

Combination Treatment of Cervical Cancer Using Folate-Decorated, pH-Sensitive, Carboplatin and Paclitaxel Co-Loaded Lipid-Polymer Hybrid Nanoparticles

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Junjian Wang¹⁻³

¹Institution of Cancer and Basic Medicine, Chinese Academy of Sciences, Hangzhou 310022, Zhejiang Province, People's Republic of China; ²Department of Gynecological Surgery, Cancer Hospital of the University of Chinese Academy of Sciences, Hangzhou 310022, Zhejiang Province, People's Republic of China; ³Department of Gynecological Surgery, Zhejiang Cancer Hospital, Hangzhou 310022, Zhejiang Province, People's Republic of China

Purpose: Cervical cancer is one of the most common causes of death among women globally. Combinations of cisplatin, paclitaxel, bevacizumab, carboplatin, topotecan, and gemcitabine are recommended as first-line therapies.

Methods: This study focuses on the development of folate-decorated, pH-sensitive lipid-polymer hybrid nanoparticles (LPNs). Loading carboplatin (CBP) and paclitaxel (PTX), LPNs were expected to combine the therapeutic effects of CBP and PTX, thus show synergistic ability on cervical cancer.

Results: FA-CBP/PTX-LPNs showed the sizes of 169.9 ± 5.6 nm, with a narrow size distribution of 0.151 ± 0.023 . FA-CBP/PTX-LPNs exhibited pH-responsive drug release, high cellular uptake efficiency ($66.7 \pm 3.1\%$), and prominent cell inhibition capacity ($23 \pm 1.1\%$). In vivo tumor distribution and tumor inhibition efficiency of FA-CBP/PTX-LPNs was the highest, with no obvious body weight lost.

Conclusion: High tumor distribution and remarkable antitumor efficiency obtained using in vitro as well as in vivo models further proved the FA-CBP/PTX-LPNs is a promising tool for cervical cancer therapy.

Keywords: cervical cancer, folate, pH-sensitive, carboplatin, paclitaxel, lipid-polymer hybrid nanoparticles

Introduction

Cervical cancer is a malignant epithelial tumor that forms in the uterine cervix.¹ It is one of the most common causes of death among women globally.² Cervical cancer treatment approaches include surgery, radiation therapy, chemotherapy, and targeted therapy.³ Chemotherapy is a powerful therapeutic approach for the cancer therapy. However, using a single therapeutic agent is not effective in eradicating cancer cells, and hence the use of combinatorial therapy is necessary and inevitable.⁴ Various combinations of cisplatin, paclitaxel, bevacizumab, carboplatin, topotecan, and gemcitabine are recommended as first-line therapies.⁵ However, conventional chemotherapy is lack of cell specificity thus may cause serious side effects: both normal cells and cancer cells may be killed together by anticancer drugs.⁶

Nanoparticles have been widely investigated in the treatment of cancer because nanoparticles have special characters that can load small molecules for biomedical applications.⁷ Therefore, various nanoparticles including polymeric nanoparticles,

Correspondence: Junjian Wang
Institution of Cancer and Basic Medicine,
Chinese Academy of Sciences, No. 1 East
Banshan Road, Gongshu District,
Hangzhou City, Zhejiang Province,
People's Republic of China
Email wangjunjian@protonmail.com

lipid nanoparticles, dendrimers, and micelles have been designed to encapsulate anticancer drugs.⁸ Lipid-polymer hybrid nanoparticles (LPNs) usually contained a polymer inner core and a phospholipid surface layer.⁹ LPNs combine advantages of both liposomes and polymers into a single platform, which is an ideal system for combinatorial delivery based on the dual-component structure.¹⁰ pH-sensitive nanoparticles have been widely used to deliver drugs in cancer therapy due to the lower pH in tumors than in normal tissues.^{11–13} In this study, pH-responsive LPNs were applied for the carboplatin and paclitaxel delivery.

Surface decorated nanoparticles (by conjugating specific ligands) could potentially be delivered to specific organs, tissues, cells, or even cellular organelles.¹⁴ Nanoparticles surface modified with different ligands may elicit specific cellular interactions, so the *in vivo* fate and efficacy of these nanoparticles can be dramatically affected.¹⁵ Folate (FA), a nonimmunogenic receptor-specific ligand, has emerged as an attractive specific ligand for targeted anticancer drug delivery.¹⁶ FA showed immense potential to target cancer cells owing to its high affinity for folate receptors, which are normally over-expressed in various human carcinomas, including cervical cancer.¹⁷ So FA decorated nanoparticle formulations were developed for targeted cancer therapy.^{18–20}

The present research focuses on the development of folate-decorated, pH-sensitive LPNs. Loading carboplatin (CBP) and paclitaxel (PTX), LPNs were expected to combine the therapeutic effects of CBP and PTX, thus show synergistic ability on cervical cancer.

