Supporting Information for

ORIGINAL ARTICLE

Membrane-camouflaged supramolecular nanoparticles for co-delivery of chemotherapeutic and molecular-targeted drugs with siRNA against patient-derived pancreatic carcinoma

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1. Supporting methods

1.1. Chemicals and reagents

Gemcitabine (GEM), erlotinib (Er), (2-Hydroxypropyl)-β-cyclodextrin (β-CD), polyethyleneimine (branched PEI, molecular weight 600 Da) and acetic acid were purchased from J&K Scientific Co. *N,N*-Diisopropylethylamine (DIPEA), pyridine, triethylamine and 4-Nitrobenzyl chloroformate were provided by TCI Reagents. These chemicals were used without further purification. Acetone, ethyl acetate, dichloromethane (DCM) and *N,N*-dimethyl-formamide (DMF) were provided by Aladdin Reagents and dried with CaH_2, and then distilled under nitrogen atmosphere before use. Other solvents used in the experiments were obtained from Aladdin Reagents, which were analytical grade and used without further purification. All aqueous solutions were prepared using ultrapure water. Antibodies against IRAK4 (#4363), α-SMA (#19245) and anti-GAPDH (#2118) were obtained from Cell Signalling Technology (MA, USA). Antibodies against hENT1 (ab182023), p-EGFR (ab32578) and EGFR (ab52894) were obtained from

Abcam (Cambridge, USA). Antibodies against NF-κB (14220-1-AP), NF-κB p65(10745-1-AP) were purchased from Proteintech (Wuhan, China). Human IL-1β ELISA Kit (KE00021) was purchased from Proteintech. D-Luciferin potassium salt was purchased from Yeasen (40902ES01, Yeasen, Shanghai, China).

1.2. Synthesis of ICG-siIRAK4/Er@GEM-SS-PC

The ICG-siIRAK4/Er@GEM-SS-PC was synthesized according to the literature[1,2], ICG (2 mg) was dissolved in 1 mL DMSO solution, and then added into the siIRAK4/Er@GEM-SS-PC (10 mg in 5 mL water solution) and stirred for 8 h in dark environment. After that, the mixture was collected and dialyzed against ultrapure water using the dialysis bag (MWCO 1000 Da) for 2 days in order to remove free ICG and remaining organic solvent.

1.3. Instrumentation

The ^{1}H nuclear magnetic resonance (^{1}H NMR) was recorded on a Bruker Avance 500 MHz spectrometer (Bruker, Karlsruhe, Germany). The mass spectrometry (MS) was obtained on an AB Sciex Triple TOF 5600+ mass spectrometer (AB Sciex, GA, USA). The particle size and zeta potential were determined by using a Malvern Nano-ZS90 particle size analyzer (Malvern Panalytical, Shanghai, China). The morphology of nanoparticles was observed on a JEM-2010HR transmission electron microscopy (JEOL, Tokyo, Japan). The UV–Vis spectra wasrecorded on a Hitachi U-3010 UV–Vis spectrophotometer (Hitachi, Tokyo, Japan). The HPLC analyses were performed on an Agilent 1260 high performance liquid chromatography (Agilent, Beijing, China). The cell apoptosis rate was examined by using the BD FACScan™ flowcytometry system (NJ, USA).

1.4. siRNA synthesis and preparation of the nano-drug siIRAK4/Er@GEM-SS-PC

The IRAK4 siRNA was synthesized as previously described[3]. Target sequences: shIRAK4#1, GCAGUUUGAUCAAGAAAUATT; shIRAK4#2, GCACAUGAGAUGCAAGAUUTT. The desired amount of Er@GEM-SS-PC was mixed with siIRAK4 in 100 μL double-distilled water (ddH2O) and incubated at room temperature for 0.5 h and formed the nano-drug siIRAK4/Er@GEM-SS-PC. The complexes were diluted with ddH$_2$O and determined the characterizations by DLS and TEM. The siRNA loading and encapsulation efficiency were quantified by using Cy5.5-labled siRNA, which exhibited the strong absorbance peak at 670 nm. The encapsulation efficiency of the siRNA was 72.3%, and the loading content of siRNA in nanoparticle was 1.34%.

1.5. Preparation of hybrid membranes and siIRAK4/Er@GEM-SS-PC-M

The cell membrane was prepared according to previous studies[4,5]. Briefly, Briefly, RAW264.7 macrophages, pancreatic cancer cell PANC-1 and SW1990 were harvested, and washed twice with PBS. Then, cell membranes were collected using trypsin-EDTA solution, where 10 mL PBS containing an EDTA-free mini protease inhibitor tablet was added (Pierce). The solution was disrupted under the sonication at 200 W for 3 min, followed by centrifuging at $3200 \times g$ for 5 min to remove the cells. The supernatant was centrifuged again at $20,000 \times g$ for 20 min to collect pellet. The pellet containing membrane was then washed twice and filtered with 0.45 μm pore size filters. The pancreatic cancer cell and RAW 264.7 membranes were mixed in a 1:1 (*w/w*) ratio, and sonicated at 37 °C for 10 min to allow their fusion. The membranes were identified by SDS-PAGE analysis of protein profile and specific cell protein marker. To prepare the fused membrane-coated nanoparticles, 200 μg of the hybrid membranes was mixed with siIRAK4/Er@GEM-SS-PC solution and stirred at 37 °C for 1 h. The mixed solution was physically extruded through a 220 nm polycarbonate membrane using an Avanti mini-extruder for seven passes to obtain the hybrid membrane-coated siIRAK4/Er@GEM-SS-PC-M NPs. To purify siIRAK4/Er@GEM-SS-PC-M from uncoated membrane components, the resulting hybrid membrane coated siIRAK4/Er@GEM-SS-PC-M solution was centrifuged at $3000 \times g$ for 30 min to remove the uncoated membrane as previous described[6]. After washing with PBS for several times, no protein in the supernatant was detected by BCA Protein Assay. The mean diameter, zeta potential, and morphology of nanoparticles were characterized by DLS and TEM, respectively.

1.6. Colony formation assay

Colony formation assay was performed according to previous study[7]. Briefly, a density of 500/per well SW1990 and PANC-1 cells were seeded in 12-well plates, respectively. The cells were incubated with GEM (5 μmol/L), Er (5 μmol/L), GEM (5 μmol/L) combined Er (5 μmol/L), siNT/Er@GEM-SS-PC-M, siIRAK4/Er@GEM-SS-PC and siIRAK4/Er@GEM-SS-PC-M in culture medium. After cells had grown for 14 days, the clones were then fixed by 4% formalin, washed in PBS, and stained with 0.1% crystal violet at room temperature. Clones containing 50 or more than 50 cells were regarded as a positive clone when observed under a microscope.

1.7. Cell apoptosis analysis

Cell apoptosis was determined by Annexin-V-FITC and propidium iodide (PI) staining using apoptosis detection kit (BD, USA) according to the manufacture's protocol. Briefly, SW1990 and PANC-1 cells were seeded in 12-well plates at 1×10^5 cells/well. After 24 h treatment with GEM (5 μmol/L), Er (5 μmol/L), GEM (1 μmol/L) combined Er (5 μmol/L), siNT/Er@GEM-SS-PC-M, siIRAK4/Er@GEM-SS-PC and

siIRAK4/Er@GEM-SS-PC-M for 24 h, cells were collected, washed twice with PBS. After that, the cells were stained by Annexin-V-FITC and PI to measure the apoptotic cells. The analysis of apoptotic cells was performed on flow cytometry FACS Aria III (BD, USA).

