

Electronic Supplementary Information

Alkaline phosphatase-triggered assembly of etoposide enhances its anticancer effect

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1. General methods

Recombinant intestinal alkaline phosphatase (ALP) was purchased from BaoMan Inc. (Shanghai) (one unit (U) is the enzyme activity that cleaves 1 μmol of the standard substrate per minute at 37 °C). Etoposide phosphate was obtained from Hangzhou Hyper Chemicals Limited. The commercially available reagents were used without performing further purification, unless noted otherwise. HPLC analyses were performed on an Agilent 1200 HPLC system that is equipped with a G1322A pump and in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column (9.6 * 250 mm, 5-Micron) or a Stable Bond Analytical Zorbax, 300SB-C18 RP column (4.6 * 250 mm, 5-Micron) with CH_3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent. The spectra of electrospray ionization-mass spectrometry (ESI-MS) were recorded on a LCQ Advantage MAX ion trap mass spectrometer (Thermo Fisher) or a time-of-flight Agilent Technologies 6224 mass spectrometer. ^1H NMR and ^{13}C NMR spectra were obtained on a 400 MHz Bruker AV III 400. Rheological measurement was performed on an AR 2000ex (TA instrument) system, 40 mm parallel plates were used during the experiment at the gap of 300 μm . Cryo-transmission electron microscopy (cryo-TEM) images were obtained on a Tecnai F20 transmission electron microscope from FEI company. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclon) supplemented with 10% fetal bovine serum at 37 °C, 5% CO_2 , and humid atmosphere. 5 week old (weighting ~ 15 g) BALB/c nude mice were used for animal experiments. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use Committee

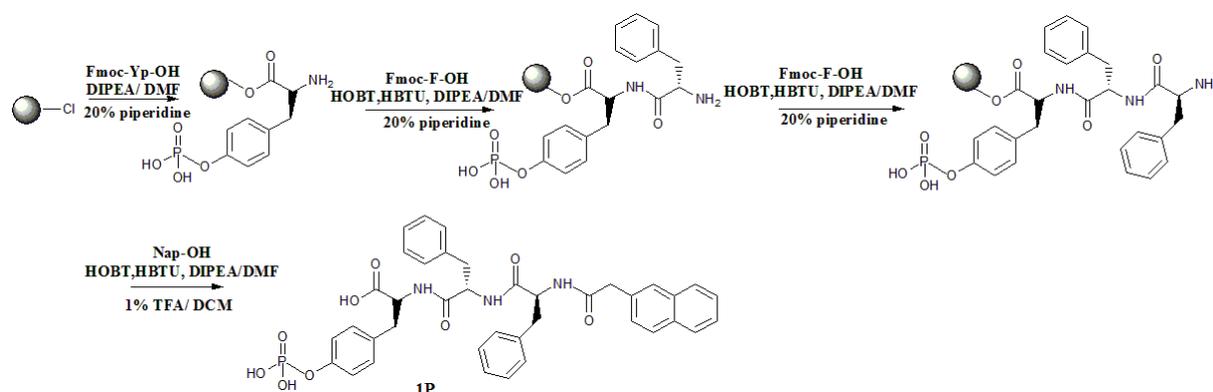
(USTCACUC1301019).

MTT assay:

The cytotoxicity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with HeLa cells. Cells growing in log phase were seeded into 96-well cell culture plate at 3×10^3 /well. The cells were incubated for 24 h at 37 °C under 5% CO₂. The solution of **1P** (100 µL/well) at concentrations of 0, 100, 200, 300, 400, and 500 µM in 100 µL of medium were added to the wells, respectively. The cells were incubated for 48 hours at 37 °C under 5% CO₂ to study the toxicity of **1P**. Then the solutions of **EP** or **EP + 1P** (1 : 6, w/w) at **EP** concentration of 5, 10, 20, and 40 µM in 100 µL of medium were added to the wells, respectively. The cells were incubated for 24, 48, or 72 hours at 37 °C under 5% CO₂. Ten µL solution of 5 mg/mL MTT dissolved in PBS buffer (pH 7.4, 10 mM) was added to each well of the 96-well plate. Then 100 µL DMSO was added to each well to dissolve the formazan after an additional 4 h of incubation. The data were obtained using an enzyme-linked immunosorbent assay (ELISA) reader (VARIOSKAN FLASH) to detect its absorption at 490 nm. The following formula was used to calculate the viability of cell growth: viability (%) = (mean of absorbance value of treatment group/mean of absorbance value of control) × 100.