Materials and Methods

Materials

Docetaxel was obtained from Sanwei Pharmaceutical Co. Ltd (Shanghai, China). Poly (D,L-lactic-co-glycolic) (PLGA, 50:50, MW 20,000) was purchased from Shandong Institute of Medical Instrument (Shandong, China). poly(vinyl alcohol) (PVA), dimethyl sulfoxide (DMSO), coumarin-6 (Cou-6), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Fetal Bovine Serum (FBS) was purchased from Invitrogen Corporation (Carlsbad, CA). Injectable soy lecithin (ISL), Dimethyl sulfoxide (DMSO), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDCI), and 1-Hydroxybenzotriazole (HOBt) were obtained from Aladdin Reagent Co. Ltd (Shanghai, China). Glyceride oleate (GO) were purchased from Sigma-Aldrich Co. (St. Louis,

MO, USA). Folate-polyethylene glycol-COOH (FA-PEG-COOH) was provided by Xi'an Ruixi Biological Technology Co., Ltd (Xi'an, China). Eagle's Minimum Essential Medium (EMEM) and human cervix adenocarcinoma cell line (HeLa cells) were obtained from the American type culture collection (ATCC[®] CCL-2[™], Manassas, VA, USA). Female BALB/c nude mice (6–8 weeks) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). The animal experiments were carried out in accordance with the UK Animals Act, 1986, and associated guidelines, EU Directive 2010/63/EU for animal experiments and were approved by the Laboratory Animal Ethics Committee of Zhejiang Cancer Hospital (No. 2019-09-001).

Synthesis of Folate-Contained, pH-Sensitive Ligands

Folate-contained, pH-sensitive ligands were synthesized by conjugating FA-PEG-COOH with GO through a hydrazone bond (adipohydrazide) (Figure 1). Firstly, DMSO was used to dissolve FA-PEG-COOH (1 mmol) and reacted with the amine groups of adipohydrazide (HZ, 1 mmol) for 12 h by adding DCC (1 mmol) and NHS (4 mmol) under nitrogen atmosphere in dark to get FA-PEG-HZ.²¹ Then, GO (1 mmol) was dissolved in DMSO and added to the FA-PEG-HZ solution, in the mean time, EDCI (0.25 mmol) and HOBt (0.25 mmol) were added to the stirring solution for 24 h to form FA-PEG-HZ-GO. FA-PEG-HZ-GO was lyophilized and characterized by ¹H-NMR analysis.

Preparation of CBP and PTX Co-Loaded LPNs

FA decorated, CBP and PTX co-loaded LPNs (FA-CBP/PTX-LPNs) were prepared by a one-step nanoprecipitation method (Figure 2).²² PCL (100 mg), CBP (10 mg), and PTX (10 mg) were dissolved in acetone (10 mL) to form the organic phase. FA-PEG-HZ-GO and ISL were dispersed in water, heated to 65–70°C to form a homogeneous dispersion. The organic phase was added dropwise to the dispersion which was stirred at 400 rpm. The HA-DOX/GA-LPHNs solution was washed for three times using a centrifugal filter with a molecular weight cut-off (MWCO) of 10 kDa to remove the acetone and free molecules and got FA-CBP/PTX-LPNs. FA decorated, single CBP or PTX loaded LPNs (FA-CBP-LPNs and FA-PTX-LPNs) were prepared by using one drug (CBP or PTX) only. FA decorated, blank LPNs (FA-LPNs) were prepared by using no drug. Undecorated CBP and PTX co-loaded

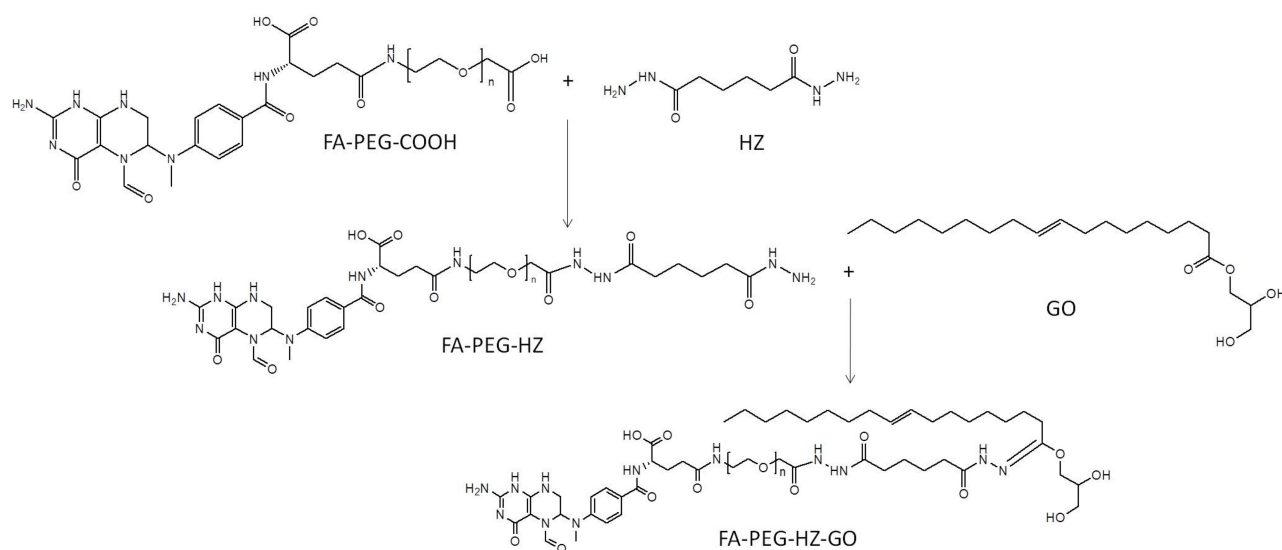


Figure 1 Synthesis of folate-contained, pH-sensitive ligands. Folate-contained, pH-sensitive ligands were synthesized by conjugating FA-PEG-COOH with GO through a hydrazone bond.