1.8. Migration assay

The migration assay was performed as previously described[8]. Briefly, SW1990 and PANC-1 cells were seeded in 12-well plates at 1×10^5 cells/well and incubated overnight to achieve an approximate confluence rate of 90%. Subsequently, the cell monolayer was scraped in a cross shape using a 200 μL pipette tip at 0 h and washed three times with PBS to ensure a scratch area without residual cells, followed by the above indicated treatment. Images were captured with a phase-contrast microscope (CKX41; Olympus Corporation, Tokyo, Japan) at 0 and 48 h. The distance between the sides of the scratch was determined by the Image-Pro Plus software in 3 random fields. The synergism analysis was carried out according to the previous methods[9,10]. Briefly, to determine drug interactions (*i.e.*, additive, synergistic, or antagonistic), we calculated the coefficient of drug interaction (CDI) using the formula CDI = AB/(A × B), where AB was the ratio of the effect of the combination to the control, and A or B was the ratio of single drug to the control. CDI value <1, =1 or >1 indicated that the drugs were synergistic, additive or antagonistic, respectively.

1.9. TUNEL assay

The apoptotic cell death was evaluated by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) method using an apoptosis detection kit (C1090, Beyotime Biotechnology, Shanghai, China). The tumor tissues from the above treated groups were embedded in paraffin. The sections were cut to 4 μm thicknesses and mounted on polylysine coated glass slides. Immunofluorescence procedures for detecting apoptotic cardiomyocytes were performed according to the manufacturer's instructions by using an Olympus microscope (Olympus, Tokyo, Japan). For each slide, five fields were randomly chosen, and using a defined rectangular field area (20× objective). The index of apoptosis was determined (number of positively stained apoptotic cells/rectangular field area). Assays were performed in a blinded manner.

## 1.10. *In vivo* toxicity evaluation

For HE staining, subcutaneous tumors and other organs were dislodged and fixed in 4% paraformaldehyde. Subsequently, the organs were stepwise dehydrated with sucrose solution of 15% and 30% for 24 h respectively. Blood was collected and centrifuged from the tumor-bearing mice to detect the level of UA (uric acid), BUN (blood urea nitrogen), albumin, ALT (alanine aminotransferase), AST (aspartate aminotransferase), and total protein in serum.

References

1. Guo ZD, Gao MN, Song ML, Li YS, Zhang DL, Xu D, et al. Superfluorinated PEI derivative coupled with [99m]TC for ASGPR targeted[19]F MRI/SPECT/PA tri-modality imaging. *Adv Mater* 2016;**28**:5898-06.

2. Ren SS, Cheng X, Chen MK, Liu C, Zhao PC, Huang W, et al. Hypotoxic and rapidly metabolic PEG-PCL-C3-ICG nanoparticles for fluorescence-guided photothermal/photodynamic therapy against OSCC. *ACS Appl Mater Interfaces* 2017;**9**:31509-18.

3. Alterman JF, Godinho BMDC, Hassler MR, Ferguson CM, Echeverria D, Sapp E, et al. A divalent siRNA chemical scaffold for potent and sustained modulation of gene expression throughout the central nervous system. *Nat Biotechnol* 2019;**37**:884-94.

4. Chen Q, Huang GJ, Wu WT, Wang JW, Hu JW, Mao JM, et al. A hybrid eukaryotic-prokaryotic nanoplatform with photothermal modality for enhanced antitumor vaccination. *Adv Mater* 2020;**32**:e1908185.

5. Ji B, Cai HQ, Yang Y, Peng FH, Song MY, Sun KJ, et al. Hybrid membrane camouflaged copper sulfide nanoparticles for photothermal-chemotherapy of hepatocellular carcinoma. *Acta Biomater* 2020;**111**:363-372.

6. Gao C, Huang Q, Liu C, Kwong CHT, Yue L, Wan JB, et al. Treatment of atherosclerosis by macrophage-biomimetic nanoparticles *via* targeted pharmacotherapy and sequestration of proinflammatory cytokines. *Nat Commun* 2020;**11**:2622.

7. Fung SW, Cheung PF, Yip CW, Ng LW, Cheung TT, Chong CC, et al. The ATP-binding cassette transporter ABCF1 is a hepatic oncofetal protein that promotes chemoresistance, EMT and cancer stemness in hepatocellular carcinoma. *Cancer lett* 2019;**457**:98-109.

8. Liu PX, Shi Y, Fan ZY, Zhou YJ, Song YY, Liu YJ, et al. Inflammatory smooth muscle cells induce endothelial cell alterations to influence cerebral aneurysm progression *via* regulation of integrin and VEGF expression. *Cell Transplant* 2018;**28**:713-22.

9. Jin JL, Gong J, Yin TJ, Lu YJ, Xia JJ, Xie YY, et al. PTD4-apoptin protein and dacarbazine show a synergistic antitumor effect on B16-F1 melanoma *in vitro* and *in vivo*. *Eur J Pharmacol* 2011;**654**:17-25.

