid nanoparticles to form buthionine sulfoximine/celecoxib@biotin-heparin/heparin/calcium carbonate/calcium phosphate nanoparticles (BSO/CXB@BNP). To investigate the reversal of MDR, the drug resistant cells (MCF-7/ADR) were pretreated by the dual-inhibitor loaded nanoparticles (BSO/CXB@BNP) followed by the treatment of doxorubicin (DOX) loaded nanoparticles (DOX@BNP). The dual-inhibitor loaded nanoparticles (BSO/CXB@BNP) exhibited greatly enhanced efficiency in down-regulation of GSH and P-gp since BSO and CXB had combined effects on the reduction of GSH and P-gp in drug resistant tumor cells. As a result, BSO/CXB@BNP exhibited a significantly improved capability in reversal of MDR compared with mono-inhibitor loaded nanoparticles (CXB@BNP and BSO@BNP). As compared with free drug resistance inhibitors, delivery of drug resistance inhibitors by functional nanocarriers could obviously improve the therapeutic efficiency due to enhanced cellular uptake and increased intracellular drug accumulation. The study on immunostimulatory effects of different treatments showed that BSO/CXB@BNP treatment resulted in the lowest concentration of interleukin 10, a cytokine related to tumor development. These results suggest the nanoparticulate drug delivery platform developed in this study has promising applications in multiple drug delivery to overcome drug resistance in tumor treatments.

1. Introduction

In cancer treatments, chemotherapy is one of the most common and effective approaches. Unfortunately, that approach is greatly limited by the development of multiple drug resistance (MDR) in cancer cells [1]. Among different MDR mechanisms, the overexpression of P-glycoprotein (P-gp), an ATP-dependent efflux pump with broad substrate specificity, to pump drugs out of cells is one of the most common reasons of MDR [2,3]. In addition, the elevated glutathione level in cells is another conventional cause of MDR. Most commonly, the content of (L-γ-glutamyl-L-cysteinylglycine) (glutathione, GSH), which plays a crucial role in protecting tissues against the deleterious effects of oxidative damage, in drug resistant cells is higher than that in nonresistant cells [4].

To reverse the drug resistance, diverse drug resistance inhibitors have been developed. Although many of these agents have been found to overcome drug resistance *in vitro*, *in vivo* results have been disappointing, mainly because of the poor aqueous solubility of inhibitors and low intracellular inhibitor concentration at targeted sites [5–7]. More importantly, drug resistance inhibitors may cause undesired side effects to normal cells. For example, P-gp plays important roles in the physiological regulation of endogenous and xenobiotic compounds in the body. Therefore, it is important to limit the exposure of normal cells and tissues to P-gp efflux inhibitors. GSH is an important antioxidant in cells, which can prevent damage of cellular components caused by reactive oxygen species, heavy metals and toxic chemical agents. The drug resistance inhibitors which down-regulate the intracellular GSH level may lead to unfavorable effects on healthy cells [8,9].

As it is well known, drug delivery systems offer various advantages as compared with single agents. Among numerous drug delivery systems, nano-sized drug delivery systems have attracted increasing scientific interest because of their capabilities

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to increase drug availability, to achieve passive tumor targeting resulted from enhanced permeability and retention (EPR) effect and active targeting by surface functionalizations, and to overcome drug resistance by "stealth" endocytosis [10–15].

Through encapsulation of drug resistance inhibitors in nano-sized carriers, drug resistance inhibitors can be delivered to tumor cells to minimize the unfavorable effects on normal tissues and cells. Although drug carriers with diverse functions have been extensively investigated for the delivery of chemotherapeutic agents, the studies on the delivery systems for drug resistance inhibitors are still limited [16,17].

The purpose of the current study is to develop multifunctional nano-sized co-delivery systems with ideal biocompatibility and biodegradability for co-delivery of multiple drug resistance inhibitors to efficiently reverse the cancer drug resistance. In our study, biotin-heparin/heparin/calcium carbonate/calcium phosphate (HPB/HP/$CaCO_3$/CaP) hybrid nanoparticles with enhanced cell internalization, good serum stability, active targeting function and pH sensitivity for multiple drug delivery were prepared. All components in the drug carrier were introduced to the nanoparticles by self-assembly in an aqueous medium under mild conditions, and the whole preparation process did not involve any organic solvent and surfactant. As a natural glycosaminoglycan, heparin involves in diverse physiological processes through interacting with proteins with heparin-binding domains [18]. In addition, heparin also exhibits various anticancer activities in tumor progression and metastasis [19,20]. In the current study, the negatively charged heparin chains endow the nano-sized drug delivery system with good stability in the presence of serum [17,20]. As a cell growth promoter, biotin (vitamin B7) has been used as tumor targeting ligands since biotin receptors are often overexpressed on the surface of rapidly proliferating cancer cells [21]. In the current study, biotin-heparin was introduced to the nanoparticles to realize biotin mediated tumor targeting delivery. The inorganic compounds, $CaCO_3$ and CaP, not only improve the drug loading and release properties but also have favorable pH sensitive dissolution behavior to facilitate the intracellular drug release [17,22].

