Electronic Supplementary Material (ESI) for Biomaterials Science.
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# Supporting Information

# A Nanomedicine Enables Synergistic Chemo/Photodynamic Therapy for Pancreatic Cancer Treatment

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**Experimental section**

*Materials:* 2-Mercaptoethanol, cis-1,2-dichloroethylene, 4-nitrophenyl chloroformate, gemcitabine and chlorin e6 was purchased from Shanghai Macklin Biochemical Co., Ltd (Shanghai, China); Fmoc-amine functionalized polystyrene resin and Fmoc amino acids were purchased from GL Biochem(Shanghai) Ltd. (Shanghai, China); Cell Counting Kit-8 (CCK-8) was purchased from Boster Biological Technology Co., Ltd(California, USA); JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide) MitoMP Detection Kit were obtained from Solarbio (Beijing, China)；Hoechst 33342, Annexin V-FITC/PI Apoptosis Detection Kit 2',7'-dichlorofluoresceindiacetate (DCFH-DA) and Alexa Fluor 488-conjugated Rabbit anti-mouse secondary antibody was purchased from Yeasen Biotechnology (Shanghai) Co., Ltd (Shanghai, China); Phosphorylated $H_2A$ histone family member X ($\gamma$-$H_2AX$) antibody, c-IAP1 antibody, Cleaved-caspase-3 （Cl.cas-3）antibody was purchased from Cell Signaling Technology Inc.,( Massachusetts, USA);Dulbecco's Modifid Eagle's Medium (DMEM) and fetal bovine serum (FBS) was purchased from Biological Industries (Beit Haemek, Israel); Deionized water was purified using a Milli-Q system (Millipore, Millford, MA, USA).

*Characterization:* $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker AVA400 spectrometer (400 and 100 MHz, respectively). Analytical HPLC was performed on an Agilent 1260 infinity II HPLC system coupled to a multiwavelength detector and equipped with a ZORBAX® 3.5 μm C18 column (150 × 4.6 mm), with a flow rate of 1 mL/min, eluting with 95% water for 3 min, then to 95% CH3CN over 22 min, and 95% CH3CN for 5 min (both with 0.1% HCO2H). Preparative HPLC was performed on an Agilent 1260 infinity II HPLC system coupled to a multiwavelength detector and equipped with an Agilent Prep-C18 10 μm column (50 × 21.2 mm), with a flow rate of 20 mL/min, eluting with 95% water for 2 min, then to 95% CH3CN over 15 min, and 95% CH3CN for 3 min (both with 0.1% HCO2H). High resolution mass spectroscopy analyses were performed on a Thermo Q Exactive Focus mass spectrometer. Transmission electron microscopy (TEM, FEI Tecnai G2 F20 S-Twin,

USA) was used to observe the morphology of the nanoparticles. Zeta potential and size distribution of nanoparticles was measured using a Zetasizer Nano ZS (Malvern, U.K.). The UV–vis absorption spectra were measured using a UV–vis spectrophotometer (PerkinElmer Lambda750, USA). The fluorescence spectra were recorded using a FL970 fluorescence spectrometer (Techcomp Group, Shanghai, China). Flow cytometry analysis was carried out on a CytoFLEX V0-B3-R1 flow cytometer (Beckman Coulter) using FlowJo software for data analysis. The absorbance of 96-well plates was read on a Multiskan Go multimode reader (Thermo Scientific). Confocal images were taken on a multiphoton confocal laser scanning microscope (CLSM) (TCS-SP8, Leica Wetzlar, Germany). In vivo fluorescent images were taken using an IVIS spectrum imaging system (Xenogen, USA).

*Synthesis of pro-apoptotic peptide:* The pro-apoptotic peptide Smac N7 (AVPIAQK) were synthesized by solid phase peptide synthesis (SPPS). Fmoc-amine functionalized polystyrene resin was used. Fmoc-protecting groups were removed by piperidine in DMF (*v*/*v* = 1:4). 1-Hydroxybenzotriazole (3 equiv.) and diisopropylcarbodiimide (3 equiv.) was used for Fmoc amino acid (3 equiv.) coupling. Cleavage of peptides (without end capping) was conducted using a mixture of TFA and $H_2O$ (95:5, *v*/*v*). The crude was precipitated and washed with cold diethyl ether twice and purified by prep-HPLC.

HRMS (ESI) for $C_{35}H_{63}N_{10}O_9$ $[M+H]^+$: calcd.: 767.47740; found: 767.47668.

HPLC (220 nm) $t_R$ = 13.27 min.