Abbreviations: FA, folate; PEG, polyethylene glycol; GO, glyceride oleate.

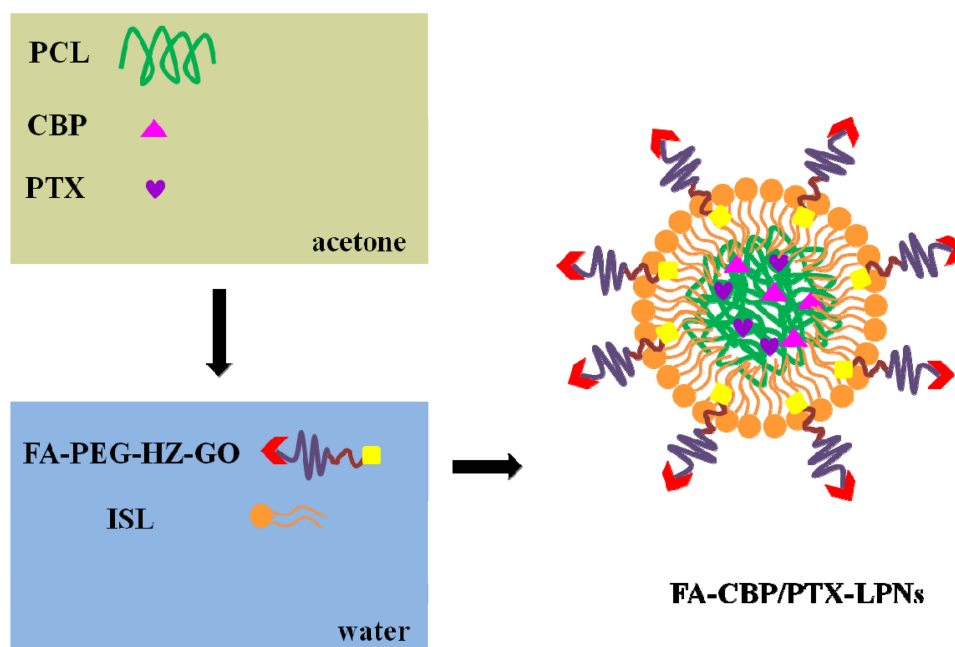


Figure 2 Schematic for nanoparticle assembly. FA decorated, CBP and PTX co-loaded LPNs (FA-CBP/PTX-LPNs) were prepared by a one-step nanoprecipitation method.

Abbreviations: FA, folate; CBP, carboplatin; PTX, paclitaxel; LPNs, lipid-polymer hybrid nanoparticles.

LPNs (CBP/PTX-LPNs) were prepared by using PEG-DSPE instead of FA-PEG-HZ-GO.

Characterization of LPNs

The average particle diameter, particle size distribution, and surface charge were determined by dynamic light scattering (DLS) technique using a Zetasizer Nano ZS (Malvern,

UK).²³ The morphology and size of the particles were observed using JEM-1010 transmission electron microscopy (JEOL, Tokyo, Japan). The encapsulation capacity of LPNs was determined by measuring the concentrations of CBP and PTX using a C-18 reverse-phase high-performance liquid chromatography (RP-HPLC) (Beckman Coulter, Brea, CA) at a flow rate of 1 mL/min.²⁴ Tert-butyl methyl

ether (5 mL) was added to the LPNs suspension and vortex for 1 min to extract the drugs and then redissolved in acetonitrile:water (10:90) solution. The solution (1 mL) was injected into the C-18 column, and CPT and PTX were detected at 227 nm after different retention times. The amounts of CBP and PTX were quantified by HPLC. The encapsulation efficiency (EE) was determined as: (Amount of entrapped drug/amount of total drug) \times 100%.

In vitro Drug Release of LPNs

In vitro CBP and PTX release behaviors of LPNs were analyzed in acetate buffer (pH 5.5) or phosphate sodium buffer (PBS buffer) (pH 7.4) medium, respectively.²⁵ FA-CBP/PTX-LPNs or CBP/PTX-LPNs were dispersed with buffer (5 mL) and sealed in a dialysis bag (MWCO: 2 kDa), which was immersed in the release medium (25 mL) shaking (100 rpm) at 37°C. At present time points, 2 mL of release medium was taken out and 2 mL of fresh medium were added. The amounts of CBP and PTX were quantified by HPLC.