10. Xu SP, Sun GP, Shen YX, Peng WR, Wang H, Wei W, et al. Synergistic effect of combining paeonol and cisplatin on apoptotic induction of human hepatoma cell lines. *Acta Pharmacol Sin* 2007;**28**:869-78
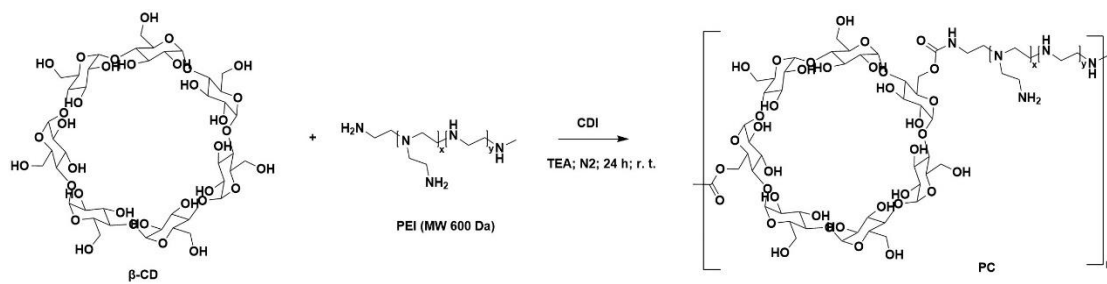
Scheme S1. Synthetic route for cationic polymer gene vector **PC**.
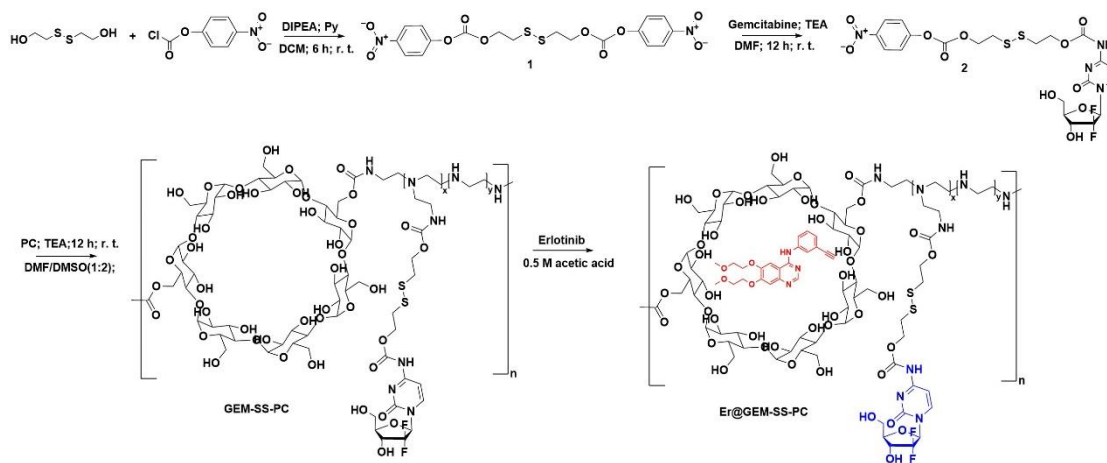
Scheme S2. Synthetic route for the co-delivery system (Er@GEM-SS-PC).
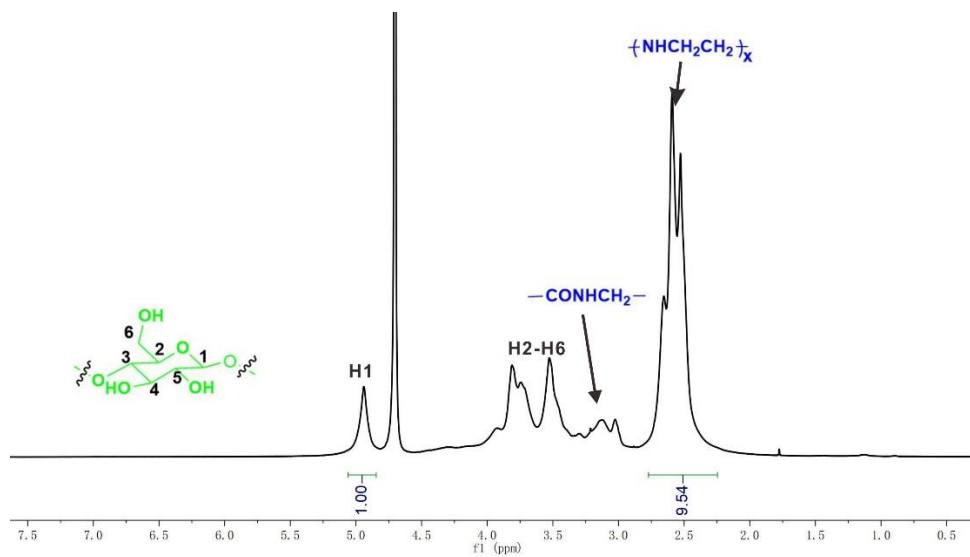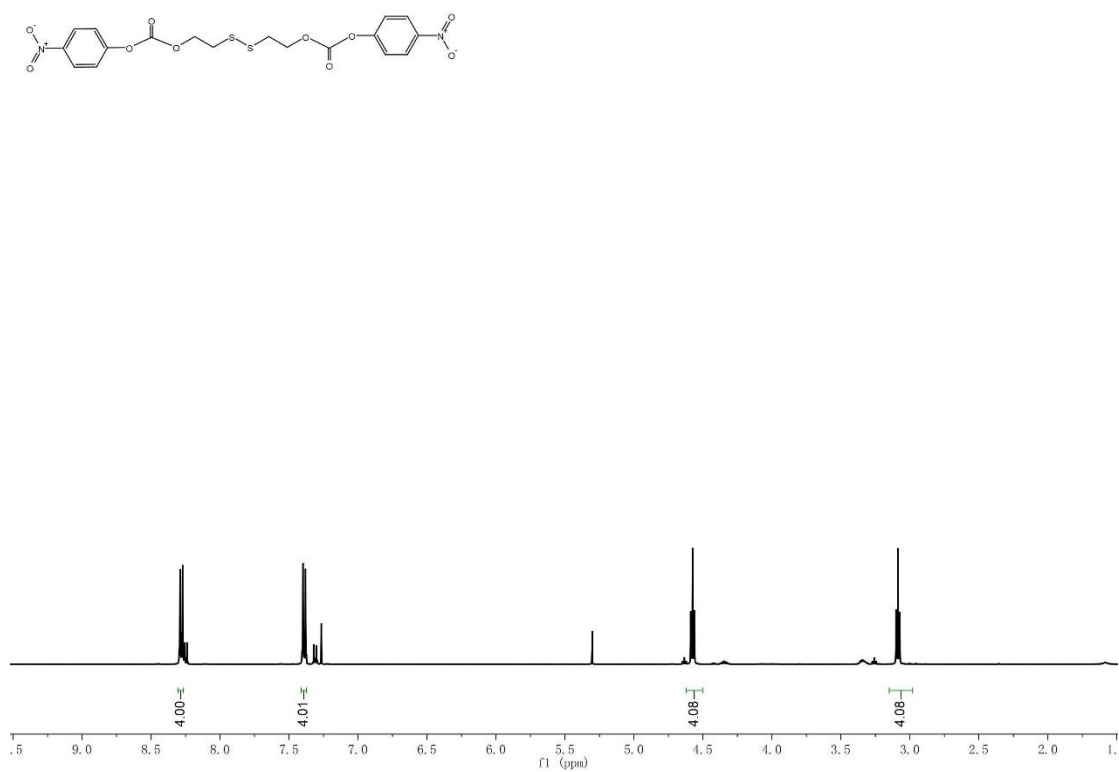
Figure S1 ^{1}H NMR analyses for **PC** (D$_2$O).
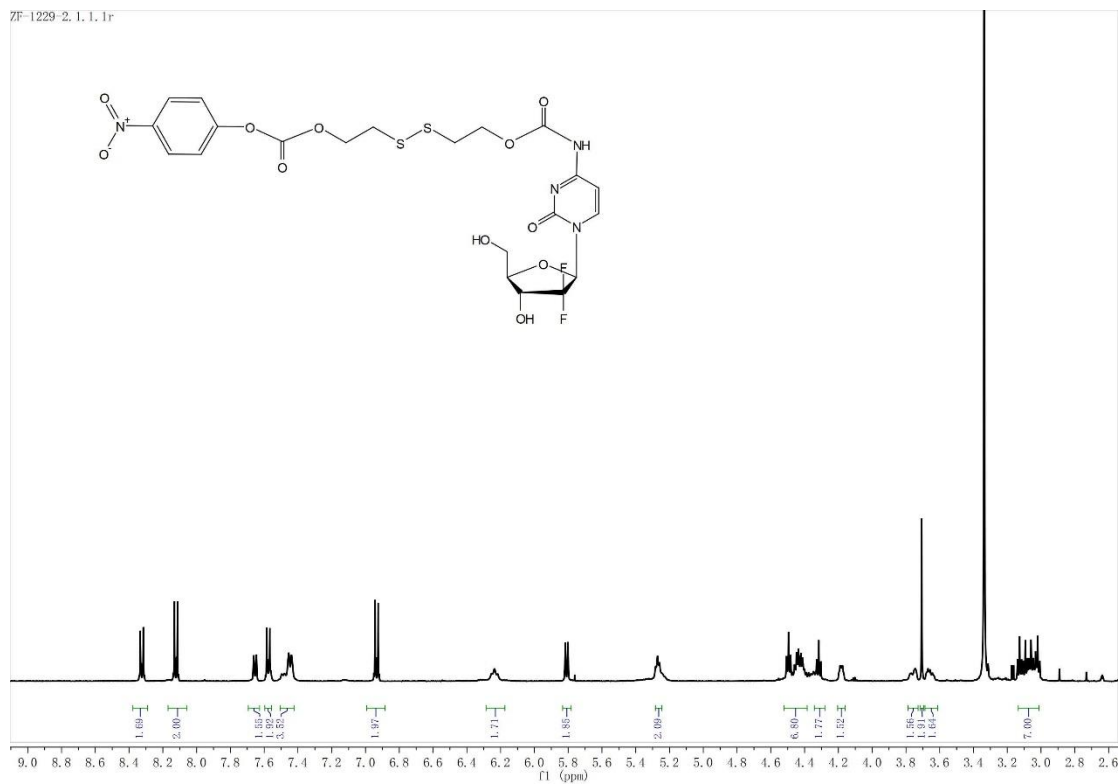