*Synthesis of compound 1*.[1] 2-mercaptoethanol (6.00 g, 76.8 mmol) and NaOH (3.07 g, 78.8 mmol) were dissolved in ethanol (30 mL) and stir at 0 ℃ for 30 min. cis-1,2-dichloroethylene (3.72 g, 38.4 mmol) in ethanol (4 mL) was added dropwisely, heated at 80 ° C for 18 h, cooled to room temperature, diluted with 20 mL of water, and washed 3 times with ether. The combined organic layer was washed twice with water, dried over anhydrous magnesium sulfate, and concentrated to obtain a crude product. The product was further purified by silica gel column chromatography using ethylacetate/hexane (7:3, *v*/*v*) as eluent to obtain the titled compound 1 (65%).

HRMS (ESI) for $C_6H_{12}NaO_2S_2$ $[M+Na]^+$: calcd.: 203.0171; found: 203.0171.

$^1$H NMR (400 MHz, MeOH-$d_6$) δ = 6.18 (s, 2H), 3.70 (t, J = 6.7 Hz, 4H), 2.85 (t, J = 6.7 Hz, 4H).

Data in agreement with the literature.[1]

*Synthesis of compound 2*.[1] Compound 1 (1.43 g, 7.9 mmol) was dissolved in DCM. triethylamine (3.64 mL, 26.2 mmol, 3.3 equivalents) was added, and the stirring was continued in an ice bath for 0.5 h. 4-nitrophenyl chloroformate (3.53 g, 17.5 mmol) in DCM (4 mL) was gradually added and stirred at room temperature for 24 h. The organic solution was washed with NaHCO3 and water for 3 times, dried with anhydrous MgSO4 and concentrated to obtain a crude product. The crude product was purified by column chromatography (ethylacetate/hexane =1:4, *v*/*v*) to obtain the desired compound 2 (40%).

HRMS (ESI) for $C_{20}H_{18}N_2NaO_{10}S_2$ $[M+Na]^+$: calcd.: 533.0295; found: 533.0297.

$^1$H NMR (400 MHz, CDCl3) δ = 8.31 (d, J = 9.2 Hz, 4H), 7.41 (d, J = 9.2 Hz, 4H), 6.23 (s, 2H), 4.47 (t, J = 6.8 Hz, 4H), 3.10 (t, J = 6.8 Hz, 4H).

Data in agreement with the literature. [1]

*Synthesis of compound 3*.[1] Compound 2 (1.20 g, 2.2 mmol) and gemcitabine (0.59 g, 2.2 mmol) were dissolved in dimethyl sulfoxide (3 mL), and triethylamine (0.31 mL, 2.23 mmol, 1 equivalent) was added. The resulting mixture was stirred at room temperature for 48 h. EA (40 mL) was added, and the organic phase was washed with water for 3 times. The solution was dried with anhydrous magnesium sulfate and concentrated to obtain a crude product. The crude product was purified by prep-HPLC to afford the desired pure product compound 3 (30%).

HRMS (ESI) for $C_{23}H_{25}F_2N_4O_{11}S_2$ $[M+H]^+$: calcd.: 635.0924; found: 635.0925.

$^1$H NMR (400 MHz, DMSO-d6) δ = 8.01 (d, J = 7.6 Hz, 2H), 7.66 (d, J = 7.6 Hz, 1H), 7.49 (s, 2H), 6.64 (d, J = 8.7 Hz, 1H), 6.29 (s, 1H), 5.80 (d, J = 7.5 Hz, 1H), 4.60 (m, 1H), 4.43 – 4.26 (m, 5H), 4.12 (m, 1H), 3.16 – 2.97 (m, 6H).

Data in agreement with the literature. [1]

*Synthesis of compound 4*. Compound 3 (1.39g, 2.2 mmol) and Smac N7 peptide (1.69g, 2.2 mmol) were dissolved in DMSO (3 mL), and triethylamine (0.31 mL, 2.23 mmol, 1 equiv) was added. The resulting mixture was kept stirring at ambient

temperature for 48 h. EA (40 mL) was added and the organic phase was washed with water for three times. The organic solution was dried using anhydrous $MgSO_4$ and concentrated to give a crude product. The crude product was purified by prep-HPLC to afford the desired pure product compound 4. (35%)

HRMS (ESI) for $C_{52}H_{82}F_2N_{13}O_{17}S_2$ $[M+H]^+$: calcd.: 1262.5356; found:1262.5357.

HPLC (220 nm) $t_R$ = 16.57 min (purity > 90%).