2. Syntheses and Characterizations of **1P** and **1**

Scheme S1. The synthetic route for **1P**.



Synthesis of 1P: Compound Nap-Phe-Phe-Tyr(H₂PO₃)-OH (**1P**) was synthesized by using solid phase peptide synthesis (SPPS) method using 2-chlorotrityl chloride resin and the corresponding Fmoc-protected amino acids with side chains properly protected. The first amino acid was loaded onto the resin at about 0.5 mmol/g of resin. After loading the first amino acid to the resin, the capping reagent (DCM : MeOH : DIPEA = 17 : 2 : 1) was used to ensure all the active sites of the resin were protected. The solution of 20% piperidine in DMF was used to remove the Fmoc group, the next Fmoc-protected amino acid was coupled to the free amino group using HBTU as the coupling reagent. The growth of the peptide chain followed the established Fmoc SPPS protocol. ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.40 (t, *J* = 7.2 Hz, 2 H), 8.22 (d, *J* = 8.2 Hz, 1 H), 7.93 (d, *J* = 8.1 Hz, 1 H), 7.86 (d, *J* = 8.5 Hz, 1 H), 7.82 (d, *J* = 8.3 Hz, 1 H), 7.53 (t, *J* = 7.5 Hz, 1 H), 7.46 (d, *J* = 7.8 Hz, 1 H), 7.41 (dd, *J* = 13.6, 6.3 Hz, 1 H), 7.35 – 7.11 (m, 15 H), 4.59 (ddd, *J* = 22.4, 13.6, 7.2 Hz, 3 H), 3.88 (d, *J* = 22.2 Hz, 2 H), 3.13 – 2.95 (m, 4 H), 2.91 – 2.74 (m, 2 H). (Figure S1). ¹³C NMR (100 MHz, DMSO-*d*₆) δ 173.20, 171.54, 171.38, 170.31, 138.21, 137.95 (4C), 133.66, 132.87, 132.38, 132.31, 130.10, 129.67, 128.70, 128.44 (3C), 128.08, 127.40, 126.67 (4C), 126.37, 125.90,

124.59, 123.90, 78.12, 54.13 (2C), 53.97, 42.16 (2C), 38.02, 36.46. (Figure S2). MS: calculated for **1P** [(M + H)⁺]: 723.23, obsvd. ESI-MS: *m/z* 724.24 (Figure S3).

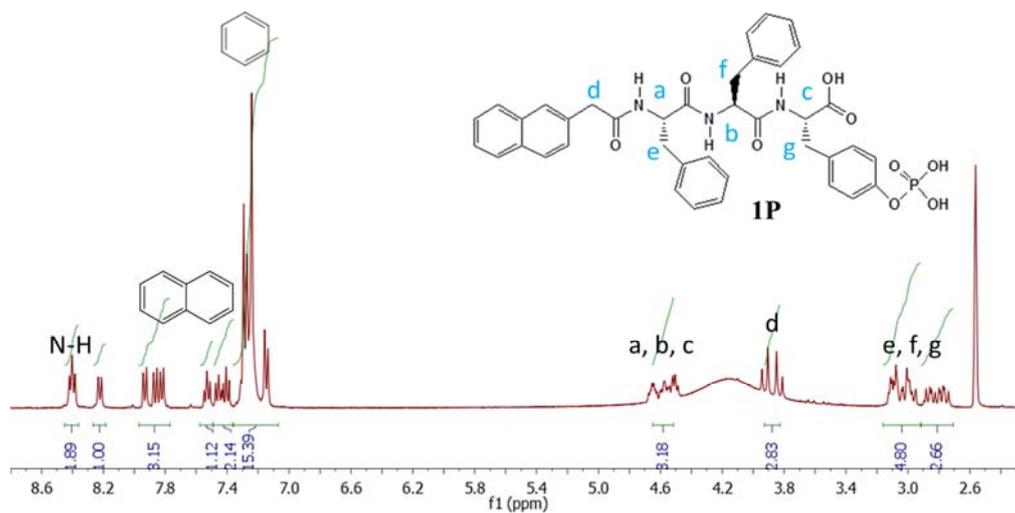


Figure S1. ¹H NMR spectrum of **1P** in *d*₆-DMSO.