Cellular Uptake of LPNs

Coumarin-6 (C6) was encapsulated in the FA-CBP/PTX-LPNs or CBP/PTX-LPNs and the uptake efficiency was evaluated on HeLa cells because C6 was a fluorescent marker that can be directly visualized and quantified.²⁶ C6 was loaded into LPNs by adding C6 (1 mg/mL of the lipid phase) into the organic phase described in the "Preparation of CBP and PTX co-loaded LPNs" section. HeLa cells were seeded in 24-well plates (5×10^4 cells/well). When 80% confluence was achieved, C6 loaded FA-CBP/PTX-LPNs or CBP/PTX-LPNs were added and incubated for 4 h (pH 7.4, 37°C). Then, the cells were washed three times with PBS and photographed using fluorescence microscopy. After trypsinized, the cells were analyzed using a flow cytometer.^{27,28}

In vitro Cytotoxicity and Synergistic Effect of LPNs

Cytotoxicity studies were carried out on HeLa cells.²⁹ HeLa cells (10^3 cells/well in 96-well plates) were seeded in EMEM adding 10% of FBS (100 μ L). After incubation for 24 h (37°C), FA-CBP/PTX-LPNs, FA-CBP-LPNs, FA-PTX-LPNs, CBP/PTX-LPNs, free CBP/PTX, and FA-LPNs were added and incubated in 5% CO₂ for 72 hrs (37°C), and cell viability was measured by MTT assay based on the manufacturer's manual. IC₅₀ values were calculated from curves constructed by plotting cell viability (%) versus drug concentration (μ M). The synergistic effect was determined

by the Combination index (CI), the CI values were calculated through the Chou-Talalay method.³⁰ The CI provides a quantitative value for synergy and is given by: $CI_{50} = (D)_a / (D_{50})_a + (D)_b / (D_{50})_b$. In this equation, a for CBP and b for PTX, (D)_a and (D)_b means the dose (concentration) of CBP and PTX in combination inhibited 50% of the cell growth; (D₅₀)_a and (D₅₀)_b means the dose (concentration) of CBP or PTX alone that inhibited 50% of the cell growth. CI < 1, = 1, and > 1 indicate synergism, additive, and antagonism effect, respectively. CI values closer to zero represent increasing synergy.

In vivo Tissue Distribution and Anticancer Ability of LPNs

Cervical carcinoma xenograft was produced by injecting HeLa cells (5×10^6 cells in 200 μ L of PBS) cells subcutaneously into the right armpit of BALB/c nude mice, which was grouped randomly (7 groups).³¹ FA-CBP/PTX-LPNs (contained 5 mg/kg CBP and 5 mg/kg PTX), FA-CBP-LPNs (contained 10 mg/kg CBP), FA-PTX-LPNs (contained 10 mg/kg PTX), CBP/PTX-LPNs (contained 5 mg/kg CBP and 5 mg/kg PTX), free CBP/PTX (contained 10 mg/kg CBP and 10 mg/kg PTX), FA-LPNs, and 0.9% saline were administered intravenously once every three days. The tumors and animal weights were measured. The tumor volume was calculated according to the equation: long axis \times short axis²/2. At 1 h and 24 h post injection, mice were sacrificed. Tissues (tumor, heart, liver, spleen, lung and kidney) were collected, decomposed on heating in nitric acid, evaporated to dryness, and redissolved in acetonitrile: water (10:90) solution.³² The amounts of CBP and PTX were quantified by HPLC.

Statistical Analysis

Mean \pm standard deviation (mean \pm SD) was used to express the data. A post hoc test (S-N-K method) was performed following ANOVA. * P < 0.05 was considered as statistical significance and ** P < 0.01 as extreme statistical significance.

Results

Characterization of FA-PEG-HZ-GO

FA-PEG-HZ-GO was characterized by ¹H-NMR: One peak at 7.1 ppm means the formation of HZ. Typical peaks of PEG were observed at 3.37–3.63 ppm, peaks at 7.77–8.10 were attributed to FA, and peaks at 1.29–1.33 belong to GO.

Table 1 Characterization of LPNs

Formulations	FA-CBP/PTX-LPNs	FA-CBP-LPNs	FA-PTX-LPNs	CBP/PTX-LPNs	FA-LPNs
Average diameter (nm)	169.9 ± 5.6	171.3 ± 5.1	166.5 ± 5.5	121.3 ± 4.1	164.1 ± 4.8
Particle size distribution	0.151 ± 0.023	0.163 ± 0.025	0.147 ± 0.031	0.129 ± 0.018	0.136 ± 0.017
Surface charge (mV)	32.9 ± 3.1	34.1 ± 3.2	33.2 ± 2.9	21.3 ± 2.6	35.3 ± 2.7
CBP EE (%)	83.1 ± 2.1	82.6 ± 2.8	N/A	80.9 ± 2.5	N/A
PTX EE (%)	84.2 ± 2.7	NA	83.6 ± 2.6	82.8 ± 2.2	N/A

Characterization of LPNs

The particle diameter, size distribution, and surface charge of LPNs were characterized and summarized in Table 1. FA-decorated LPN formulations showed the sizes of about 170 nm, while undecorated CBP/PTX-LPNs had a diameter of 121.3 nm. Narrow size distributions were found for all the formulas (between 0.129 and 0.163). Positive surface charges of LPNs were observed, with over 80% of EE.