*Molecular dynamics simulations details* The system, including water with GVS and Ce6 was generated using PACKMOL[2] in a cube box of 7 nm × 7 nm × 7 nm. The number of molecules in the box was determined according to the molar ratio. Generally, the water box consists of 10000 water molecules, 10 GVS molecules, and 10 Ce6 molecules. The original structure of molecules, such as GVS and Ce6, was drawn in Chemdraw and optimized with B3LYP theory and def2-SVP basis[3] by the ORCA package.[4] The topology information of the molecules was generated using the CGenFF server (https://cgenff.umaryland.edu/). For all MD simulations, the GROMACS 2021v4 was used and performed at 298.15 K and 1 atm by a CHARMM36 force field.[5] The constructed simulation systems were energy-minimized to remove unwanted contact using the steepest descent method.[6] Then, the system was equilibrated for 10 ns in the constant pressure−constant temperature (NPT) ensemble. Finally, a production run of 100 ns was carried out under the constant temperature (NVT) ensemble. The simulation processes were visualized and analyzed using VMD package with Tcl language. [7] The average noncovalent interaction (aNCI) among GRS, Ce6 and water was studied using Multiwfn according to a previous reported reduced density gradient (RDG) method.[8]

*Irradiation induced degradation of Ce6-GVS NPs*. The Ce6-GVS NPs were exposed to 660 nm light irradiation at 20 mW/cm$^2$. The degradation of GVS at 0, 2, 4, 6, 8 and 10 min was monitored using an analytical HPLC.

*The composition determination of Ce6-GVS NPs.* The prepared Ce6-GVS NPs was freeze-dried, redissolved in DMSO and the nanoparticle composition was determined by analytical HPLC. The relative ratio of Ce6 and GVS was calculated using the integration of HPLC peaks and compared to the standard curves of pure

compounds.

*Cell culture.* Panc-1 and 3T3-L1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin (100 units $mL^{-1}$) and streptomycin (100 μg $mL^{-1}$). Cells were cultured in a humid atmosphere containing 5% $CO_2$ at 37 ℃.

*Cellular uptake.* Panc-1 cells were seeded in an 8-well confocal chamber at a density of $2 \times 10^4$ cells per well. After 24 h, the cells were incubated with free Ce6, Ce6/GVS or Ce6-GVS NPs (Ce6 = GVS = 5 μM) for 8 h. The cell nuclei were stained with Hoechst 33342 and imaged using a CLSM with an oil-immersion/dry objective lens.

Flow cytometry (FCM) was used to quantify the cellular uptake. Panc-1 cells were incubated with free Ce6, Ce6/GVS or Ce6-GVS NPs (Ce6 = GVS = 5 μM) for 8 h. Subsequently, the cells were rinsed, harvested and analyzed by flow cytometry.

*Cytotoxicity analysis.* The cells were seeded in a 96-well plate ($5 \times 10^3$ cells/well) and incubated for 24 h. The cells were treated with GVS prodrug, free Ce6, Ce6/GVS or Ce6-GVS NPs at various concentrations, with or without 660 nm illumination. After 24 h, the medium was replaced with fresh medium containing CCK8 (0.5 mg/mL, 100 μL). The cells were further incubated for 2 h at 37 ℃ and analyzed by a microplate reader at 450 nm wavelength. Untreated cells were used as a control.

*Combination index evaluation.* The combination index (CI) was used to evaluate the comprehensive effect of drug interactions,including synergistic effect (CI < 1), additive effect (CI = 1), and antagonistic effect (CI > 1).The combination index ($CI_{50}$) was measured using the following equation:

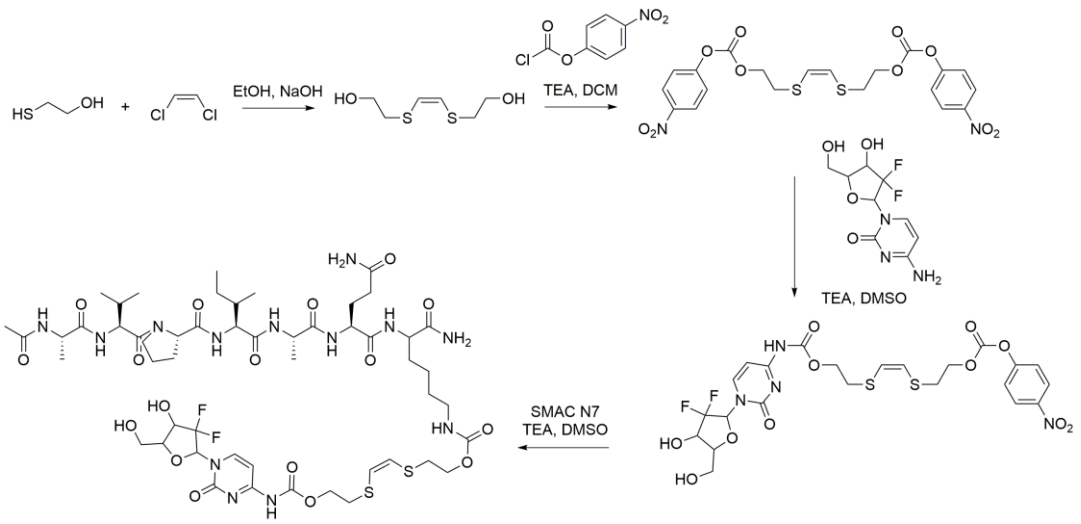
$$CI_{50} = \frac{(IC50)A}{(IC50)'A} + \frac{(IC50)B}{(IC50)'B} + \frac{(IC50)C}{(IC50)'C}$$