Two drug resistance inhibitors, celecoxib (CXB) to down-regulate P-gp expression and buthionine sulfoximine (BSO) to inhibit GSH synthesis were co-loaded in our nano-sized delivery system to reverse MDR in drug resistant tumor cells. CXB is a potent nonsteroidal anti-inflammatory drug that exhibits analgesic and antipyretic activities, which can block the activity of COX-2 enzyme. As a COX-2 inhibitor, CXB can enhance the sensitivity of cancer cells by down-regulating P-gp expression [23–28]. BSO is a potent and selective inhibitor of GSH synthesis, which selectively interacts with the enzyme γ-glutamylcysteine synthetase [29]. BSO is able to enhance the cytotoxic effect of various drugs in cancer cells [30,31]. Our study shows the P-gp and GSH levels in drug resistant cells can be efficiently down-regulated after being treated by the dual-inhibitor co-delivery system. As a result, a significantly improved tumor cell inhibition can be achieved if the cells are further treated by doxorubicin loaded nanoparticles, indicating that the dual-inhibitor co-delivery system can effectively reverse drug resistance. Our study demonstrates the co-delivery of multiple drug resistance inhibitors by polymer/inorganic hybrid nanoparticles is a promising strategy to reverse drug resistance in tumor treatments.

2. Experimental

2.1. Materials

Heparin (HP) (sodium salt, M_w = 6000–20000 g/mol, 185 USP units/mg) was supplied by Aladdin Chemistry Co. Ltd. (Shanghai, China). Celecoxib (CXB) and heparin-biotin (HPB) (sodium salt, M_w = 15000 g/mol, degree of substitution = 10%) were obtained from Sigma-Aldrich. Doxorubicin hydrochloride (DOX) was from Zhejiang Hisun Pharmaceutical Co. Ltd. (China). Buthionine sulfoximine (BSO) was supplied by Santa Cruz Biotechnology Co., Ltd. (Shanghai, China). Calcium chloride ($CaCl_2$), anhydrous sodium carbonate (Na_2CO_3), sodium phosphate dibasic dodecahydrate ($Na_2HPO_4{\cdot}12H_2O$) and dimethyl sulfoxide (DMSO) supplied by Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) were of analytical grade and used as received. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Amresco.

Drug resistant MCF-7/ADR cells were from KeyGEN Biotech Co. Ltd. (Nanjing, China) and were cultured in RPMI-1640 (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mg/ml $NaHCO_3$, and 100 U/ml antibiotics (penicillin–streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere. To maintain the drug resistance, MCF-7/ADR cells were co-incubated with DOX with a concentration of 1 μg/ml. Nonresistant MCF-7 cells and HeLa cells were obtained from China Center for Typical Culture Collection (Wuhan, China). MCF-7 cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 mg/ml $NaHCO_3$, and 100 U/ml antibiotics (penicillin–streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere. HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS, 2 mg/ml $NaHCO_3$, and 100 U/ml antibiotics (penicillin–streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Preparation of blank nanoparticles and drug loaded nanoparticles

HPB/HP/$CaCO_3$/CaP nanoparticles (BNP) were prepared by following procedures. 20 μl of Na_2CO_3 solution (0.02 M) and 15 μl of Na_2HPO_4 solution (0.02 M) were mixed to obtain solution **A**. 35 μl of $CaCl_2$ solution (0.02 M), 36 μl of HP solution (4 mg/ml) and 4 μl of HPB solution (4 mg/ml) were mixed and stirred to obtain solution **B**. Then solution A was added into the solution **B** dropwise. The mixture was stirred for 5 min at room temperature to obtain HPB/HP/$CaCO_3$/CaP nanoparticles (abbreviated as BNP).

For comparison, HP/$CaCO_3$/CaP nanoparticles (abbreviated as NP) were prepared in the absence of HPB by a similar procedure. During the preparation, 40 μl of HP solution (4 mg/ml) was used. Other conditions were the same as that for the preparation of BNP.

DOX loaded HPB/HP/$CaCO_3$/CaP nanoparticles were prepared by as follows. 20 μl of Na_2CO_3 solution (0.02 M) and 15 μl of Na_2HPO_4 solution (0.02 M) were mixed to obtain solution **C**. 35 μl of $CaCl_2$ solution (0.02 M), 36 μl of HP solution (4 mg/ml), 4 μl of HPB solution (4 mg/ml), and 12 μl of DOX (2 mg/ml) were mixed and stirred to obtain solution **D**. Then solution **C** was added into the solution **D** dropwise. The mixture was stirred for 5 min to obtain DOX loaded HPB/HP/$CaCO_3$/CaP nanoparticles (abbreviated as DOX@BNP). The solution containing nanoparticles was centrifuged at 15,000 rpm for 5 min and the non-encapsulated drug in the supernatant was removed.

For comparison, DOX loaded HP/$CaCO_3$/CaP nanoparticles (abbreviated as DOX@NP) were prepared by a similar procedure in the absence of HPB.

BSO and CXB co-loaded HPB/HP/$CaCO_3$/CaP nanoparticles were prepared by as follows. 100 μl of Na_2CO_3 solution (0.02 M) and 75 μl of Na_2HPO_4 solution (0.02 M) were mixed to obtain solution **E**. 175 μl of $CaCl_2$ solution (0.02 M), 180 μl of HP solution (4 mg/ml), 20 μl of HPB solution (4 mg/ml), 3.2 μl of CXB solution (10 mg/ml), and 98.3 μl of BSO solution (4 mg/ml) were mixed and stirred to obtain solution **F**. Then solution **E** was added into the solution **F** dropwise. The mixture was stirred for 5 min to obtain BSO and CXB co-loaded HPB/HP/$CaCO_3$/CaP nanoparticles (abbreviated as BSO/CXB@BNP). The solution containing nanoparticles was cen-