Supporting information

Delivery of gefitinib loaded nanoparticles for effectively inhibiting prostate cancer progression

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Materials and methods

Cell culture and reagents

The human PCa cell line 22RV1 was purchased from CAS Center for Excellence in Molecular Cell Science (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin. Additionally, PCR analysis was performed to ensure the absence of mycoplasma contamination.

The preparation process of nanomaterials

Preparation and Characterization of PLGA NPs and GEF-Loaded PLGA NPs: PLGA NPs and GEF or fluorescent-dye-loaded PLGA NPs were prepared through a nanoprecipitation method. As aforementioned, PLGA polymer, DSPE-PEG2k and drug/dye mixed in DMSO at predetermined mass ratio were dropwise added into ultra-water under stirring, followed by the ultrafiltration process. The nanoparticle size and their distribution were recorded by DLS (Malvern, UK). Transition electron microscopy (TEM, JEOL, Japan) was used to intuitively confirm the size and structure of the NPs. In stability test, nanoparticles were dispersed in PBS or PBS with 10% FBS and the particle size were monitored by DLS in triplicate at different time intervals.

The encapsulation efficiency (EE) and drug-loading capacity (DLC) of drug-loaded PLGA NPs were measured by HPLC. Dissolve 10 μL of drug-loaded PLGA NPs in 950 μL of acetonitrile and centrifuge at 13,000 rpm for

10 minutes. Then filtered solution was injected into the HPLC column for the detection of drug content under an UV absorption of 254 nm. The calculated formulas were as follows:
(EE) × wt% = mass of encapsulated drug/mass of drug added 100% (1)
(DLC) ×wt% =mass of encapsulated drug/the total mass of NPs 100% (2)

In Vitro Drug Release Profiles

The drug release profiles were tested by the dialysis method. In brief, 1 mL GEF@PPLGA NPs were added into each dialysis bag (MWCO = 3500 Da), and then immersed these dialysis bags into 10 mL of each different buffers (pH = 5.0 and 7.4) at 37 °C with stirring at 100 rpm. The GEF concentration from the release medium was quantified by HPLC. Each sample was run in triplicate.

Cellular uptake assay22RV1

Confocal laser scanning microscopy and flow cytometry (CLSM) were used to study the uptake and distribution of the PLGA NPs in the cells. cells were seeded in six-well plate at a density of 2 × 105 and then incubated for1 days. Coumarin 6 loaded PLGA NPs (C6@PLGA NPs) were added and incubated for 0.5, 1, 2, 4 and 8 hours.

The cells were washed twice with precooled PBS and perform flow cytometry analysis. In addition, the cells were washed twice with precooled PBS and fixed with 4% formaldehyde at room temperature for 15 minutes. After washing twice with PBS, the cells were further stained with DAPI and detected by confocal laser scanning microscopy to qualitatively analyze intracellular uptake.

Distribution of PLGA NPs

22RV1 cells were seeded on cover glass at approximately 60% confluence. Next, the medium was replaced with C6-loaded NPs in serum-free medium. After incubation for 0.5, 1, 2, 4 and 8 hours, the cells were washed twice with PBS and then treated with Hoechst 33342 (5 μg/ml) and LysoTracker Red (100 nM) sequentially. Finally, the cells were visualized by confocal laser scanning microscope.

In vitro cytotoxicity assay

22RV1 cells were incubated in 96-well plates at 2000 cells per well for 24 h. Then, the cells were treated with different concentrations of GEF, PLGA NPs and GEF@PLGA NPs for 24 and 48 hours. Subsequently, 10 μL of Cell Counting Kit 8 (CKK8) was added to each well and incubated at 37℃ for 2 hours. The absorbance was measured at 450 nm with a microplate reader.

Apoptosis assay

22RV1 cells were seeded in six-well plates at a density of 2 × 105 cells per well and cultured for 24 h. The cells were next treated with PBS, GEF, PLGA NPs and GEF@PLGA NPs for 24 h. Then, the cells were collected, and apoptosis was detected using an Annexin V/FITC and PI apoptosis detection kit.