where $(IC_{50})'X$ and $(IC_{50})X$ represent the concentration of drug used in combination to achieve 50% effectiveness and concentrations of single drug to achieve the same effect, respectively.

*Western blot.* Panc-1 cells were seeded in 6-well plates and incubated for 24h. GVS prodrug, free Ce6, Ce6/GVS or Ce6-GVS NPs (Ce6 = GVS = 5 μM) were added and incubated for 8 h. After 10 min irradiation at 660 nm, the cells were incubated for 24 h. The cells were lysed in RIPA lysis buffer (1% sodium deoxycholate, 1% NP-40,

0.1% SDS, 150 mM NaCl, 25 mM Tris-HCl and 1% protease inhibitor). Protein samples were incubated with sodium dodecyl sulfate (SDS) loading buffer, and boiled for 5 min. Equal amounts of proteins were loaded and separated by 10% SDS-polyacrylamide gel and were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with individual primary antibodies for 24 h at 4 ℃, and further incubated with HRP-conjugated appropriate secondary antibodies for 2 h at room temperature. After washing with TBS-T, the protein bands were detected using an enhanced chemiluminescence (ECL) systems. The band intensities were calculated using an ImageJ software.

**Supplementary figures**




**Scheme S1.** Scheme of the synthesis of ROS-responsive peptide-drug-conjugate-based prodrug(Gem-Vinyldithioether-Smac N7, GVS).