In vitro Drug Release of LPNs

The drug release behaviors of FA-CBP/PTX-LPNs and CBP/PTX-LPNs in pH 7.4 and 5.5 are different (Figure 3). CBP/PTX-LPNs showed almost the same release pattern in the different pH values; however, FA-CBP/PTX-LPNs exhibited faster release at pH 5.5. FA-CBP/PTX-LPNs showed over 80% of CBP and PTX release at 24 h, while at pH 7.4 the time was delayed to 48 h.

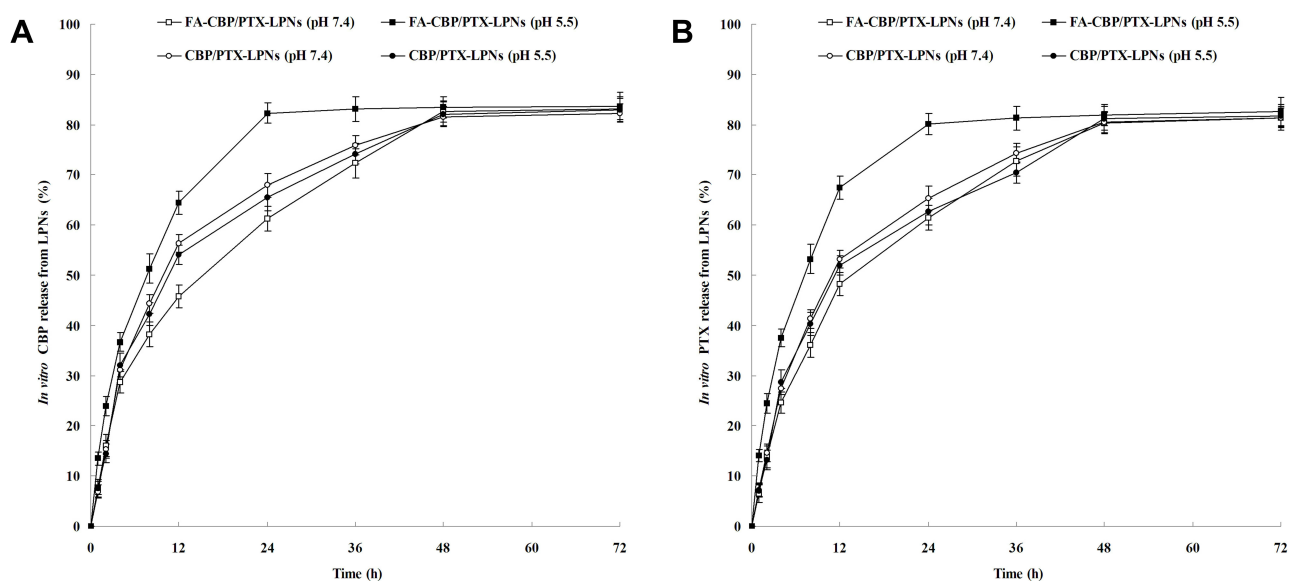


Figure 3 In vitro CBP (A) and PTX (B) release of LPNs. The drug release behaviors of FA-CBP/PTX-LPNs and CBP/PTX-LPNs were observed at pH 7.4 and 5.5. Data presented as mean ± SD (n=5).

Abbreviations: FA, folate; CBP, carboplatin; PTX, paclitaxel; LPNs, lipid-polymer hybrid nanoparticles.

Cellular Uptake of LPNs

Figure 4 displays the cellular uptake efficiency results of FA-CBP/PTX-LPNs or CBP/PTX-LPNs. The HeLa cell uptake of FA-CBP/PTX-LPNs ($66.7 \pm 3.1\%$) was remarkably higher than CBP/PTX-LPNs ($31.6 \pm 2.2\%$) ($P < 0.01$).

In vitro Cytotoxicity and Synergistic Effect of LPNs

FA-CBP/PTX-LPNs showed remarkably higher cytotoxicity than CBP/PTX-LPNs (Figure 5A, $P < 0.05$), suggested that the use of FA contained pH-sensitive ligand could promote the delivery of the drugs encapsulated within nanoparticles.⁴³ Significant improvements of cytotoxicity by CBP/PTX-LPNs in comparison to free CBP/PTX illustrated that the LPNs improved the drug delivery, thus gaining better efficiency than free drug solution. Evaluation of drug–drug interaction is important in

