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Encapsulation of verapamil and doxorubicin by MPEG-PLA to reverse drug resistance in ovarian cancer

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ABSTRACT

Purpose: Ovarian cancer is usually treated with transurethral resection or systemic chemotherapy in clinic. However, the development of drug resistance in ovarian cancer is frequently observed in ovarian cancer patients, leading to failure of tumor inhibition and recurrence. In this study, we aimed to efficiently reverse the drug resistance and enhance the anticancer effects by co-delivery of chemotherapeutic agents and multi-drugs resistant proteins inhibitor in ovarian cancer treatment.

Methods: The cell viability was measured by using MTT or flow cytometry (Annexin V/PI staining) under different culture conditions. Western blot was used to detect the expression of P-gp. We employed confocol to visualize the drug distribution under different culture systems. Using flow cytometry, we examined the drug absorption. MPEG-PLA was used to load chemotherapeutic drugs. We also applied mice model to evaluate the killing ability and side effects of free or methoxy poly (ethylene glycol)-poly (l-lactic acid) (MPEG-PLA) loaded drugs.

Results: We found that pre-treatment of verapamil, a multi-drugs resistant proteins inhibitor, could efficiently reverse the drug resistant in ovarian cancer. To further improve the pharmacokinetics profiles and avoid the systemic toxicity caused by agents, we encapsulated verapamil and doxorubicin (DOX) by polymeric nanoparticles MPEG-PLA. Co-delivery of verapamil and DOX by nano-carrier revealed reduced drug resistance and enhanced anticancer effects compared with the free drug delivery. More importantly, accumulated drugs, prolonged drug circulation and reduced systemic were observed in nanoparticles encapsulation group.

Conclusion: Co-delivery of verapamil and chemotherapeutic drugs by MPEG-PLA efficiently reversed the drug resistance, resulting in enhanced anticancer effects along with reduced systemic toxicity, which provides potential clinical applications for drug resistant ovarian cancer treatment.

1. Introduction

Ovarian cancer is one of the most common malignancies in women with a leading cause of death in gynecological tumors [1,2]. Due to clinical advances in surgical treatment and chemotherapy, many ovarian cancer patients show positive prognosis in early stage. However, in advanced metastatic carcinoma, especially in recurrence after chemotherapy, chemotherapeutic agents show limited curative effects along with severe systemic toxicity and drug resistance development [3–5]. Hence, innovative approaches to reverse drug resistance and to enhance anticancer effects are urgent needs for ovarian cancer treatment in clinic.

It has been demonstrated that the development of drug resistance is controlled by various curial factors [6], including the overexpression of multi-drug resistance protein like p-glycoprotein (P-gp) [7], up-regulation of anti-apoptotic protein Bcl-2 [8], activation of pro-survival signaling pathways and so on [9]. Among these mechanisms, the up-regulation of P-gp encoded by the *ABCB1* is the most commonly encountered in chemo-resistant patients. P-gp, a promiscuous drug transporter, has a remarkable capacity to bind and transport a wide array of cytotoxicity drugs from cytoplasm to extracellular environment, resulting in the lower intracellular drug concentration and drug resistance development [7]. Verapamil, a derivative of papaverine, has been reported to serve as an inhibitor of P-gp *in vitro* [10]. Several chemotherapeutic agents are reported to be more efficient in tumor suppression when combined with verapamil [11–13]. However, the low

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bioavailability and potential systemic toxicity limited the application of verapamil in tumor therapy.

In recent years, polymeric nanoparticles are considered as the favorable drug carriers to encapsulate chemotherapeutic agents [14]. Soluble nanoparticles could efficiently facilitate the drugs aggregation in tumor sites by the enhanced permeability and retention effects and the controlled drug release induced by pH, resulting in enhanced anticancer effects and reduced systemic toxicity *in vivo* [14,15]. MPEG-PLA, an amphiphilic polymer, has attracted increasing attention due to its biodegradable ability and satisfactory drug delivery efficiency. In addition, MPEG-PLA has been known to form inclusion complex with various chemotherapeutic agents, which shows extensive applications and the ability of solubilizing insoluble compounds [16].

Hence, we co-encapsulated verapamil and doxorubicin (DOX) in MPEG-PLA nanoparticles to reverse the drug resistance in ovarian cancer. The characterization, anticancer effects and systemic toxicity of the prepared nanoparticles were evaluated detailed in our study. We firstly used MPEG-PLA nanoparticles to co-deliver the DOX and P-gp inhibitor to reverse the drug resistances in ovarian cancer. Compared to previous nanoparticles, MPEG-PLA reveals high biocompatibility and superior safety. We also demonstrated the enhanced anticancer effects of verapamil and DOX loaded nanoparticles, with a sufficient evaluation of safety *in vivo*. Our results revealed that co-delivery of verapamil and chemotherapeutic agents encapsulated by MPEG-PLA nanoparticles might serve as an innovative approach in drug resistant ovarian cancer therapy.