Figure S2 ^{1}H NMR spectrum of **1** (in CDCl$_3$).

Figure S3 ^{1}H NMR spectrum of **2** (in DMSO-d_6).
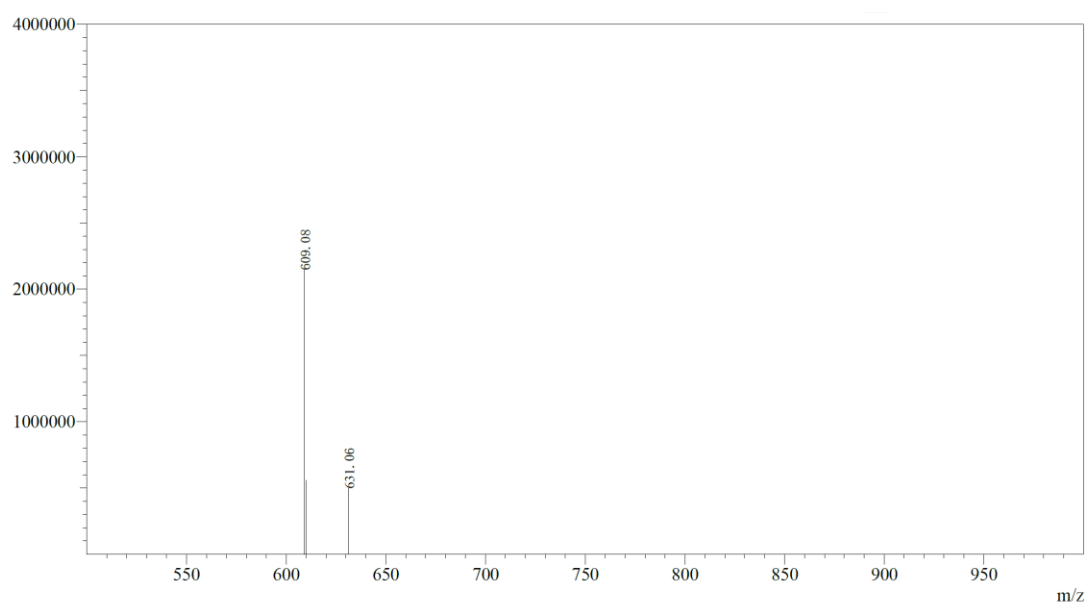
Figure S4 MS spectrum of **2**.
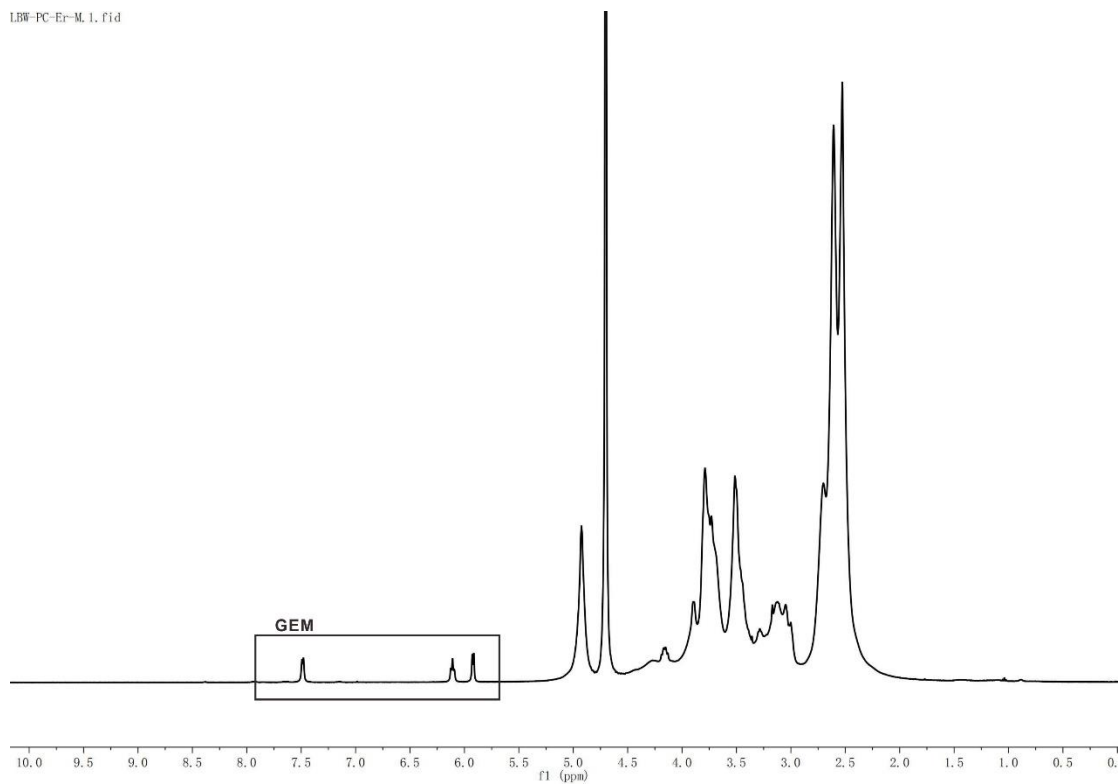
Figure S5 ^{1}H NMR spectrum of GEM-SS-PC (in CD$_3$OD-d_4).
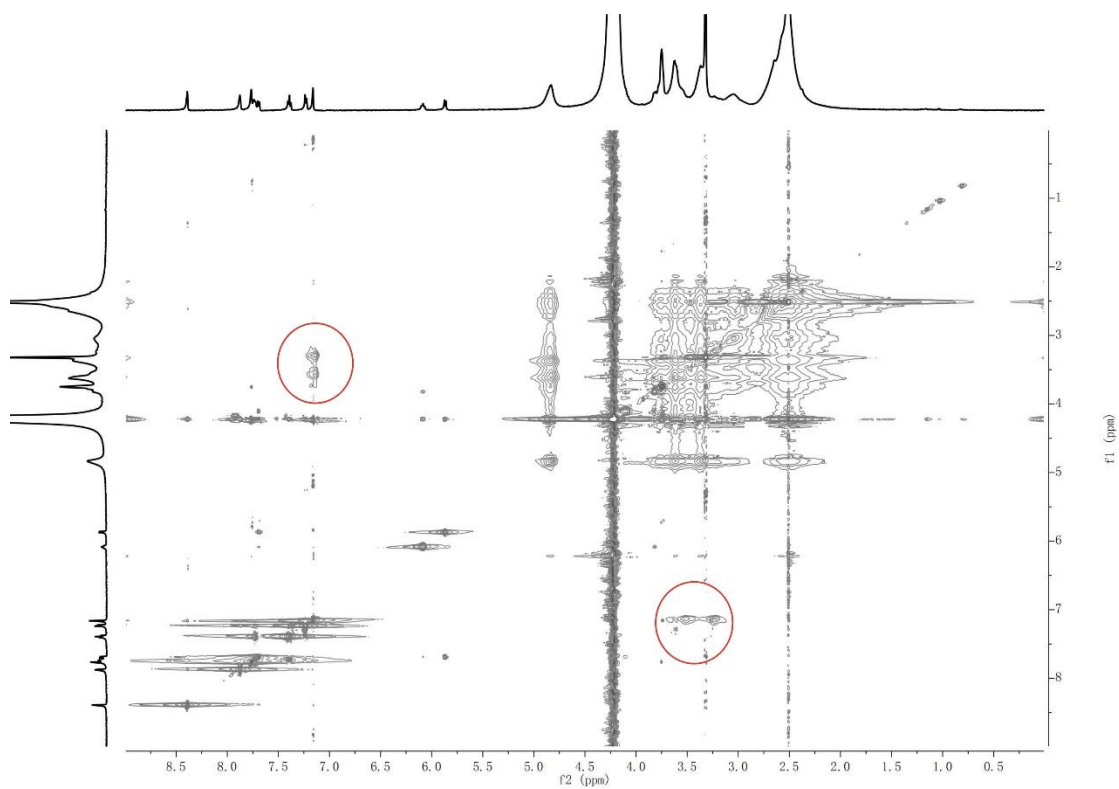
Figure S6 2D-NOESY spectrum of Er@GEM-SS-PC (in DMSO-d_6).
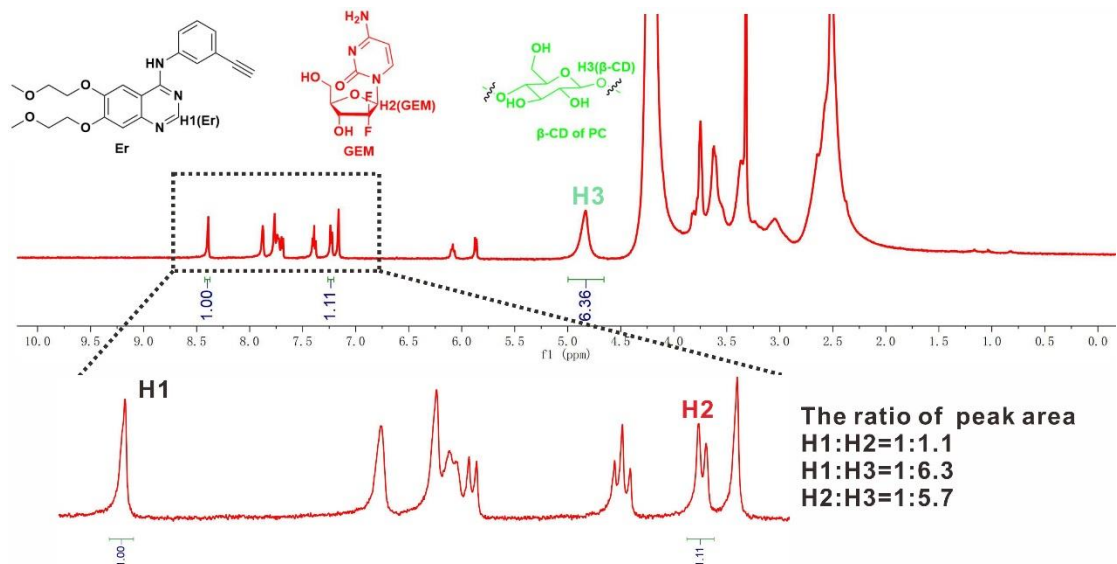
Figure S7 ^{1}H NMR analyses for the mole ratio of Er and GEM in Er@GEM-SS-PC.
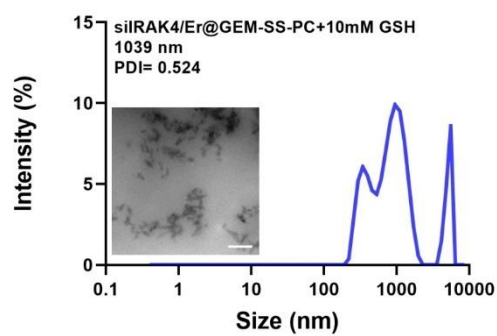
Figure S8 The TEM image for the siIRAK4/Er@GEM-SS-PC incubated with 10 mmol/L GSH. Scale bar, 500 nm.
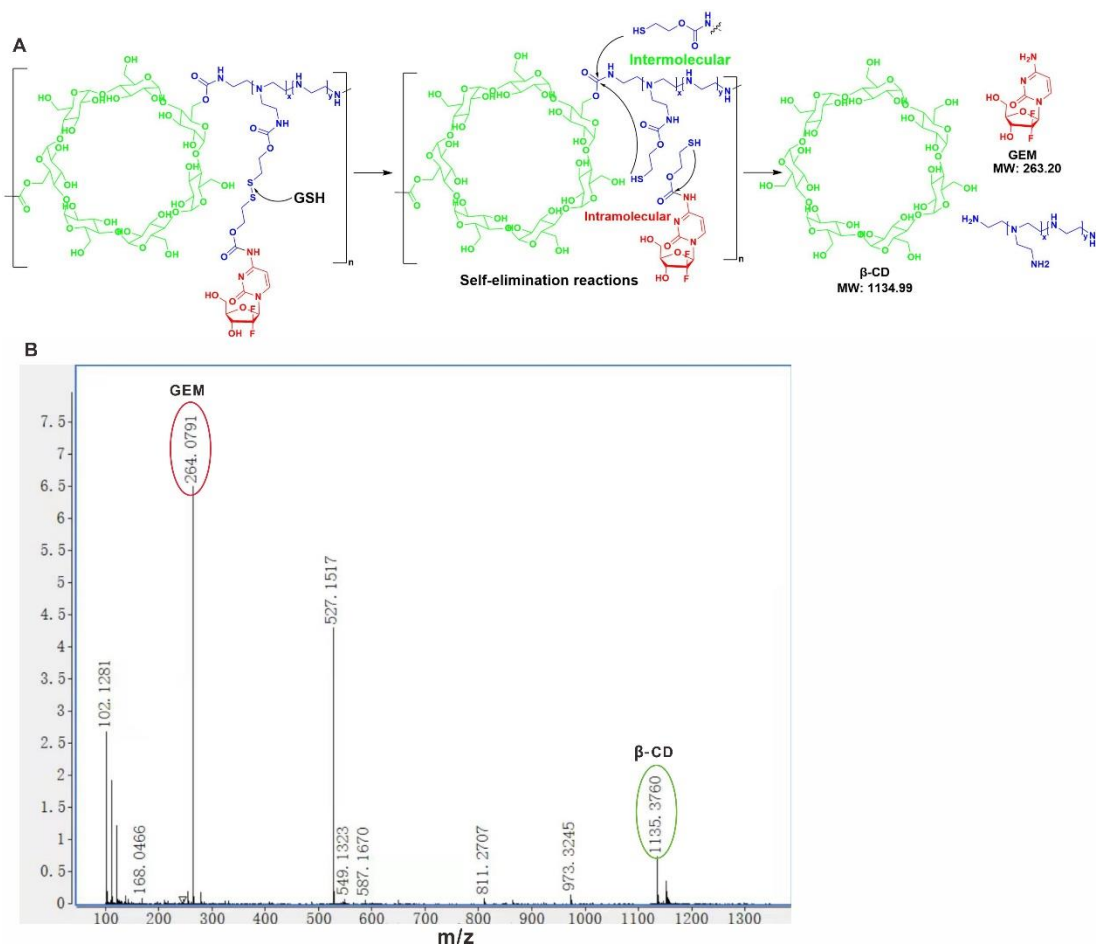
Figure S9 (A) The intramolecular or intermolecular self-eliminations reactions of siRNA@GEM-SS-PC was trigger by high level of GSH. (B) HR-MS (ESI) analyses for siRNA@GEM-SS-PC upon incubation with 10 mmol/L GSH for 12 h
