Calcium Phosphorus Nanoparticles Coloaded with Paclitaxel and ADAM-10-siRNA for the Treatment of Triple Negative Breast Cancer

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1. Introduction

Triple negative breast cancer (TNBC) refers to breast cancer that is not positive for estrogen (ER), progesterone (PR), and human epidermal growth factor receptor (Her-2) leading to a poor clinical treatment effect [1–3]. TNBC patients account for approximately 15-20% of diagnosed breast cancer patients for whom endocrine therapy and targeted therapy are both ineffective [4]. Triple-negative breast cancer is extremely special, and it is prone to metastases to the liver, lung, brain, and other organs, endangering other parts, and can be life-threatening in severe cases [5]. Treatment of TNBC is a huge challenge.

Chemotherapy is still one of the main treatment strategy and the most used drug for TNBC [6]. PTX is the first-line drug for the clinical treatment of breast cancer [7, 8]. After the tumor cells are exposed to paclitaxel, a large number of microtubules will be accumulated in the cells. The accumulation of these microtubules interferes with various functions of the cells, especially the cell division stops in the mitotic phase and blocks the normal division of the cells. It is also widely used as monotherapy or combination therapy in the treatment of TNBC [9]. However, due to the poor water solubility, nonspecific, and low bioavailability of PTX which were the key challenge for selective targeting therapy, it is easy to form the body’s chemotherapy resistance [10–12]. Therefore, there is an urgent need to develop targeted and effective therapeutic approaches to inhibit TNBC.

A disintegrin and metalloproteinase 10 (ADAM10) is a member of ADAM sheddases, which belong to matrix metalloproteinases (MMPs) [13]. ADAM family members are uniquely structured cell surface proteins with potential adhesion and protease domains [14]. This gene encodes a member of the ADAM family that cleaves a variety of proteins, including TNF-α and E-cadherin [15]. Alternate splicing results in multiple transcript variants encoding distinct proteins that may undergo similar processing [16]. In recent years, the role of ADAM10 in breast cancer has gradually
attracted attention, especially the human epidermal growth factor receptor 2 (HER2) enriched subtype [17]. ADAM10 is thought to be one of the major proteases that cleave and shed the HER2 receptor ectodomain [18, 19]. It is reported that ADAM10 inhibitor can inhibit breast tumor metastasis and suppress drug resistance [20]. Free siRNA is easily degraded, and lipofectamine is the main method for delivering siRNA, but it has strong cationic toxicity, so it cannot be widely used in clinical practice [21, 22]. On this basis, we used gene interference technology and nanotechnology to simultaneously encapsulate the small interfering RNA (siRNA) of ADAM10 with the chemotherapeutic drug PTX to inhibit the growth of breast cancer.

In this project, copolymer DSPE-PEG2K was used to encapsulate PTX, and then, calcium phosphate adsorbed siADAM10 to deposit on the outside, forming CaP-PTX/siADAM10 NPs of core-shell structure [23–25]. CaP-PTX/siADAM10 NPs can be passively targeted by TNBC through the enhanced permeability and retention (EPR) effect due to leak of vasculature damaged lymphatic system [26, 27]. The PEG chains in the outer layer of the nanoparticles prevent the immune system from engulfing them and thus increase their systemic circulation time. The calcium-phosphorus shell is rapidly degraded under acidic conditions to release siADAM10, which promotes the drug sensitivity of MDA-MB-231 cells to PTX [28]. The internal PTX is then released to promote tumor apoptosis to exert an antitumor effect.

2. Materials and Methods

2.1. Materials. DSPE-PEG2K was purchased from Xi’an ruixi Biological Technology (China, Xi’an). ADAM10-siRNA (siADAM10) and human GAPDH-siRNA (siGAPDH) were purchased from RiboBio (China, Guangzhou). siADAM10 sequences are as follows: 5′-GCAGACTCTGAGGAAGTTGT-3′ (forward) and 5′-ACAGGACACAGGAAGACCG-3′ (reverse). siGAPDH sequences are as follows: 5′-CGAGATCCTGAGGAAGTTGT-3′ (forward) and 5′-TTACACCATGACCACTA-3′ (reverse). Paclitaxel (PTX) was purchased from Sigma-Aldrich (USA). Anti-BAX and anti-Bcl-2 antibodies were purchased from Cell Signaling Technology (USA). MDA-MB-231 was bought from ATCC (Rockville, MD, USA). Fetal bovine serum (FBS), DMEM, and antibiotics (penicillin/streptomycin) were purchased from Gibco.