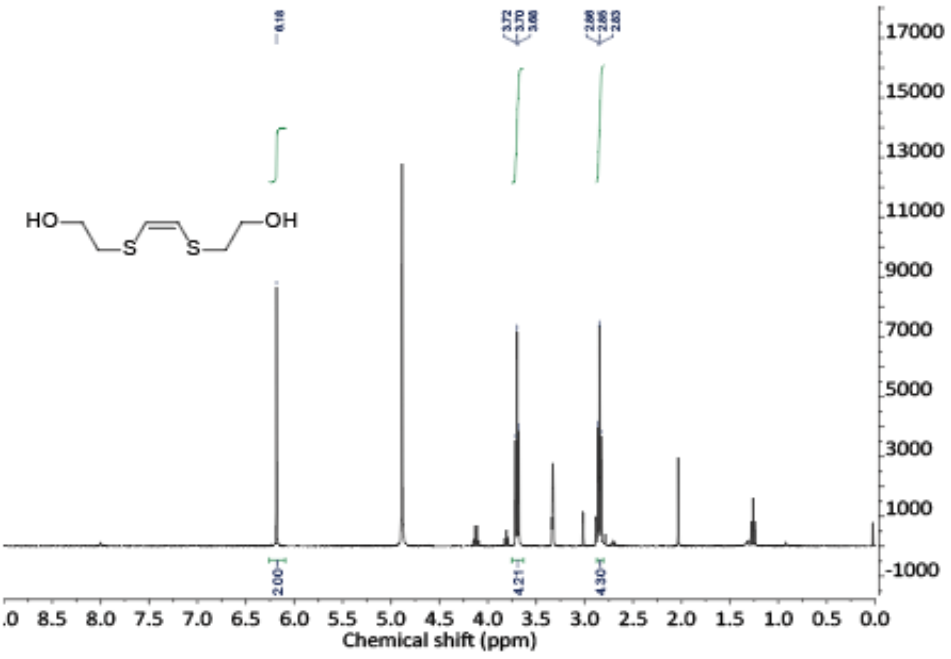



**Figure S1.** $^{1}H$ spectrum of compound 1 recorded in MeOH-$d_6$.

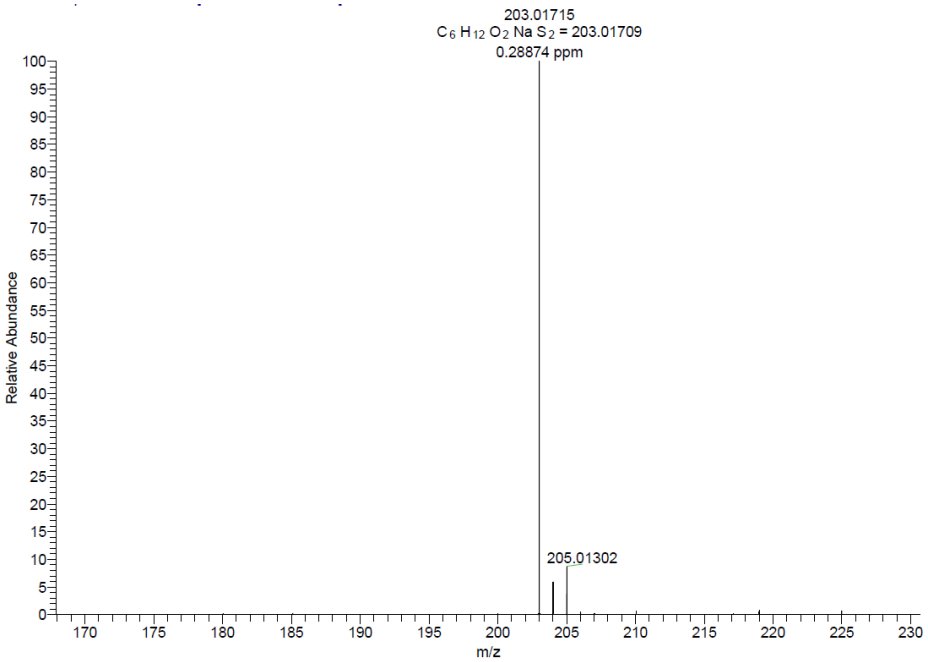



**Figure S2.** HRMS spectrum of compound 1.

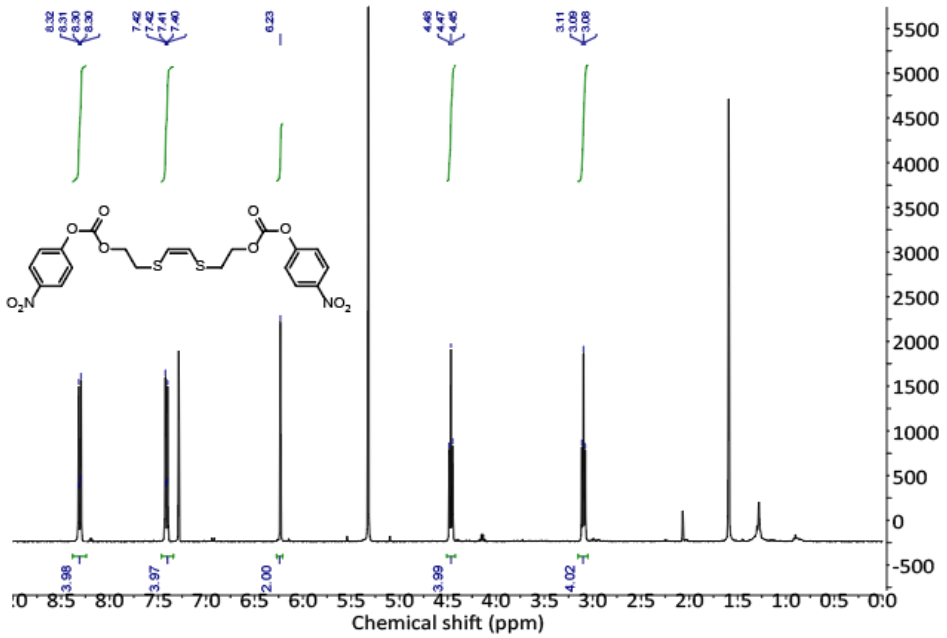



