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An experimental study of ovarian cancer imaging and therapy by paclitaxel-loaded phase-transformation lipid nanoparticles combined with low-intensity focused ultrasound

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ABSTRACT

Drug-loaded phase-transformation lipid nanoparticles (NPs) combined with low-intensity focused ultrasound (LIFU) for ultrasound molecular imaging and therapy, which is a very promising drug carrier and can provide both physical and chemical therapeutics, simultaneously. We successfully prepared the paclitaxel (PTX) loaded anti-LHRHR targeted phase-transformation lipid nanoparticles (PTX-*anti*-LHRHR-PTNPs) for ovarian cancer in this study combined with LIFU has the following characteristics: On the one hand, it showed smaller size and greater stability than blood cells, which significantly prolonged its half-life in the body, and can actively target ovarian cancer OVCAR-3 cells, and smoothly penetrate the endothelial gap into the tumor site for specifically killing the ovarian cancer cells. Thereby, the special drug carrier improved the therapeutic effect and reduced toxic and side effects, maximized the protection of normal tissues and minimized adverse reactions. On the other hand, PTX-*anti*-LHRHR-PTNPs can be targeted to focus after being injected intravenously and remain in the tumor target tissue for a long time. At the same time, liquid-gas phase-transformation can occur under LIFU triggering, resulting in more ideal and sustained ultrasound imaging effects. Then acoustic contrast agent is used to develop the molecular level of ultrasound scattering, so as to evaluate the diseased tissue from the molecular level.

1. Introduction

Improving the early diagnosis and treatment of ovarian cancer and reducing the toxic and side effects of chemotherapeutic drugs are the current research hotspots. Ultrasonic molecular imaging is targeted ultrasound contrast agent as probe, which can show inflammation, thrombosis and tumor angiogenesis at the molecular level for the targeted treatment to target sites [1–3]. Compared with traditional CT and MRI, ultrasonic molecular imaging has the advantages of non-invasive, non-toxic, non-radioactive pollution, real-time dynamic and so on [4,5]. Ultrasonic molecular probe can be used not only for diagnosis, but also as a new drug or gene carrier. Simultaneously, based on the intrinsic chemical properties of the molecular probes surface, the specific ligands or antibodies can be attached to the surface of the contrast agent by covalent or non-covalent attachment methods [6–8]. A considerable number of studies have found that 80% of ovarian cancer cells have a high expression level of luteinizing hormone-releasing hormone (LHRH), but almost non expression in normal cells, which provided the possibility of targeting ovarian cancer [9]. Sun et al. [10] used receptor-ligand binding to prepare LHRH-MBs in anticipation of targeting ovarian cancer. However, the half-life of LHRH is very short (2–3min) *in vivo*, and it is easy to cleave. For this reason, we chose the luteinizing hormone-releasing hormone receptor antibody (anti-LHRHR), and used the antigen-antibody binding method

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to specifically bind the LHRH receptor antibody to the LHRH receptor.

A large number of literature have verified that ultrasound-targeted microbubble destruction (UTMD) technology combined with the targeted drug-loaded microbubbles can perform targeted tumor therapy, providing a new treatment method for malignant tumors [11–13]. However, microbubble ultrasound contrast agent such as SonoVue, whose particle size is larger and called micron microbubble (2–5 μm). Because it is difficult to penetrate the tumor vascular endothelium (380–780 nm) to reach the tumor cells or tissues to achieve molecular level imaging, it can only be used as a blood pool contrast agent, and the microbubble has a short time *in vivo*, which limits its application in ultrasonic molecular imaging [14,15]. R.E.Apfel first proposed a new type of ultrasound carrier in 1998, which is now widely known as phase-transformation droplets. And it is expected to make up for the shortcomings of microbubbles in the treatment. PTX-*anti*-LHRHR-PTNPs were injected into the blood circulation through intravenous injection, which were bound to the LHRH receptor on OVCAR-3 cells of ovarian cancer gathered to the tumor. Subsequently, when the tumor was irradiated by LIFU, resulting in liquid-gas phase-transformation of PFP wrapped in the phospholipid bilayer. It is gradually transformed into a nanometer micro bubble with gas as the core, increasing the acoustic impedance of its surrounding tissue, thus greatly enhancing the effect of ultrasonic development. At the same time, PTX was released, killing ovarian cancer cells efficiently and specifically, and reducing the toxic and side effects on normal tissues. In summary, this study aims to prepare the PTX-*anti*-LHRHR-PTNPs, which could be stable, uniform particle size, ultrasound imaging and treatment, for the early diagnosis and treatment of ovarian cancer experimental study.