2.2. Preparation of Nanoparticles. PTX NPs were prepared by ultrasonic emulsification and self-assembly of DSPE-PEG2K, which was amphiphilic block copolymers [29, 30]. In brief, DSPE-PEG2K was dissolved in trichloromethane with PTX; then, water was added to get mixture via ultrasonic. Organic phase was removed by rotary evaporation. 500 μg/ml siADAM10 was mixed with 500 mM CaCl2, then, 500 μl PTX NP solution was added in with quick mixing. At last, buffer solution (pH 7.4) containing 140 mM NaCl, 50 mM HEPES, and 15 mM Na2HPO4 was added into the mixture, which was stirred for 30 min. Then, CaP-PTX/siADAM10 NPs were purified by ultracentrifugation [29].

2.3. Characterization of CaP-PTX/siADAM10 NPs. The size and zeta potential of CaP-PTX/siADAM10 NPs in aqueous solution were detected by DLS instrument (Malvern Zetasizer Nano S-90). The morphology was analyzed by TEM (Hitachi H-7500). The sample solution was stained with 2 wt% phosphotungstic acid aqueous solution for 30 sec.

2.4. Binding Assay. To verify the function of anti-ICAM as target of nanoparticle, we incubated MDA-MB-231 cells with PBS, free Cy5-siRNA, and CaP-Cy5-siRNA NPs for 30 min at 4°C. Then, cells were washed three times with PBS. Flow cytometry was used to analyze Cy5-positive fluorescence intensity.

2.5. Drug Release In Vitro. CaP-PTX/siADAM10 NPs were added into dialysis bags (Millipore, USA) in PBS for release profile research at pH 7.4, 6.0, and 5.0 for 24 h.

2.6. Cell Uptake. MDA-MB-231 cells were seeded into a 24-well plate at a density of 1×105, cultured with DMEM complete medium, and replaced with basal medium after 36-48 h, respectively, at 2 h and 6 h. Cy5-siRNA was used to label nanoparticles. Cells were washed twice with PBS and then fixed with 4% paraformaldehyde (300 μl for 20 min). Next, paraformaldehyde was discarded and cells were washed once more with PBS. Hoechst 33342 was used to stain the nucleus for 20 min. The slides were numbered, and 8 μl/slide of glycerol was added dropwise, and the samples were observed with a laser confocal fluorescence microscope (Beckman Coulter, Gallios).

2.7. Cell Viability Detection In Vitro. CCK-8 assay was used to evaluate the cell viability of CaP-PTX/siADAM10 NPs on MDA-MB-231. MDA-MB-231 cells were plated into 96-well plate and incubated for 12 h. Then, cells were treated with PBS, PTX, CaP-PTX/siNC NPs, and CaP-PTX/siADAM10 NPs separately for 48 h.

2.8. Quantitative Real-Time PCR Assay (qRT-PCR). To quantify the expression level of ADAM10, RT-qPCR assay was used to analyze the gene silence ability. The data was calculated and analyzed by delta-delta Ct (2−ΔΔCt). ΔCt = the Ct value of the target gene – Ct value of GAPDH.

2.9. Western Blot Analysis. The protein of cells was collected and detected by BCA Protein Assay Kit (Beyotime). Other methods were followed standard methods. The primary antibodies to bcl-2 and bax were bought from Cell Signaling Technology (USA).

2.10. Animal Model. About 1×106 MDA-MB-231 cells were injected into the fourth mammary fat pad of balb/c nude mice to build orthotopic breast cancer model. After 2 weeks, tumors were grown to 100 mm3.

2.11. Drug Distribution Analysis. To research the biodistribution of nanodrug delivery system, orthotopic breast cancer model mice were administrated with free DiR and CaP-DiR/siNC NPs via tail vein injection (DiR, 0.4 mg/kg). Then, the mice were imaged at 8 h, 24 h, and 48 h time point by small animal live imaging system (Caliper, Hopkinton,
MA). After the last imaging, the mice were sacrificed, and their main organs (heart, liver, spleen, lung, and kidney) and tumors were collected and imaged.