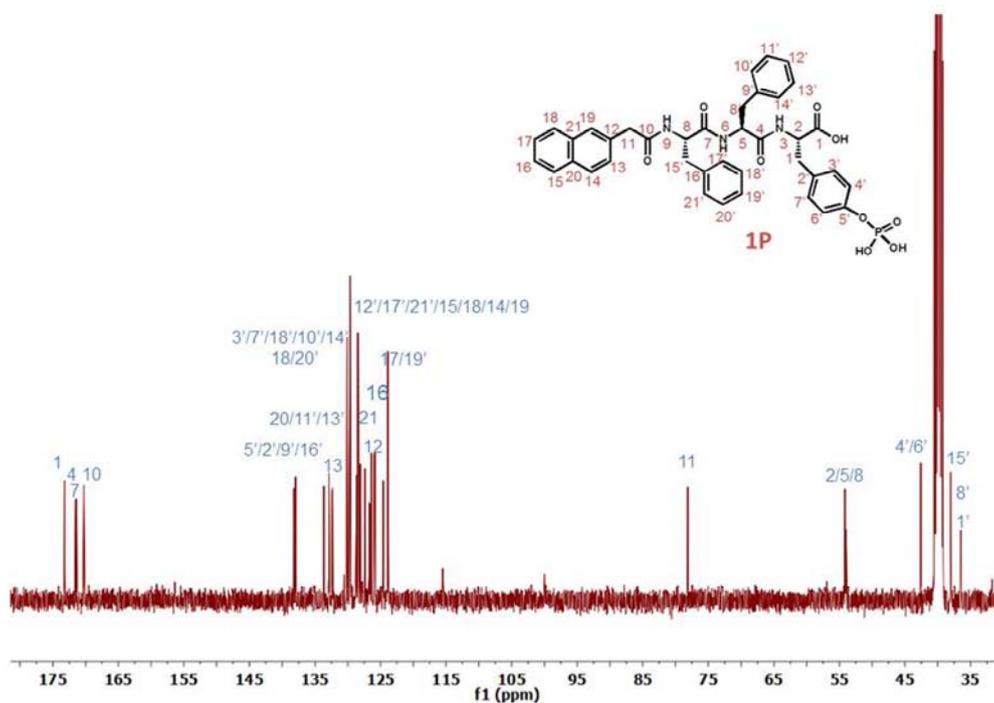


Figure S2. ¹³C NMR spectrum of **1P**.