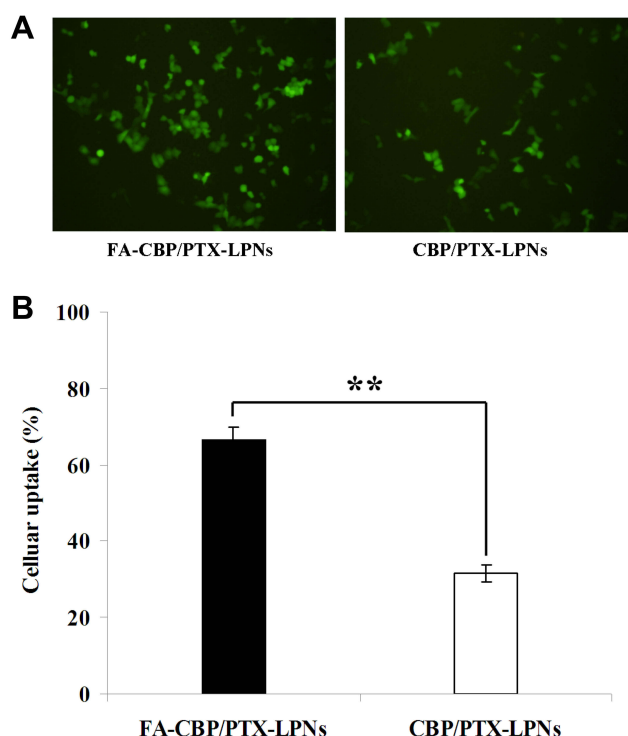


Figure 4 Cellular uptake efficiency of FA-CBP/PTX-LPNs and CBP/PTX-LPNs. (A) photographed using fluorescence microscopy and (B) analyzed using a flow cytometer. Data presented as mean \pm SD (n=5), **P < 0.01.

Abbreviations: FA, folate; CBP, carboplatin; PTX, paclitaxel; LPNs, lipid-polymer hybrid nanoparticles.

combination with cancer chemotherapy, and combination index (CI) was introduced by Chou and Talalay for quantification of synergistic or antagonistic effect.⁴⁴ FA-CBP/PTX-LPNs, CBP/PTX-LPNs, and free CBP/PTX showed CI_{50} value <1, showing synergy effects (Figure 5B).

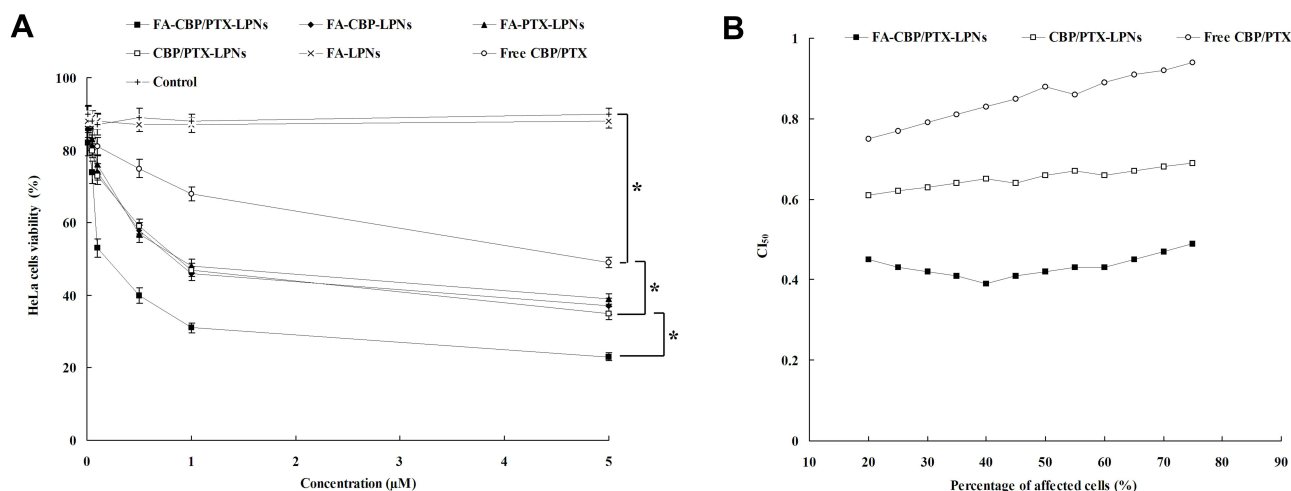


Figure 5 In vitro cytotoxicity (A) and synergistic effect (B) of LPNs evaluated on HeLa cells by MTT assay. Data presented as mean \pm SD (n=5), *P < 0.05.

Abbreviation: LPNs, lipid-polymer hybrid nanoparticles.

In vivo Tissue Distribution and Anticancer Ability of LPNs

In vivo tissue distribution results are shown in Figure 6. At 1 h, LPNs showed less accumulation in the kidney and heart than free drugs ($P < 0.05$). At 24 h, the distribution of FA-CBP/PTX-LPNs in tumor was the highest, higher than CBP/PTX-LPNs and free CBP/PTX ($P < 0.01$). This phenomenon could be explained by the enhanced permeability and retention (EPR) effect on the tumor site that lets the LPNs accumulated easily in the tumor.⁴⁵ The tumor tissue concentrations of LPNs kept up high at 24 h post administration, indicating the sustained-release behavior of the LPNs, which may attribute to the presence of PEG chain on the surface of particles.⁴⁶ The most significant in vivo tumor inhibition efficiency was observed when FA-CBP/PTX-LPNs formula was administrated (Figure 7), which was better than FA-CBP-LPNs ($P < 0.05$) and CBP/PTX-LPNs ($P < 0.01$). CBP/PTX-LPNs illustrated better antitumor ability than free CBP/PTX ($P < 0.01$), the latter showed remarkable ability over the control group ($P < 0.05$). On day 21, the images of the tumor are taken and presented in Figure 7B. There was no obvious body weight loss in LPN groups. No animal death was observed in all the tested groups. These outcomes proved the system may be considered as a safe carrier to deliver the anti-cancer drugs. These could be explained by the LPNs constructed could exhibit high structural integrity, stability during usage, and sustained-release capability. FA-decorated pH-sensitive LPNs might be compatible with the lipid structured cell membrane, which allows the system to