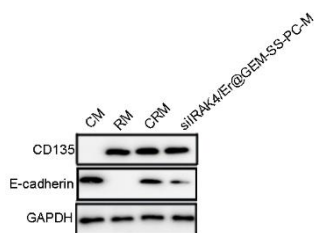
Figure S10. Western-blotting analysis of cell membrane proteins of PANC-1, RAW264.7, hybrid membranes and hybrid membrane coated nanoparticles (NPs). cancer cell membrane (CM), RAW264.7 macrophage membrane (RM), hybrid membranes (CRM) and siIRAK4/Er@GEM-SS-PC-M.
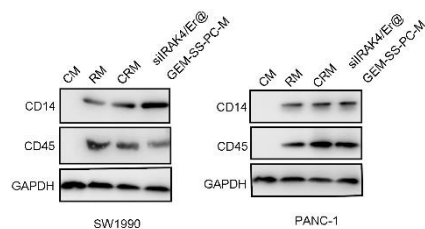
Figure S11 Western-blotting analysis of CD45 and CD14 on PANC-1, RAW264.7, hybrid membranes and hybrid membrane coated nanoparticles (NPs). cancer cell membrane (CM), RAW264.7 macrophage membrane (RM), hybrid membranes (CRM) and siIRAK4/Er@GEM-SS-PC-M.
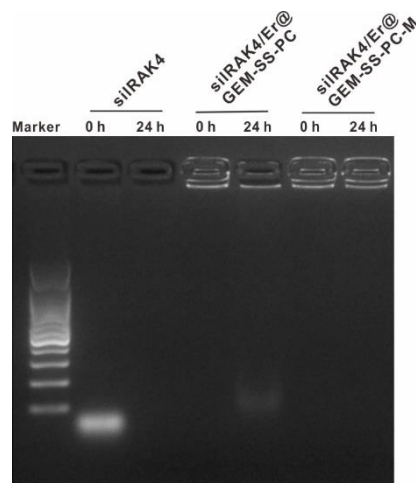
Figure S12 The free siIRAK4, siIRAK4/Er@GEM-SS-PC and siIRAK4/Er@GEM-SS-PC-M were incubated in serum at 37 °C for up to 24 h. The stability of siIRAK4 was measured electrophoretic gel assay.
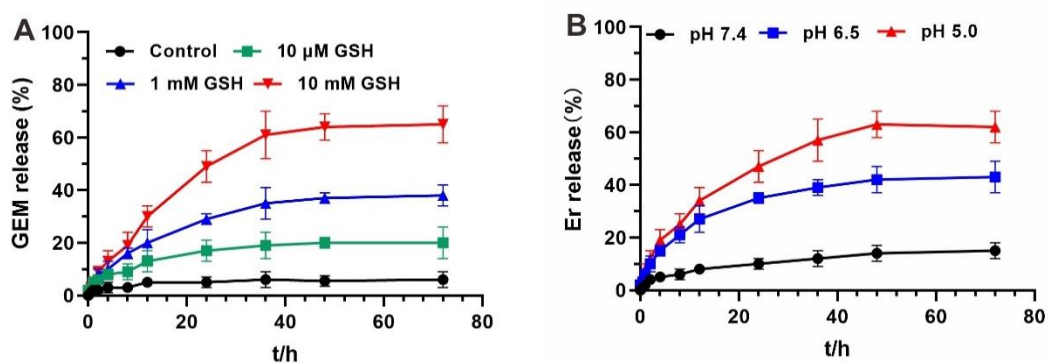