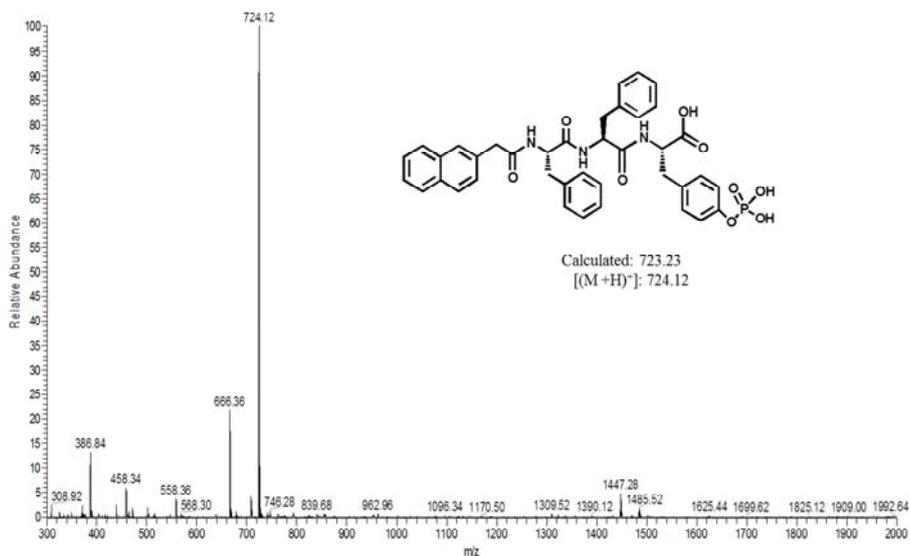
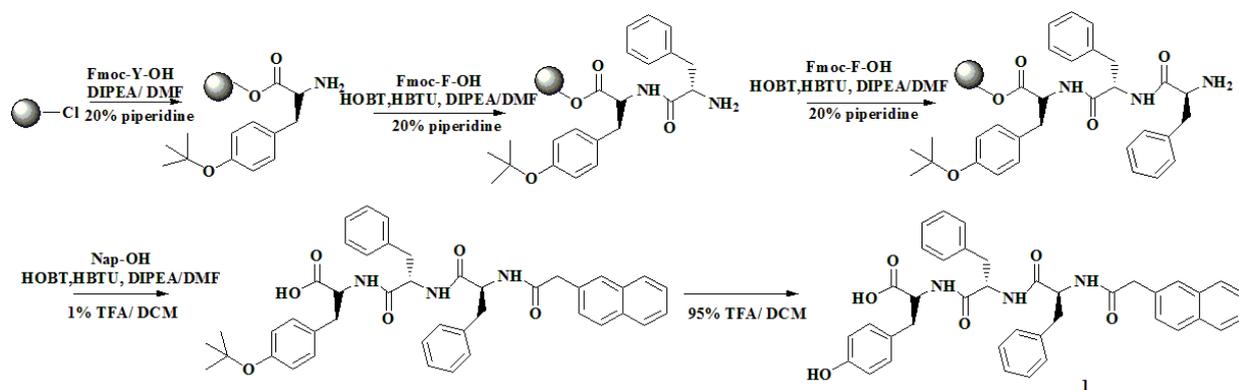


Figure S3. ESI/MS spectrum of **1P**.

Scheme S2. The synthetic route for **1**.



Synthesis of 1: Compound Nap-Phe-Phe-Tyr-OH (**1**) was synthesized after deprotection of the protecting group from Fmoc-Phe-Phe-Tyr(tBu)-OH, which was synthesized with SPPS and purified by HPLC. ^1H NMR (400 MHz, $\text{DMSO-}d_6$) δ 8.29 (t, $J = 12.7$ Hz, 2 H), 8.14 (d, $J = 8.1$ Hz, 1 H), 7.87 (d, $J = 8.3$ Hz, 1 H), 7.78 (dd, $J = 17.6, 8.3$ Hz, 2 H), 7.47 (t, $J = 7.4$ Hz, 1 H), 7.42 – 7.29 (m, 2 H), 7.17 (dd, $J = 16.1, 10.4$ Hz, 13 H), 6.87 (d, $J = 8.1$ Hz, 2 H), 4.50 (ddd, $J = 21.1, 13.3, 6.1$ Hz, 3 H), 3.81 (dd, $J = 35.9, 15.1$ Hz, 2 H), 3.08 – 2.83 (m, 4 H), 2.82 – 2.65 (m, 2 H) (Figure S4). ^{13}C NMR of **1** (100 MHz, $\text{DMSO-}d_6$) δ (ppm): ^{13}C NMR (101

MHz, DMSO-*d*₆) δ 173.19, 171.52, 171.36, 170.26, 154.05, 138.21, 137.95, 133.66, 132.87, 132.38, 132.31, 130.10, 129.67, 128.70, 128.44, 128.08, 127.40, 126.67, 126.37, 125.90, 124.59, 123.90, 78.12, 54.13, 53.97, 38.02, 36.46 (Figure S5). MS: calculated for **1** [(M - H)⁻]: 643.27, obsvd. ESI-MS: *m/z* 642.26. (Figure S6).