**Figure S3.** [1]H spectrum of compound 2 recorded in CDCl3.

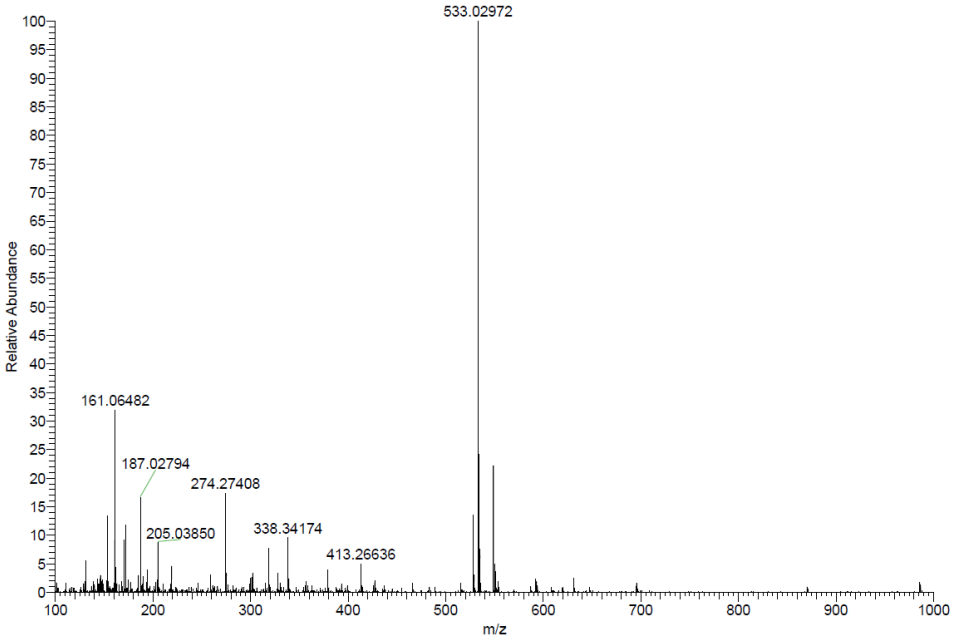



**Figure S4.** HRMS spectrum of compound 2

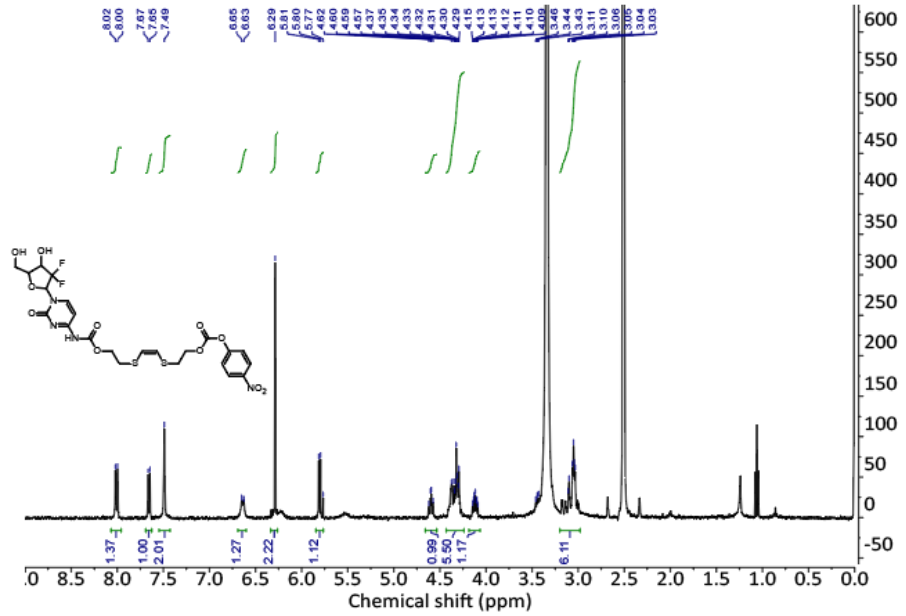



**Figure S5.** $^{1}$H spectrum of compound 3 recorded in DMSO-d6.

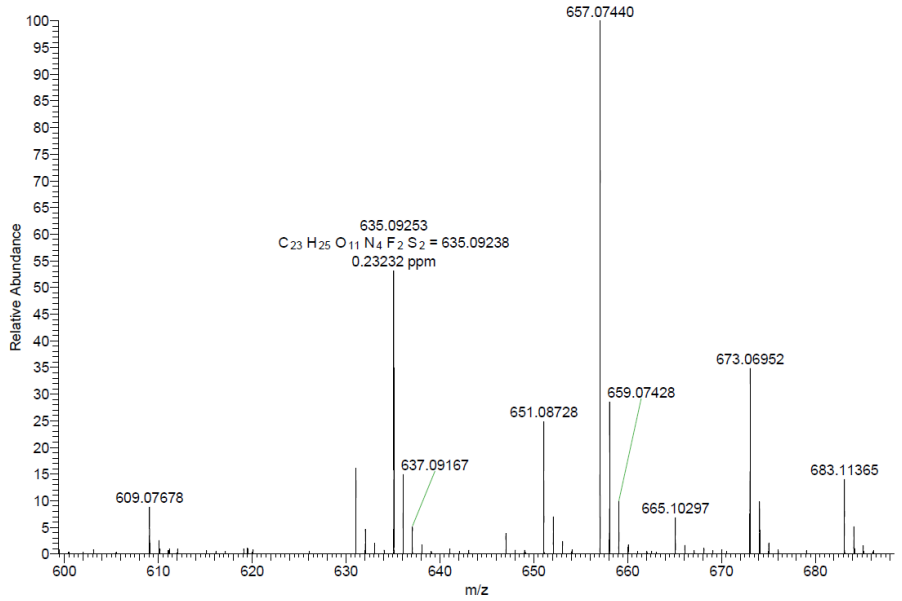