Figure S13 (A) The cumulative release profiles (as measured by HPLC) for GEM from siIRAK4A/Er@GEM-SS-PC-M, which was triggered by different GSH concentration (0–10 mmol/L). (B) The cumulative release profiles (as measured by HPLC) for Er from siIRAK4/Er@GEM-SS-PC by different pH value (5.0, 6.5 and 7.4). ($n = 3$)
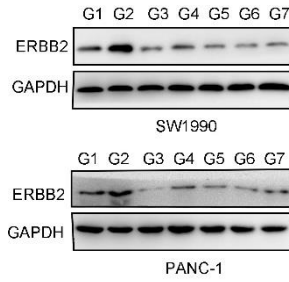
Figure S14 The SW1990 and PANC-1 cells were incubated with GEM, Er, free GEM and Er, siNT/Er@GEM-SS-PC, siIRAK4/Er@GEM-SS-PC and siIRAK4/Er@GEM-SS-PC-M for 24 h. The protein expression of ERBB2 was determined by Western Blot. G1, PBS; G2, GEM; G3, Er; G4, free GEM and Er; G5, siNT/Er@GEM-SS-PC; G6, siIRAK4/Er@GEM-SS-PC; G7, siIRAK4/Er@GEM-SS-PC-M.
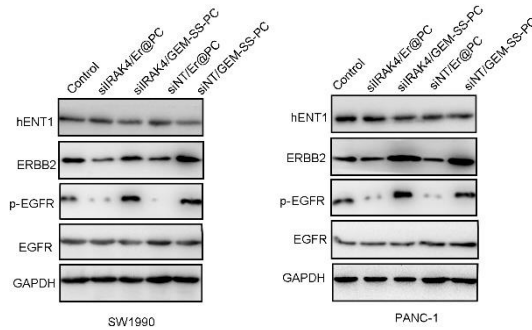