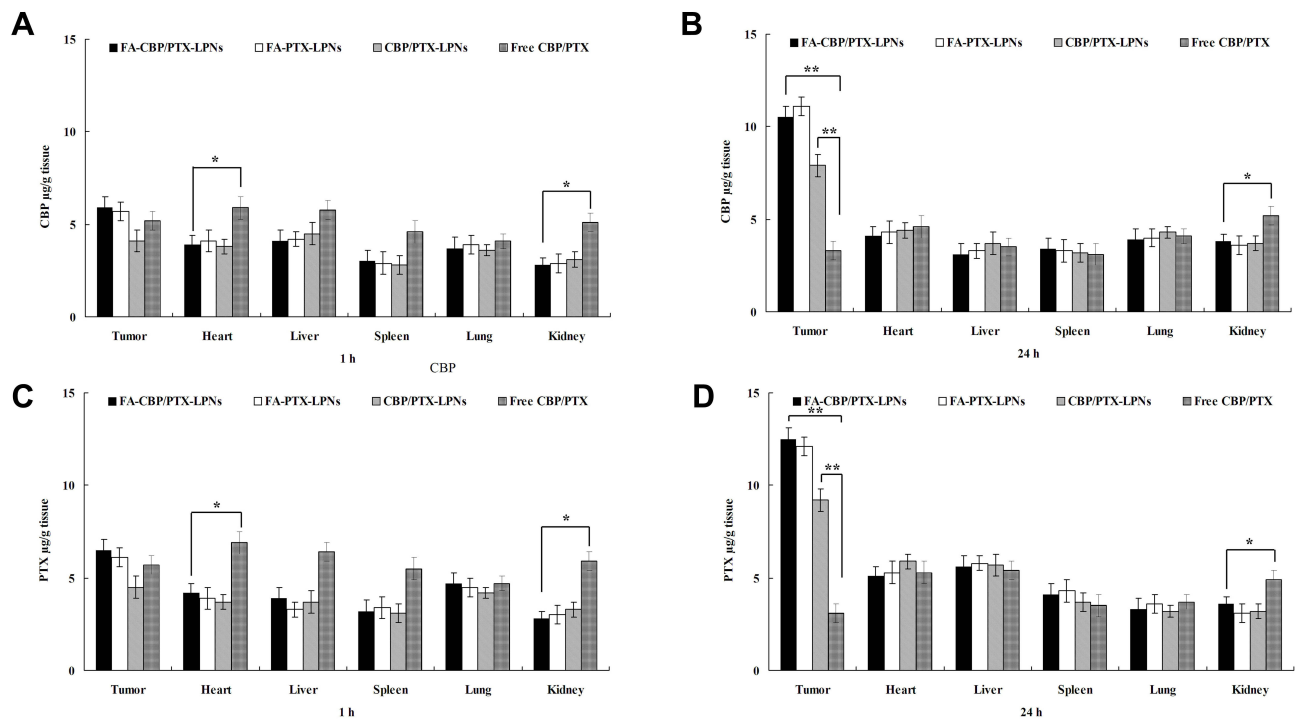


Figure 6 In vivo tissue distribution of CBP (A, B) and PTX (C, D) investigated at 1 h (A, C) and 24 h (B, D) of administration. *P < 0.05 and **P < 0.01. Abbreviations: CBP, carboplatin; PTX, paclitaxel.