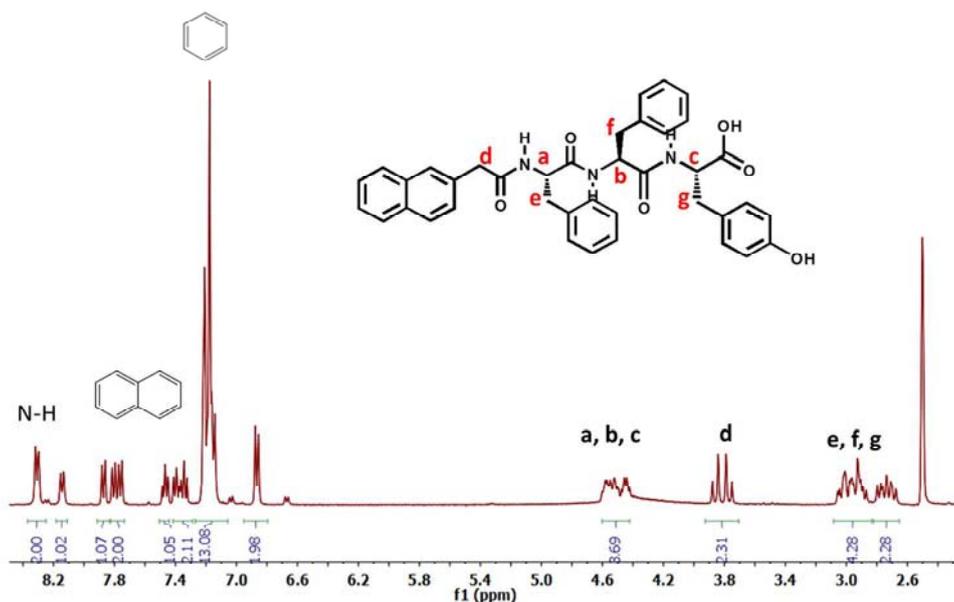


Figure S4. ¹H NMR spectrum of **1**.

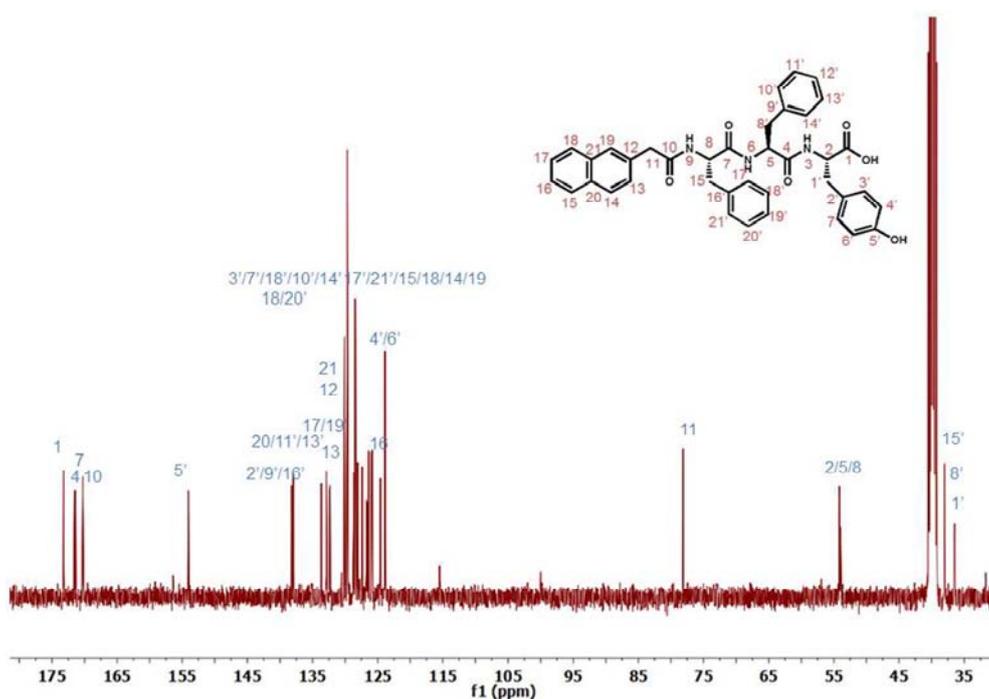


Figure S5. ¹³C NMR spectrum of **1**.