**Figure S6.** HRMS spectrum of compound 3

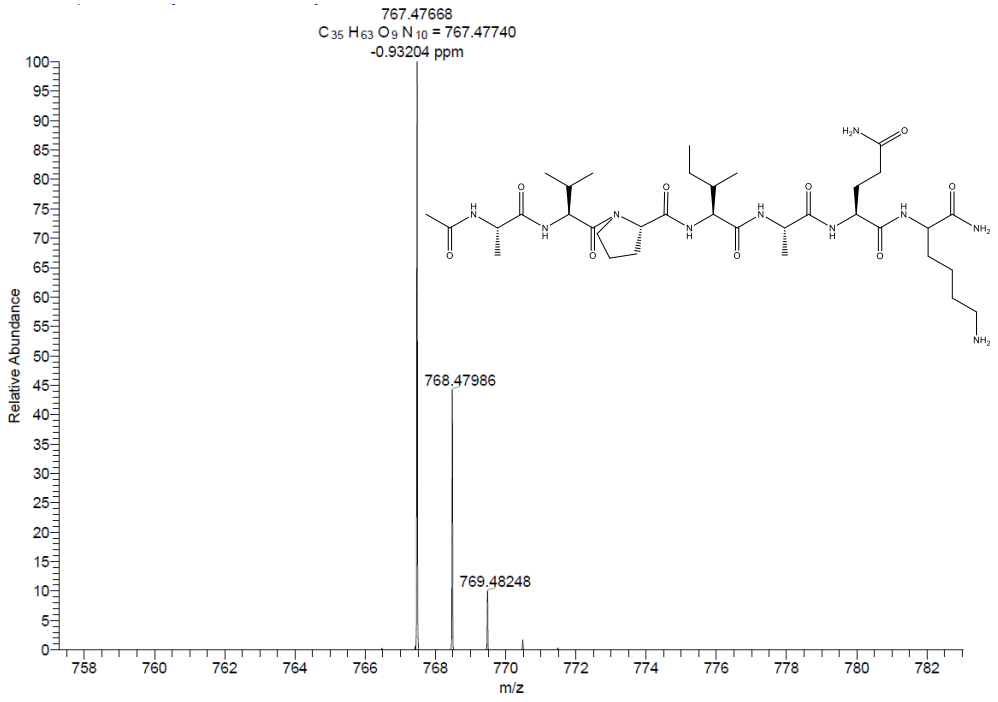



**Figure S7.** HRMS spectrum of Smac N7 (AVPIAQK).

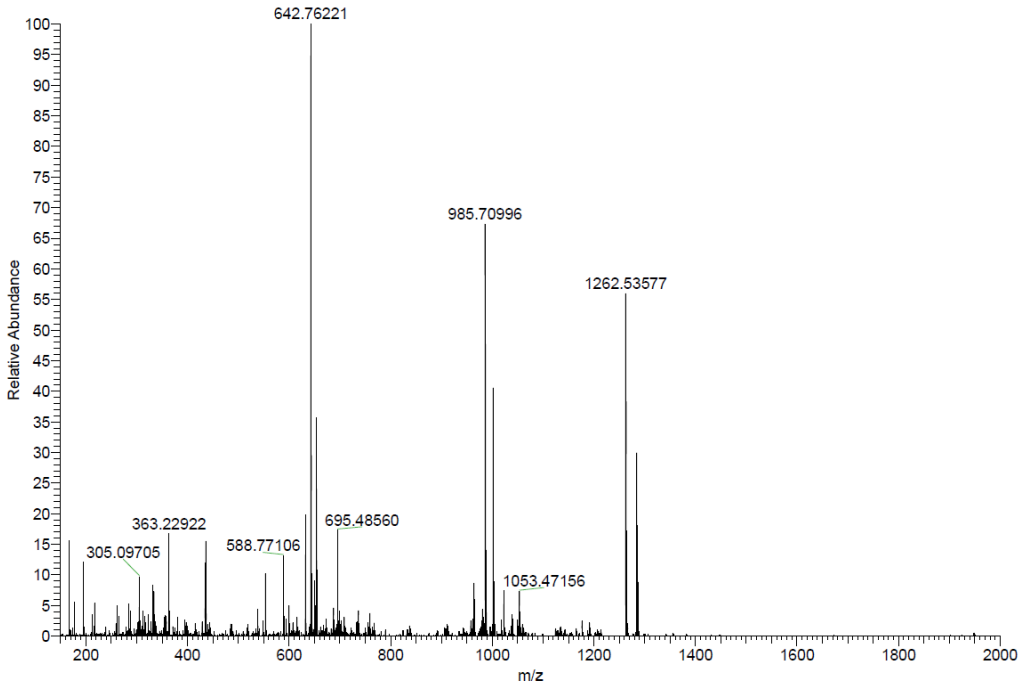



**Figure S8.** HRMS spectrum of compound 4 (**GVS**).

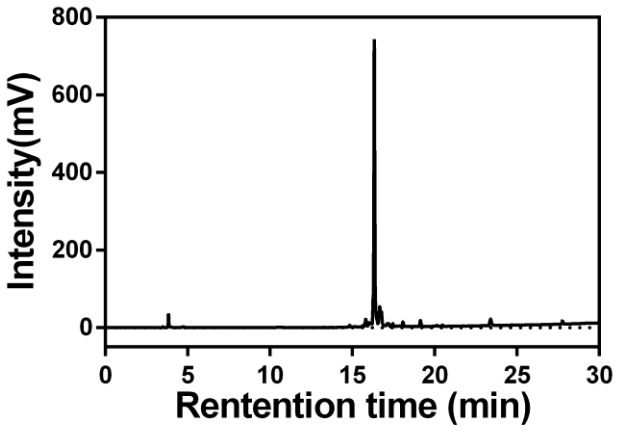