Figure S15 The SW1990 and PANC-1 cells were incubated with control, siIRAK4/Er@PC, siNT/GEM-SS-PC, siNT/Er@PC, and siNT/ GEM-SS-PC for 24 h. The protein expression of hENT1, p-EGFR and EGFR was determined by Western blot.
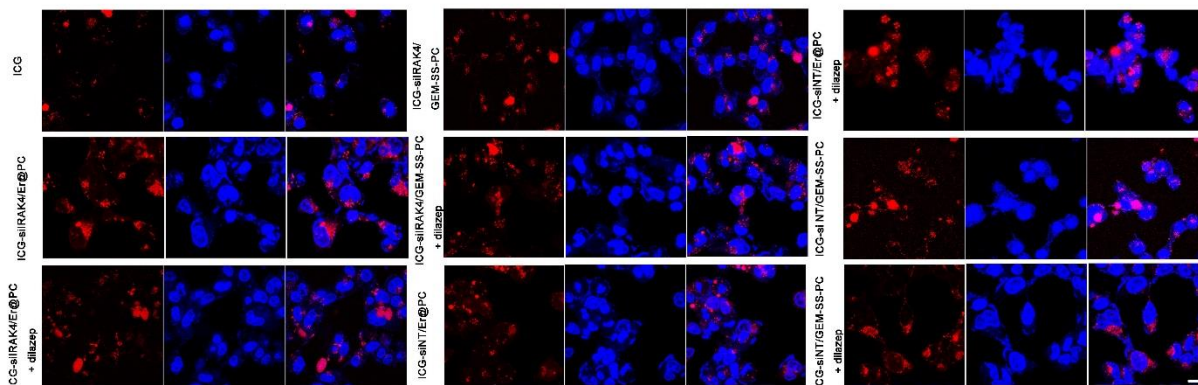
Figure S16 The SW1990 and PANC-1 cells were pre-incubated with or without dilazep (a hENT1 inhibitor) and then treated with the free ICG or ICG-labeled control, siIRAK4/Er@PC, siNT/GEM-SS-PC, siNT/Er@PC, and siNT/ GEM-SS-PC for 24 h. Confocal images of the above treated cells. Scale bar, 10 μm.
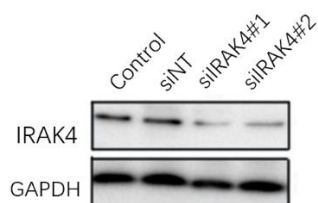
Figure S17 The knockdown efficiency of siIRAK4#1, siIRAK4#2 and siNT (siNT refers to scrambled siRNA) in PANC-1 cells. (siIRAK4#1, siIRAK4#2 refers to two different target sequences)
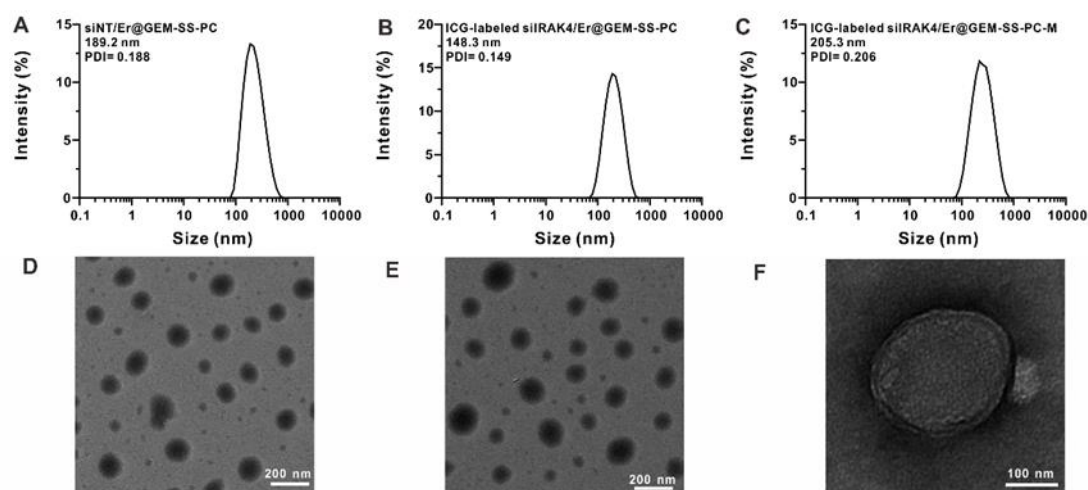

Figure S18 The diameter distribution as determined by DLS and the TEM image for siNT/Er@GEM-SS-PC, ICG-labeled siIRAK4/Er@GEM-SS-PC and ICG-labeled siIRAK4/Er@GEM-SS-PC.
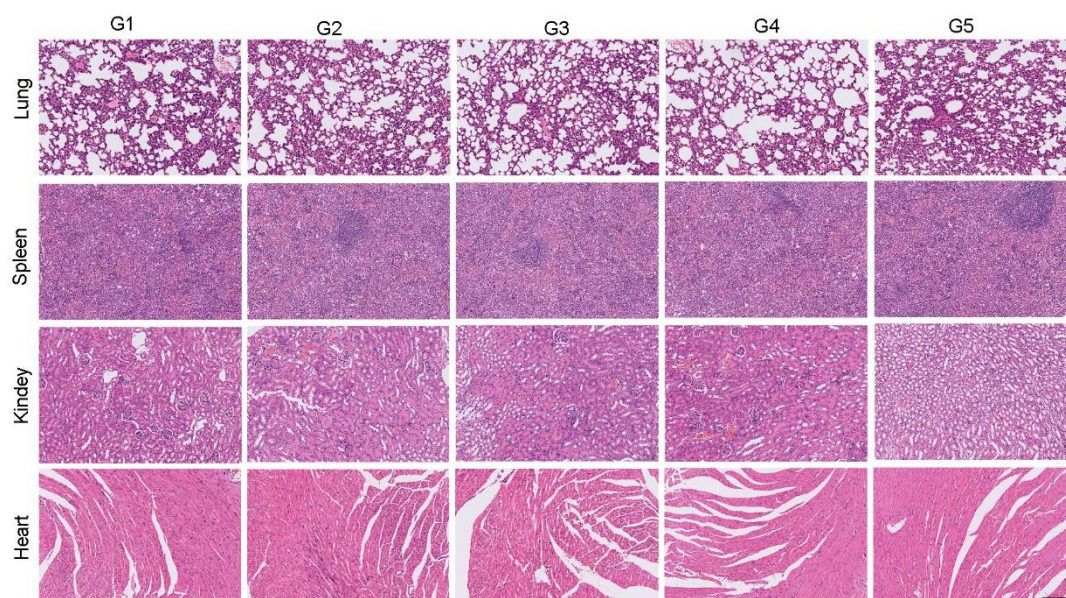