2.12. Pharmacokinetics of CaP-PTX/Cy5-siRNA NPs. In order to confirm the stability of the in vivo fluorescence intensity of the nanoparticles, we carried out the verification
in rats. The rats were randomly divided into two groups, each group of 5 rats, and the free Cy5-siRNA and CaP-PTX/Cy5-siRNA NPs were injected into the tail vein, respectively (100 nM/kg siRNA). Blood samples of rats were collected at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, and 48 h, respectively, and the fluorescence intensity of siRNA was measured by fluorescence spectrophotometer (HITACHI, F-7000).

### 2.13. Therapeutic Effects In Vivo.

When the volume of tumors reached 100 mm³, the mice were divided into four groups and administrated with PBS, PTX, CaP-PTX/siNC NPs, and CaP-PTX/siADAM10 NPs (2.5 mg/kg) for twice a week [31, 32]. Changes in mouse body weight and tumor volume were recorded at each administration. TUNEL and H&E were utilized to evaluate the therapeutic efficacy.


Main organs (heart, liver, spleen, lung, kidney) and serum were collected after the last treatment to examine the toxicity and liver and kidney function by H&E staining.

### 2.15. Statistical Analysis.

All statistical significance analyses were performed via t-test by Graph Prism 8.0. \( \ast \ P < 0.05, \ast\ast \ P < 0.01, \ast\ast\ast \ P < 0.001 \).

### 3. Results and Discussion

#### 3.1. Preparation and Characterization of Nanoparticles.

As shown in Figure 1(a), PTX NPs were prepared by self-assembly. Calcium phosphate adsorbs ADAM10 siRNA (siADAM10) and deposits on the outer layer of PTX NPs to form CaP-PTX/siADAM10 NPs, which were used for tail vein administration in mice. The CCK-8 experiment confirmed

![Figure 2: Cell uptake and therapy effect to MDA-MB-231 cells in vitro. (a) Cell uptake of CaP-Cy5-siRNA NPs by MDA-MB-231 cells evaluated by CLSM. (b) Cell viability of MDA-MB-231 treated by PBS, PTX, CaP-PTX/siNC NPs, and CaP-PTX/siADAM10 NP analysis of by CCK-8 for 72 h. (c, d) Expression of bcl-2 (c) and bax (d) level was detected by western blot. (e) Quantity analysis of (c). (f) Quantity analysis of (d). (g) ADAM10 expression level was evaluated by RT-qPCR. Scale bar, 50 μm. Data as means ± SD (\( \ast \)P < 0.05, \( \ast\ast \)P < 0.01, \( \ast\ast\ast \)P < 0.001).](https://example.com/figure2.png)
that the blank NPs and blank calcium-phosphorus NPs had no obvious cytotoxicity to MDA-MB-231 cells, indicating that the calcium-phosphorus drug loading system has good biological safety (Figure S1). The diameter of CaP-PTX/siADAM10 NPs was about $10^{3.5} \pm 1.6$ nm, and the surface charge was $-10^{9.5} \pm 2.3$ mV (Figures 1(b) and 1(c)). Transmission electron microscopy (TEM) results showed that the CaP-PTX/siADAM10 NPs were uniformly spherical (Figure 1(d)).

Then, we carried out binding assay to detect the cell targeting of nanoparticles by flow cytometry; the fluorescence intensity of Cy5 was much stronger compared to free Cy5-siRNA (Figure 1(e)). In addition, we tested the stability of CaP-PTX/siADAM10 NPs at different temperatures (4, 25, and 37°C), and the results showed that there was no significant change in particle size within 72 h (Figure 1(f)). Under acidic conditions, the calcium phosphate shell is rapidly degraded to release the siRNA, and the PTX inside the nanoparticles is subsequently released. Therefore, we investigated the release amounts of siRNA and PTX from nanoparticles under different pH conditions. The results showed that at 48 h, at pH 6.0 and 5.0, the release amounts of PTX and siRNA were significantly higher than those at pH 7.4. And the release amount of siRNA is higher than that of PTX, indicating that the release of siRNA located in the outer layer of the nanoparticle is greater than that of PTX located in the inner layer (Figure 1(g)).