Transwell migration and invasion assay

A migration assay was performed by filling the bottom well of the cell culture insert with RPMI 1640 medium containing 10% FBS. The insert wells were covered with polyethylene terephthalate (PET) membranes with 8μm pores, and 5 × 104 cells/well in serum-free RPMI 1640 were added to the top culture insert. The cell culture insert chamber was incubated for 48 h at 37℃ to allow the possible migration of cells through the membrane into the bottom chamber. Membranes were stained using crystal violet. The cells in the bottom chamber were counted using an Olympus IX71 inverted microscope. The PET membranes were covered with Matrigel Basement Membrane Matrix for the cell invasion assay.

In vivo biodistribution assay

The animals used in this study was approved by the Institutional Animal Care and Use Committee, Sun Yat-Sen University (approval no. SYSU-IACUC-2021-000041). A total of 2 × 106 22RV1 cells were either combined or not combined with the same quantity of CAFs in a 100 μl solution of PBS mixed with Matrigel (Corning, #354234) at a 1:1 proportion. Subsequently, this mixture was subcutaneously administered into male nude mice of the BALB/c, aged 4 weeks. When the tumor size reached about 100 mm3, mice were injected with Dir or Dir@PLGA NPs through the caudal vein. The distribution and density of Dir were detected using an IVIS Imaging System at different time points. Finally, mice were dissected, and the major organs and tumors were obtained to further detect the distribution and density of Dir.

Blood compatibility

Fresh blood, collected from male SD rats, needed to be washed with PBS until the supernatant could be clear. The aforesaid red blood cells was diluted in PBS to form the erythrocyte suspension, then, the suspension was lived with water (positive control), PBS (negative control), PLGA-NPs and GEF@PLGA NPs at different concentrations for 3 h at 37℃. In the end, through the centrifugation for 10 mins at 3000 rpm, the supernatant of each sample could be got and used with absorbance detection at 540 nm using a microplate reader, Hemolysis rates were calculated as follows: Hemolysis%= (Absample - Ab negative)/ (Abpositive - Ab negative) x 100%.

In vivo antitumor efficacy

The animals used in this study was approved by the Institutional Animal Care and Use Committee, Sun Yat-Sen University (approval no. SYSU-IACUC-2021-000041). A total of 2 × 106 22RV1 cells were either combined or not combined with the same quantity of CAFs in a 100 µl solution of PBS mixed with Matrigel (Corning, #354234) at a 1:1 proportion. Subsequently, this mixture was subcutaneously administered into male nude mice of the BALB/c, aged 4 weeks. When the tumor volume reached approximately 50 - 100 mm3, the mice were randomly divided into four groups. Mouse models were treated by tail vein injection of 100 uL PBS, PLGA NPs, GEF, GEF@PLGA NPS suspension (GEF 3 mg/kg), respectively. During the treatment period, the formulations were injected every 3 d for a total of four injections, and the body weight and tumor volume of each mouse were recorded every 2 d. At the end of the treatment, serum was collected for evaluating blood biochemical parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (CRE). The mice were euthanized, their tumors were photographed and weighed, and the tumor sections were fixed in 4% paraformaldehyde (PFA) for H&E section staining. Expression levels of Ki67, caspase-3, and cleaved caspase-3 were detected to assess the proliferation and apoptosis of tumor tissues following standard procedures using immunohistochemistry. The primary organs of the mice in each group (heart, lung, spleen, liver, and kidney) were collected and fixed in 4% PFA for H&E section analysis. Immunohistochemistry images were acquired and processed using a Nikon Ni-U microscope and NIS-Elements software.

Statistical analyses

Two-tailed unpaired Student's t-test and one-way ANOVA were used to determine significance. P value < 0.05 was considered statistically significant. GraphPad Prism 8.0 were used for the statistical analyses. Quantified values were presented as the mean ± SD. Statistical significance was set at $P < 0.05$ (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).