Figure S19 Toxicity evaluation after tumor treatment with PBS, GEM with Er, siNT/Er@GEM-SS-PC, siIRAK4/Er@GEM-SS-PC and siIRAK4/Er@GEM-SS-PC-M. G1, PBS; G2, GEM and Er; G3, siNT/Er@GEM-SS-PC; G4, siIRAK4/Er@GEM-SS-PC; G5, siIRAK4/Er@GEM-SS-PC-M. The tissue slices from major organs were analyzed by HE staining at Day 21 after orthotopic pancreatic tumor implantation. Scale bar, 50 μm.
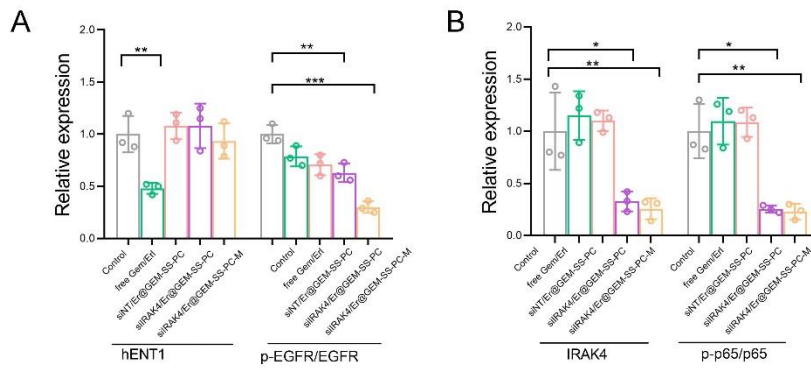
Figure S20 (A) Quantitative analysis of protein expression of hENT1 and the phosphorylation of EGFR by Western blot. (B) Quantitative analysis of protein expression of IRAK4 and the phosphorylation of p65 by Western blot. ($n = 3$)
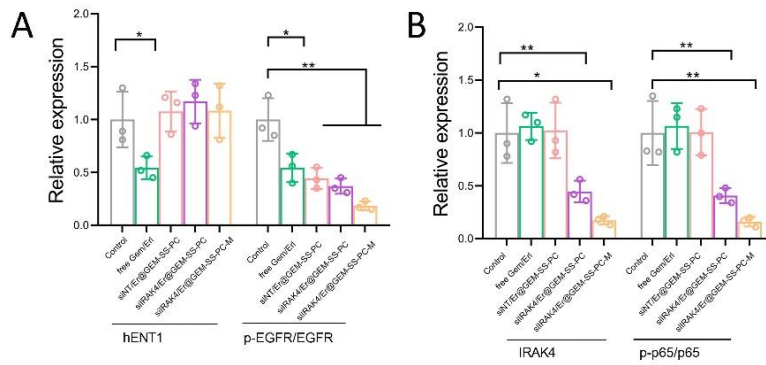

Figure S21 (A) Quantitative analysis of protein expression of hENT1 and the phosphorylation of EGFR by Western blot. (B) Quantitative analysis of protein expression of IRAK4 and the phosphorylation of p65 by Western blot. ($n = 3$).
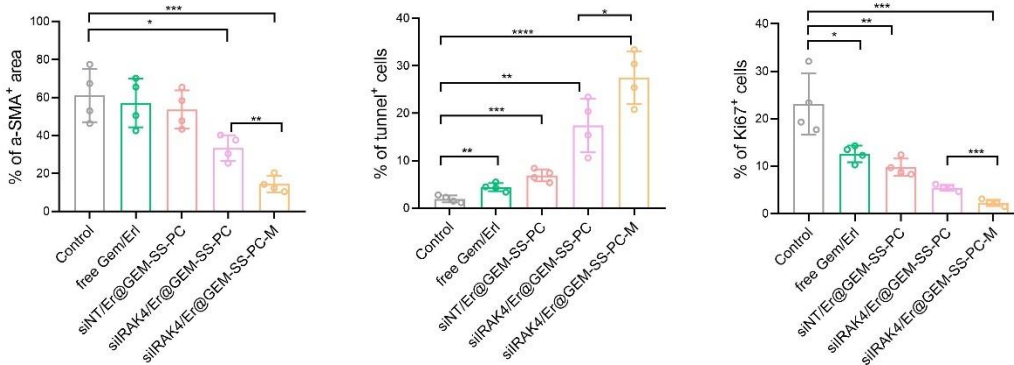

Figure S22 Quantitative analysis of α-SMA, Ki67, TUNNEL positive area in tumors. ($n = 4$).
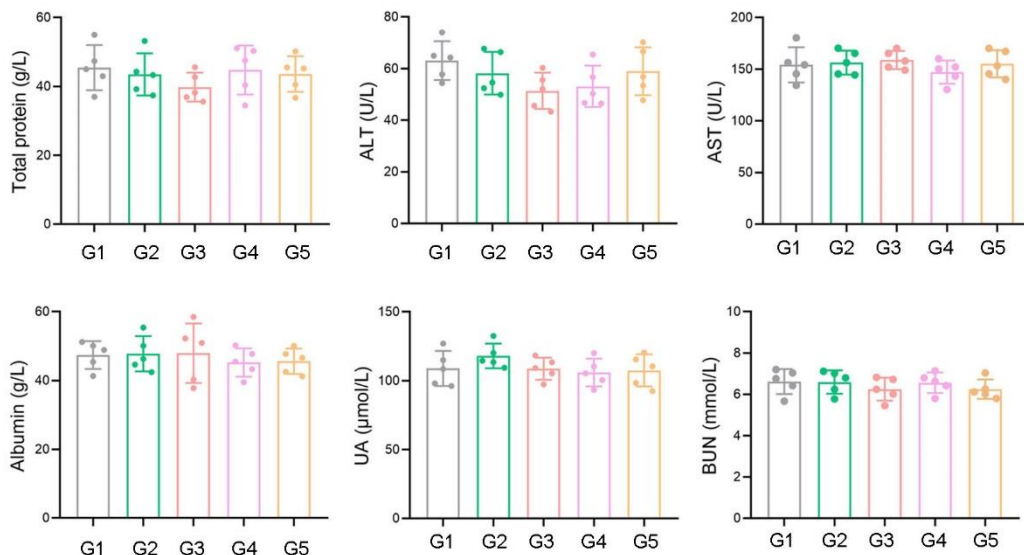
Figure S23 Hematological evaluation of patient-derived pancreatic tumor-bearing mice. The figures showed the levels of total protein, AST (aspartate aminotransferase), ALT (alanine aminotransferase), albumin, UA (uric acid), and BUN (blood urea nitrogen) in serum at Day 45 after patient-derived pancreatic tumor implantation. G1, PBS; G2, GEM and Er; G3, siNT/Er@GEM-SS-PC; G4, siIRAK4/Er@GEM-SS-PC; G5, siIRAK4/Er@GEM-SS-PC-M. Data represent mean ±S.D. ($n = 4$).
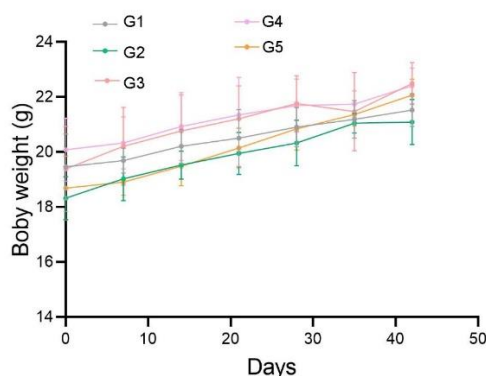

Figure S24 The body weight of patient-derived pancreatic tumor-bearing mice. G1, PBS; G2, GEM and Er; G3, siNT/Er@GEM-SS-PC; G4, siIRAK4/Er@GEM-SS-PC; G5, siIRAK4/Er@GEM-SS-PC-M. Data represent mean ± S.D. ($n = 5$).

Table S1 The mole ratio of different constituents (Er, GEM and PC) in Er@GEM-SS-PC

Group	Ratio of peak area[a]	Molar ratio
H1:H2 (Er:GEM)	1: 1.1	1: 1.1
H1:H3 (Er:PC)	1: 6.3	1.1: 1
H2:H3 (GEM:PC)	1: 5.7	1.25: 1

[a]The peak area determined by the integration of relevant peaks from ^{1}H NMR