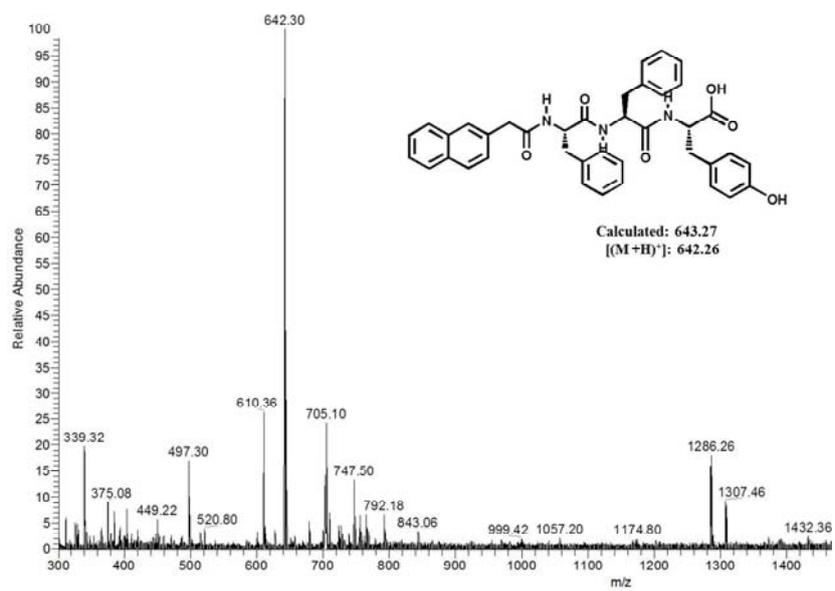


Figure S6. ESI/MS spectrum of **1**.

3. Supporting figures and tables

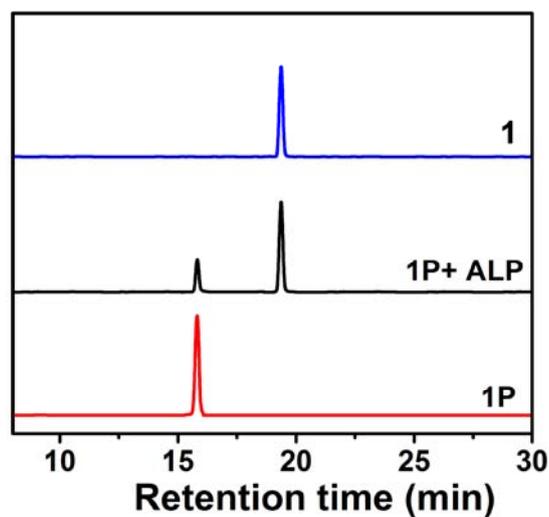


Figure S7. HPLC traces for precursor **1P** (red), **1P** incubated with ALP at 37 °C for 6 h (black), and **1** (blue). Wavelength for detection: 268 nm.

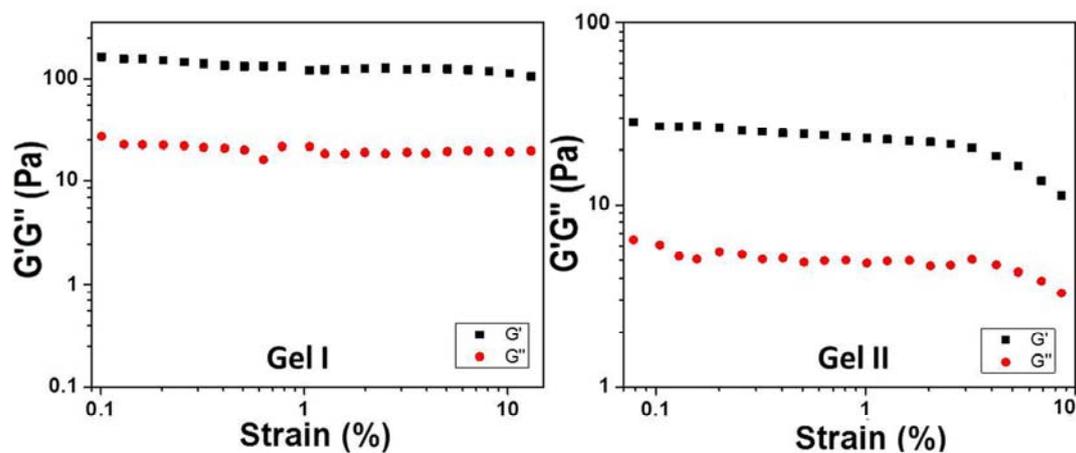


Figure S8. . Dynamic strain of storage modulus (G') and the loss modulus (G'') of **Gel I** (left) and **Gel II** (right) at the frequency of 1.0 Hz, Condition: pH 7.4, 25 °C.