2. Materials and methods

2.1. Regents and cell lines

MPEG-PLA (MPEG: PLA molar ratio = 50:50, molecular weight = 4000 g/mol) were purchased from Daigang (Jinan, China). DOX and verapamil were purchased from Sigma (San Francisco, USA). Other chemicals and solvents were purchased from Solarbio (Beijing, China). Rat myocardial cell line H9c2 was purchased from American Type Culture Collection (Maryland, USA) and supplemented with DMEM culture medium (Gibco, California, USA) with 10% fetal calf serum (Gibco, California, USA), at 37 °C in a 5% CO_2 atmosphere. Human ovarian cancer cells A2780 and SKOV3 were purchased from American Type Culture Collection (Maryland, USA) and supplemented with RPMI-1640 complete culture medium (Gibco, California, USA) containing 10% fetal calf serum (Gibco, California, USA), at 37 °C in a 5% CO_2 atmosphere. The DOX resistant ovarian cancer cell lines SKOV3/DOX^R and A2780/DOX^R were established by culturing cells with DOX in a dose-escalation manner using long-term exposure intervals. Initial cultures of A2780 or SKOV3 were supplemented with 1 nM DOX. After sensitive clones were no longer present and surviving A2780 or SKOV3 cells repopulated the flask, the concentration of doxorubicin was increased to 2, 4, 10, 25, 50, 100, and 200 nM. The process of acquired drug resistance took 6 months for A2780/DOX^R cell line establishment and 7 months for SKOV3/DOX^R cells.

2.2. Cell viability analysis

Cell viability was determined by MTT assay kit (Solarbio, Beijing, China) and Annexin V/PI staining kit (BD, NY, USA). Briefly, 2000 cells were seeded into 96-well culture plates for 12 h to adhere. Next, cells were treated with different concentrations of chemotherapeutic drugs for 2 h pre-treatment with or without 5 μM verapamil. After 48 h, cell growth was measured after addition of 10 μl 0.5 mg/ml MTT solution. After 4 h incubation at 37 °C, the medium was replaced with 100 μl dimethylsulfoxide and vortexed for 10 min. Absorbance (A) was measured at 570 nm.

The Annexin V/PI staining kit was performed as guided. Briefly, the treated cells were collected and washed with PBS. Then the cells were resuspended by 100 μl staining buffer with Annexin V/PI. The cells were kept in room temperature for 15 min. An flow cytometry (BD, NY, USA) was used to analyze the apoptosis of cells.

2.3. Western blot

Whole cell lysates were prepared from A2780, A2780/DOX^R, SKOV3 and SKOV3/DOX^R cells (10^6 cells each group) and 10 μg proteins were separated by SDS-PAGE at 100 V for 2 h. Separated proteins were then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% BSA in TBS containing 0.1% Tween-20 for 1 h at room temperature. Then the membranes were incubated with anti-P-gp antibody (Abcam, 1:1000, Cambridge, UK) or anti β-actin antibody (Abcam, 1:1000, Cambridge, UK) overnight at 4 °C. The membranes were washed three times and incubated with HRP-conjugated secondary antibodies (Abcam, 1:1000, Cambridge, UK). Proteins were visualized by ECL Western blotting substrate (Thermo, MA, USA).

2.4. Preparation of DOX and verapamil loaded nanoparticles

DOX loaded MPEG-PLA nanoparticles preparation: To obtain the DOX loaded MPEG-PLA nanoparticles, 95 mg MPEG-PLA and 5 mg DOX were co-dissolved in 2 ml dichloromethane, followed by evaporation under reduced pressure in a rotary evaporator at 60 °C. Next, the film was rehydrated in 500 ml PBS (pH = 7.4), allowing the self-assembly of DOX loaded MPEG-PLA nanoparticles.

DOX and verapamil loaded MPEG-PLA nanoparticles preparation: 95 mg MPEG-PLA, 5 mg DOX and 1 mg verapamil were co-dissolved in 2 ml dichloromethane, followed by evaporation under reduced pressure in a rotary evaporator at 60 °C. Next, the film was rehydrated in 500 ml PBS (pH = 7.4), allowing the self-assembly of nanoparticles.

Drug loading (DL) and encapsulation efficiency (EE) of co-delivery nanoparticles were calculated from the following formulas:

Drug loading (DL) = Weight of drug in nanoparticles/weight of nanoparticles.

Encapsulation efficiency (EE) = Weight of drug in nanoparticles/weight of drug total used.

2.5. Particle size analysis and morphology study

The particle size of nanoparticles was detected by Malvern Nano ZS90 (Malvern Instruments, UK). The measuring progress was carried out under the temperature of 25 °C. The morphology of the prepared nanoparticles was studied with transmission electron microscope (Hitachi Ltd., Tokyo, Japan). Briefly, distilled water was used to dilute the nanoparticles and the samples were placed on a copper grid covered with nitrocellulose. Samples were negatively stained with phosphotungstic acid and dried at room temperature.

2.6. Drugs release study

In vitro drug release behaviors of DOX, and verapamil in nanoparticles were determined by a dialysis method. Briefly, 2 ml DOX or verapamil in PBS, and DOX and verapamil loaded MPEG-PLA nanoparticles were placed in dialysis bags (molecular weight cutoff, 3.5 kDa). The dialysis bags were incubated in 20 ml PBS containing 2 ml FBS (37 °C and pH = 5.0 or 7.4) in a 50 ml tube with shaking at 100 rpm. At predetermined time points, 2 ml of release media was collected for further analysis, and the incubation mediums need to be replaced with fresh incubation mediums. The amount of released drugs was quantified by high-performance liquid chromatography (HPLC, Waters 2695; Waters Corporation, USA). Analysis of verapamil was carried out using a Varian HPLC system equipped with a ternary pump