2. Materials and methods

2.1. Preparation of NPs

Add DSPE-PEG2000-Biotin (2 mg), DPPC (5 mg), DPPA (1 mg), cholesterol (1 mg) and PTX (3 mg) to a 50 ml round bottom flask. Continue adding 9 ml Methyl alcohol and trichloromethane (Methyl alcohol: trichloromethane = 1:2), oscillate until the organic solvent is clarified. The round bottom flask was fixed on a rotary evaporator (50 °C, 120 rpm/min) and vacuum-rotated for 2 h to remove the organic solvent and dry the phosphatide and paclitaxel mixture into thin and uniform membrane. The phospholipid membrane was hydrated by the addition of PBS (2 ml) to give a milky white suspension. Subsequently, 100 μl PFP was added into the suspension, the milky white PTX nano-emulsion containing PFP was prepared by acoustic vibration method. Centrifuging for 5 min at 4 °C, 8000 rpm, washing 3 times, resuspending in 2 ml PBS and storing at 4 °C for later use. Before the experiment, it was taken out of the refrigerator and added to the 20 μl Biotin-Streptavidin (5 mg/ml). The EP tube was placed on a thermostatic oscillator (ice bath water), oscillated and incubated for 30 min, and centrifuged for 3 times and the PTX-PTNPs were obtained. Add 10 μl of biotinylated LHRHR antibody to the prepared PTX-PTNPs, oscillated and incubated for 30 min, and centrifuged for 3 times and the PTX-*anti*-LHRHR-PTNPs was obtained.

2.2. Characterization of NPs

Firstly, the surface morphology and distribution of NPs were observed under optical microscopy. Secondly, the laser particle potentiometer is used to detect the particle size and potential of NPs. Thirdly, the structure of NPs was observed by transmission electron microscopy.

The high performance liquid chromatography (HPLC) was used to detect the encapsulation efficiency (EE, which is defined as the percentage of PTX encapsulated in NPs from the total amount of PTX used initially) and drug-loading efficiency (LE, which is defined as the percentage of PTX encapsulated in the NPs versus the total amount of NPs) of PTX-loaded NPs.

The *in vitro* sustained release capacity of PTX-PTNPs were detected by dialysis method. The first step, the previously prepared PTX-PTNPs was taken out, centrifuged, resuspended in PBS and 1 ml of the suspension was taken into the dialysis bag (Molecular weight cutoff: 8000Da). The second step, the two ends of the dialysis bag were occluded, which immersed in a blue bottle containing 150 ml of sustained release medium (30% ethanol, 0.01% Tween-80, 0.02% sodium azide). The third step, the experimental divided into PTX-PTNPs group (control group) and PTX-PTNPs + LIFU group. For the PTX-PTNPs + LIFU group, the experimental conditions were as follows: the dialysis bag was placed on an agar gel, the focusing probe was about 2.8 cm away from the dialysis bag, pulse wave (5 W, 20 min; working 5 s, intermittent 5 s). The control NPs do not need to be treated. The fourth step, the blue bottle was placed in a constant temperature vibrator for continuous oscillation (37 °C, 120 rpm). Taken out 1 ml of dialysate at different time points and stored in −20 °C refrigerator, then 1 ml of fresh sustained release medium was added. The fifth step, the content of PTX in sustained release dialysate at different time points was detected by HPLC method. The last step, the cumulative release percentage (%) of PTX in the two groups of PTX-PCNPs at different time points was calculated, and the PTX release time curve was plotted.

2.3. Cell culture

The human ovarian cells (A2780 cells, OVCAR-3 cells) were cultured in RPMI-1640 medium (Gibco Invitrogen, UK) plus 10% FBS at 5% CO_2 atmosphere and at 37 °C. Passaging every 2–3 days, and taking the logarithmic growth phase cells for experiment.

2.4. Cytotoxicity, killing efficiency and targeting efficiency

A2780 cells were plated 1×10^4 cells/well in 96-well plates, the cells were cultured for 24 h and then treated with 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1.0 mg/ml, 1.5 mg/ml PTNPs, respectively. At the same time, the RPMI-1640 medium control group and the untreated normal cell control group (blank group) were set up. After 24 h, 10 μl CCK8 reagents were added to each hole and incubated at 37 °C for 2 h. The absorbance (A) of each hole was measured at the wave length of 450 nm with a full automatic enzyme labeling instrument, and cell vitality was calculated. Cell vitality (%) = $(A_{\text{Experimental group}} - A_{\text{Blank group}})/(A_{\text{Control group}} - A_{\text{Blank group}}) \times 100\%$.

According to the processing factors of cells, the experiment was divided into PTX, PTX-*anti*-LHRHR-PTNPs and PTX-*anti*-LHRHR-PTNP + LIFU. Based on the concentration of PTX, each group was further divided into 0.0375 mg/ml; 0.075 mg/ml; 0.15 mg/ml; 0.3 mg/ml; 0.45 mg/ml. It is emphasized that the LIFU instrument treatment probe coated with the couplant is placed at the bottom of the culture plate (5 MHz, 3 min). Then, the cell proliferation was detected by CCK8 method to reflect the *in vitro* killing effect of PTX-*anti*-LHRHR-PTNPs combined with LIFU on ovarian cancer cells. The specific operation steps of the CCK8 method are the same as above.

The cells were seeded in a petri dish at a density of 1×10^4 cells/well, and the experiment was carried out after the cells were attached 24 h. Taking a dish of A2780 cells, pre-adding a saturated amount of LHRHR antibody, and incubating for 20 min. After repeated washing with PBS, added PTX-PTNPs solution carrying