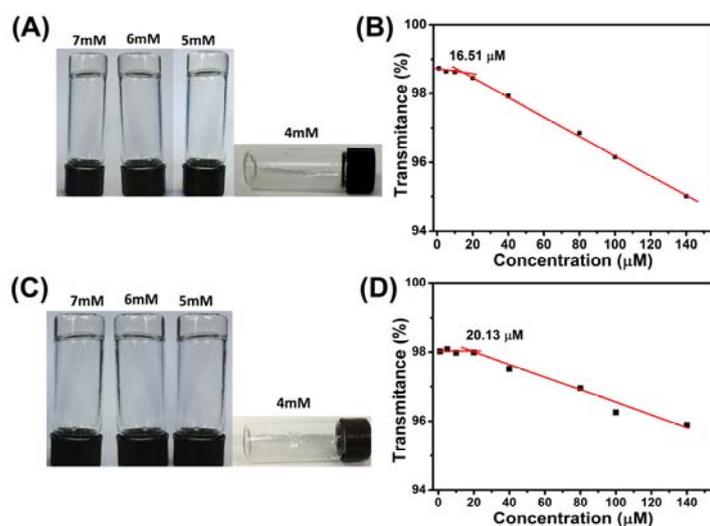


Figure S9. (A) Critical gelation concentration (CGC) determination. Inverted tube test indicated that the CGC for Gel I was 4.5 ± 0.5 mM. (B) Gelator concentration-dependent transmittance at 600 nm of dilutions of Gel I. (C) Inverted tube test indicated that the CGC for Gel II was 4.5 ± 0.5 mM. (D) Gelator concentration-dependent transmittance at 600 nm of dilutions of Gel II.

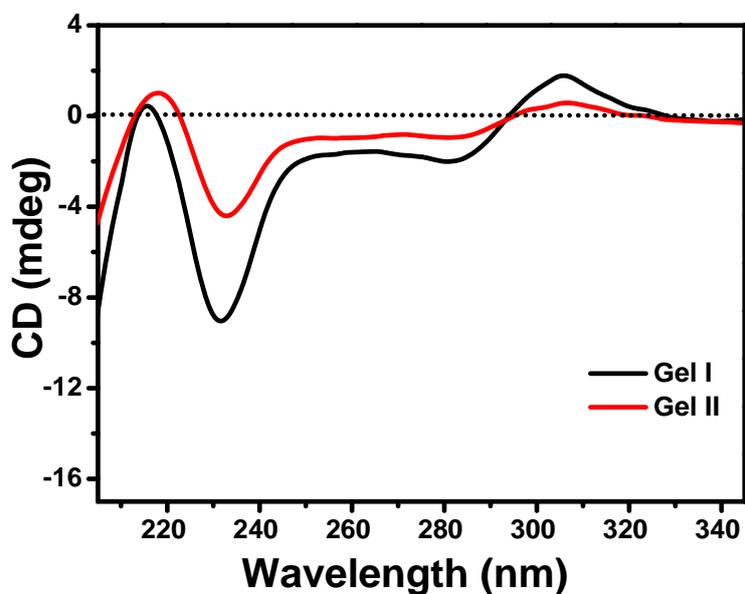


Figure S10. Circular dichroism (CD) spectra of Gel I (black) and Gel II (red).