3.2. Antitumor Effects In Vitro. Nanoparticle uptake ability by MDA-MB-231 cells affects the function of the encapsulated drug. Thus, we detected cellular uptake of CaP-Cy5-siRNA NPs by CLSM at different time points. Red fluorescence of Cy5 was higher when MDA-MB-231 cells were incubated with CaP-Cy5-siRNA for 6 h compared to 2 h, indicating that the nanoparticles were uptaken by cells effectively (Figure 2(a)). CCK-8 assay was utilized to evaluate cell viability of MDA-MB-231 cells treated with PBS, PTX, CaP-PTX/siNC NPs, and CaP-PTX/siADAM10 NPs at 24, 48, and 72 h. As shown in Figure 2(b), cell toxicity of PTX was upregulated when encapsulated by nanodrug delivery system with siNC. CaP-PTX/siADAM10 NPs achieved highest inhibition of cell growth at 72 h. We then detected the expression level of apoptosis by western blot and found that CaP-PTX/siADAM10 NPs effectively inhibited antiapoptotic proteins (bcl-2 protein) (Figures 2(c) and 2(e)) and activated proapoptotic proteins (bax protein) (Figures 2(d) and 2(f)) expression to promote the permeability of mitochondrial membrane and promote cell apoptosis. In addition, we performed a CCK-8 assay and proved that the knockdown of

![Figure 3](image-url)
Figure 4: Therapeutic efficiency of CaP-PTX/siADAM10 NPs in vivo. (a) Weight change of mice in different groups. (b) Tumor volume statistics among therapeutic period. (c) Photo of tumors ex vivo. (d) Tumor inhibition ratio of different groups compared to PBS. (e) TUNEL staining of tumors ex vivo. (f) H&E staining of tumors extracted from mice. Scale bar, 50 μm. Data as means ± SD (*P < 0.05, **P < 0.01, ***P < 0.001).
Figure 5: Biological safety of calcium phosphorus nanodrug delivery system. Three days after the last injection, blood was collected for biochemical analysis. (a–d) Plasma levels of AST (a), ALT (b), CREA (c), and UREA (d) analysis. (e) H&E staining of organs after the last treatment to evaluate side effects. Scale bar, 50 μm. Data as means ± SD (*P < 0.05, **P < 0.01, ***P < 0.001).
level of ADAM10 in MDA-MB-231 cell suppressed cell viability (Figure S2). In Figure 2(g), expression level of ADAM10 was the lowest in the CaP-PTX/siADAM10 NP group by RT-qPCR. Besides, the IC$_{50}$ value of PTX in MDA-MB-231 cell with siADAM10 was dramatically downregulated (Figure S3). These results demonstrated that PTX and siADAM10 coloaded in CaP-PTX/siADAM10 NPs synergistically promote cell apoptosis.

3.3. Biodistribution Investigation. Whether the drug can accumulate to the tumor site determines its efficacy and toxic side effects on other organs. To investigate the tumor targeting of CaP-PTX/siADAM10 NPs, we established the MDA-MB-231 mouse breast cancer model. We utilized DiR to label nanoparticles. Our nanodrug delivery system had suitable particle size to achieve enhanced permeability and retention (EPR) effect. The results showed that the calcium-phosphorus drug-loading system could target and aggregate to the tumor site, and compared with the free drug, the amount of drug aggregation gradually increased with the accumulation of time (Figure 3(a)). Fluorescence quantitative statistics also confirmed this result (Figure 3(c)). Free DiR is rapidly cleared, so fluorescence intensity drops rapidly. After 48 hours, we observed the isolated organs from mice by fluorescence scanning, and the images showed that CaP-DiR/siNC NPs mainly accumulated in the tumor site, while free DiR mainly accumulated in the liver (Figures 3(b) and 3(d)). Besides, the tumors were taken out and minced at 8 h, 24 h, and 48 h. The results of flow cytometry showed that the fluorescence intensity of the nanoparticle group was significantly stronger than that of free DiR (Figure S4).

In addition, concentration of Cy5-siRNAs in blood at different times after drug injection was tested using fluorescence spectrophotometer, as shown in Figure S5. Our nanoparticles exhibited long circulation. Thus, our CaP delivery system has a suitable particle size, and the outer layer of PEG can increase its systemic circulation time, avoid the phagocytosis of nanodrugs by the immune system, so as to target and accumulate at the tumor site, increase the drug concentration at the action site, and reduce the side effects of chemotherapy drugs on other organs of the body.