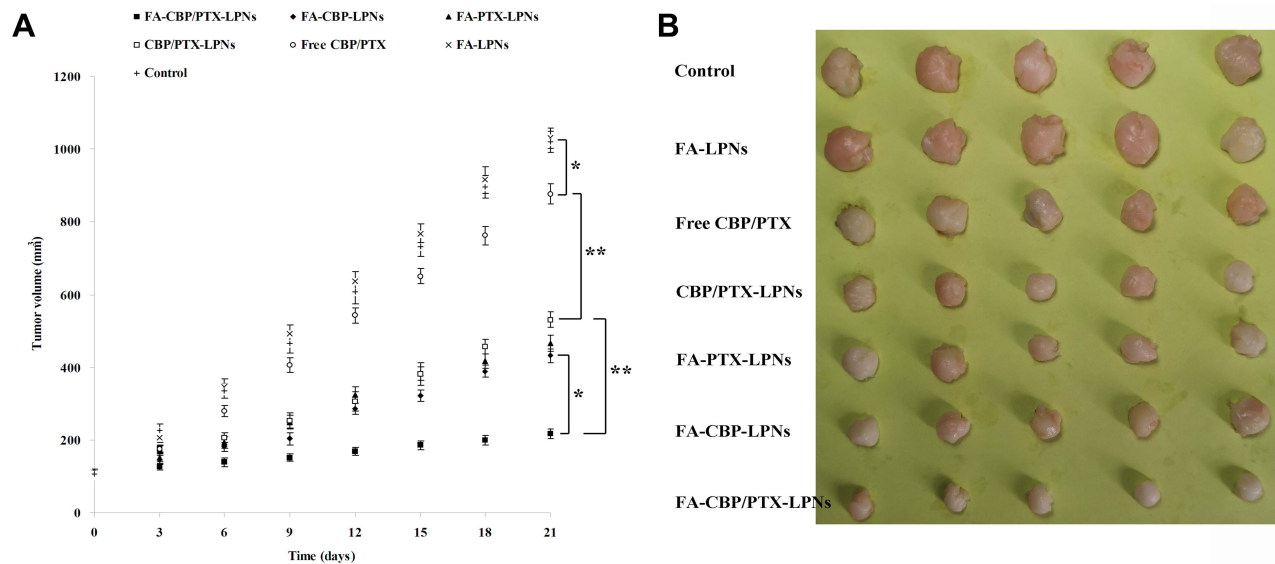


Figure 7 In vivo anti-tumor effect of the LPNs evaluated in terms of tumor volume (A) and tumor images (B). Data presented as mean ± SD (n=5), *P < 0.05 and **P < 0.01. Abbreviation: LPNs, lipid-polymer hybrid nanoparticles.

fuse to the cell membrane and deliver drugs to the tumor more efficiently.⁴⁷

Discussion

FA can bind to the folate receptor (FR) with high affinity and enter the FR-elevated malignant cells by receptor-

mediated endocytosis, and meanwhile avoid being endocytosed by those normal cells that express a low level of FRs.³³ Thus, in this study, FA was conjugated to the distal ends of FA-PEG-HZ-GO. PEG modification on the surface of nanoparticles could shield the surface from aggregation, opsonization, and phagocytosis, prolonging systemic

circulation time.³⁴ According to the pH difference between tumor tissues and normal tissues,³⁵ pH-response FA-PEG-HZ-GO ligand was designed for the nanoparticle preparation.

FA-CBP/PTX-LPNs were prepared by a one-step nanoprecipitation method. The conventional two-step method was the most common method employed in the early phase of LPN development by mixing the preformed polymeric nanoparticles preformed lipid vesicles through electrostatic interactions.³⁶ However, the main drawback of the two-step method was not efficient in terms of time and energy expenses. In contrast, the one-step method involves a single mixing of lipid with polymer to allow them self-assemble LPNs via nanoprecipitation. The size of prepared LPNs ranged from 121 to 170 nm, which could be efficiently internalized into cells.³⁷ Size and drug loading efficiency did not change after co-loading with C6 dye. PCL was used as the polymer material to encapsulate the hydrophobic drugs. Over 80% of EE may prove the fine drug entrapment efficiency of these systems.³⁸

FA-CBP/PTX-LPNs showed over 80% of CBP and PTX release at 24 h, while at pH 7.4 the time was delayed to 48 h. Wan et al observed similar release pattern for both decorated and undecorated nanoparticles, they argued that the modification of ligands on the nanoparticles' surface did not change the release behavior of drugs.³⁹ The same phenomenon was found in this research that similar release behavior was found in FA-CBP/PTX-LPNs and CBP/PTX-LPNs at pH 7.4. At pH 5.5, the drug release from CBP/PTX-LPNs had not been changed. On the contrary, FA-CBP/PTX-LPNs exhibited faster release at pH 5.5 than at pH 7.4. The results are in accordance with the observation of Yang et al, which indicated that an acidic environment could accelerate the breakdown of self-assemblies, further giving rise to faster release of the drugs.⁴⁰

In the study carried out by Tang et al, the cellular uptake and cellular internalization mechanisms of nanoparticles in HeLa (high expression of FA receptor) cells were quantitatively detected by the fluorescence method.⁴¹ In the FA receptor-overexpressing HeLa cells, the internalization of FA-decorated nanoparticles was significantly more efficient than undecorated ones due to FA receptor-mediated endocytosis and cellular macropinocytosis involved in the cellular internalizations of nanoparticles in HeLa cells. Also, the pH-triggered payload release leads to the increased cumulation of drugs in the cells.⁴²

Conclusion

In summary, FA-decorated, pH-sensitive LPNs were constructed for the co-delivery of CBP and PTX. FA-CBP/PTX-LPNs exhibited pH-responsive drug release, high cellular uptake efficiency, and prominent cytotoxicity. The high tumor distribution and remarkable antitumor efficiency of FA-CBP/PTX-LPNs further proved this system could be used as a promising tool for the targeted treatment of cervical cancer.

Disclosure

The author reports no conflicts of interest in this work.

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