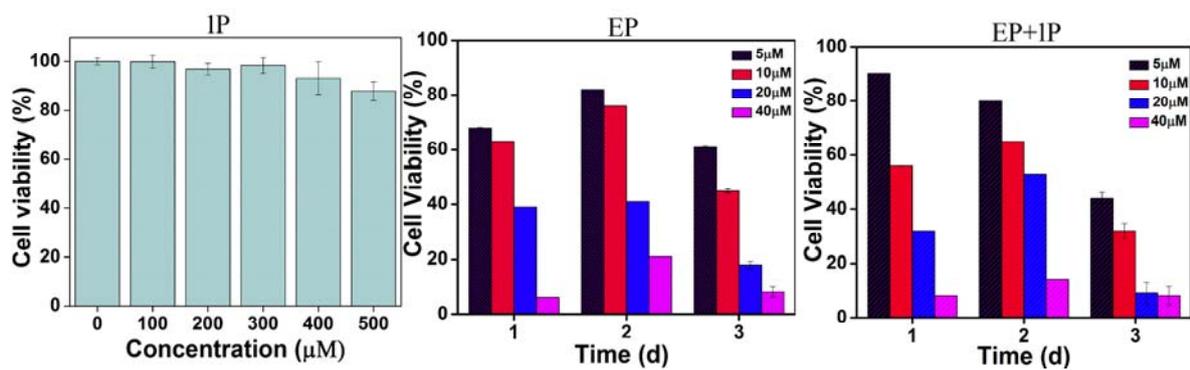


Figure S11. Left, MTT assay of HeLa cells incubated with **1P** at different concentrations for 48 h. Middle and right, MTT assay of HeLa cells incubated with **EP** or **EP + 1P** (1 : 6, w/w) at different **EP** concentrations for different times. Each value represents the average value from three independent experiments.

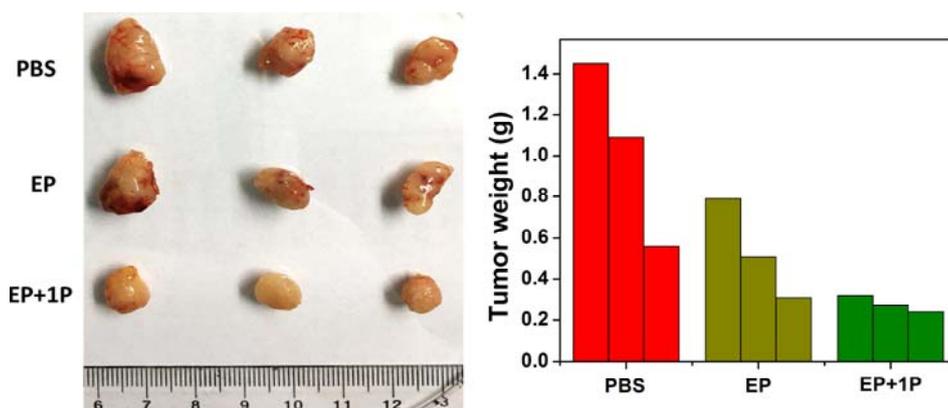


Figure S12. Left, *ex vivo* images of the tumors dissected from the tumor-bearing nude mice at day 10 after being intratumorally injected with PBS, 5 mg/kg **EP**, or 3 and 5 mg/kg **EP** with 30 mg/kg **1P** (Q2D \times 5 doses). Right, tumor weight of the tumors in the left.

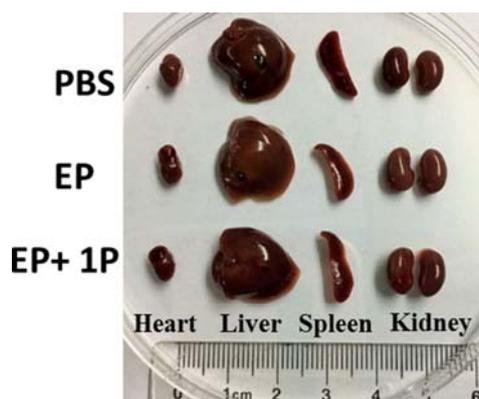


Figure S13. *Ex vivo* images of the fresh organs extracted from 3 groups of nude mice after being dissected at day 10.

Table S1: HPLC condition for purification of **1P** and **1**.

Time (min)	Flow (mL/min)	H ₂ O% (0.1% TFA)	CH ₃ CN% (0.1% TFA)
0	12.0	50	50
3	12.0	50	50
35	12.0	5	95
37	12.0	5	95
38	12.0	50	50
40	12.0	50	50

Table S2: HPLC condition for the analysis of composition of Gel **I** in Figure S7.

Time (min)	Flow (mL/min)	H ₂ O% (0.1% TFA)	CH ₃ CN% (0.1% TFA)
0	3.0	70	30
3	3.0	70	30
35	3.0	10	90
37	3.0	10	90
38	3.0	70	30
40	3.0	70	30