3.4. Therapeutic Effects In Vivo. We established the MDA-MB-231 mouse orthotopic breast cancer model to investigate the therapeutic effect of CaP-PTX/siADAM10 NPs. Tumor-bearing mice were randomly divided into four groups of four mice each. PBS, PTX, CaP-PTX/siNC NPs, and CaP-PTX/siADAM10 NPs (2.5 mg/kg) were administered by tail vein, respectively, twice a week. Mice in the PBS and free PTX groups decreased significantly compared to the CaP-PTX/siNC NP and CaP-PTX/siADAM10 NP groups (Figure 4(a)). The tumor volume of each group of mice was monitored and calculated during the treatment process, and we found that compared with the PTX and CaP-PTX/siNC NP groups, the tumors in the CaP-PTX/siADAM10 NP group of mice grew slowly and had the smallest volume (Figure 4(b)). Three days after the last treatment, the mice were sacrificed, and the tumor was taken out to take pictures (Figure 4(c)) and calculate the tumor inhibition rate. The tumor inhibition rate of the CaP-PTX/siADAM10 NP group reached 80.1% (Figure 4(d)). At the same time, the TUNEL results of tumor tissue showed that the apoptosis rate of tumor tissue in the CaP-PTX/siADAM10 NP group was the highest (Figure 4(e)). H&E results also confirmed this result (Figure 4(f)). In conclusion, calcium phosphorus nanoparticles coloaded with PTX and siADAM10 have the best tumor suppressive effect.

3.5. Biosafety Evaluation of CaP-NPs In Vivo. In order to investigate the toxic and side effects of calcium-phosphorus nanodrug loading system on normal tissues and organs, we collected the serum of mice at the end of the treatment, measured the relevant indicators of their liver and kidney functions, and made HE tissue sections for the heart, liver, spleen, lung, and kidney. Observe the pathological condition. In the PTX group, levels of aspartate transaminase (AST), alanine transaminase (ALT), urea (UREA), and creatinine (CREA) were significantly higher than the PBS group. Besides, these levels in CaP-PTX/siNC NPs and CaP-PTX/siADAM10 NPs had no significant difference. This is because the encapsulation of nanoparticles significantly reduced the toxic and side effects of the chemotherapeutic drug PTX (Figures 5(a)–5(d)). The results of H&E also showed that the liver and kidney of the PTX group were all pathologically damaged, while there were no observed damage in other groups (Figure 5(e)). In conclusion, our calcium-phosphorus nanodrug delivery system can reduce the toxic and side effects of chemotherapeutic drugs, increase tumor targeting, and improve the effect.

4. Conclusion

In summary, we successfully constructed a targeting and effective therapeutic platform CaP-PTX/siADAM10 NPs with a core-shell-distinct structure for TNBC therapy. CaP-PTX/siADAM10 NPs could target tumor site through the enhanced permeability and retention (EPR) effect so that avoiding side effects to other organs. The PEG chains in the outer layer of the nanoparticles prevent the immune system from engulfing them and thus increase their systemic circulation time. The siADAM10 outer shell from the CaP-PTX/siADAM10 NPs is first released to exert its gene inhibitory effect to increase the sensitivity of tumor cells to chemotherapeutic drugs, and then, the inner PTX is released to exert its tumor-killing effect increasing the apoptosis rate. Consequently, CaP-PTX/siADAM10 NPs were an effective platform to treat TNBC.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors have declared that there were no competing interests.
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Supplementary Materials

Supplementary information is available from Hindawi online system. Figure S1: biosafety of blank DSPE-PEG NPs and blank calcium phosphorus NPs. Figure S2: effect of siADAM10 on the viability of MDA-MB-231 cells. Figure S3: when combined with siADAM10, the IC_{50} value of paclitaxel to MDA-MB-231 cells. Figure S4: flow cytometry analysis of DiR fluorescence intensity in ex vivo tumors. Figure S5: pharmacokinetics of CaP-PTX/Cy5-siRNA NPs. (Supplementary Materials)

References