**Figure S9.** HPLC spectrum of synthesized compound 4 (**GVS**).

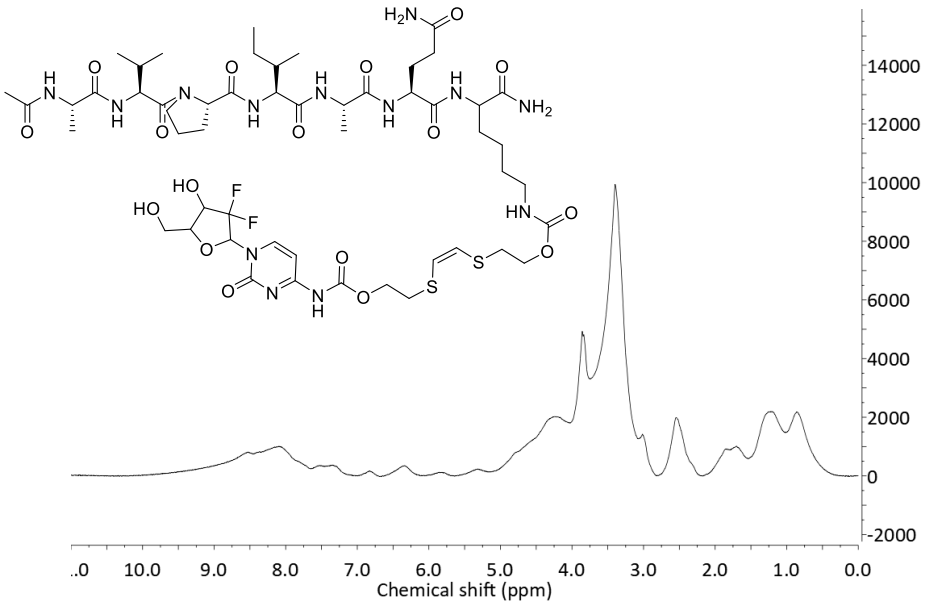



**Figure S10.** $^{1}$H spectrum of compound 4 (**GVS**).

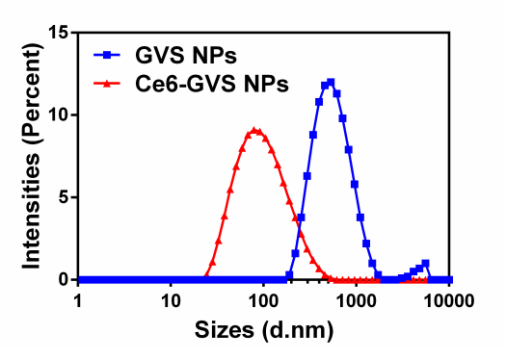



**Figure S11.** The hydrodynamic size distributions of the GVS NPs and Ce6-GVS NPs.

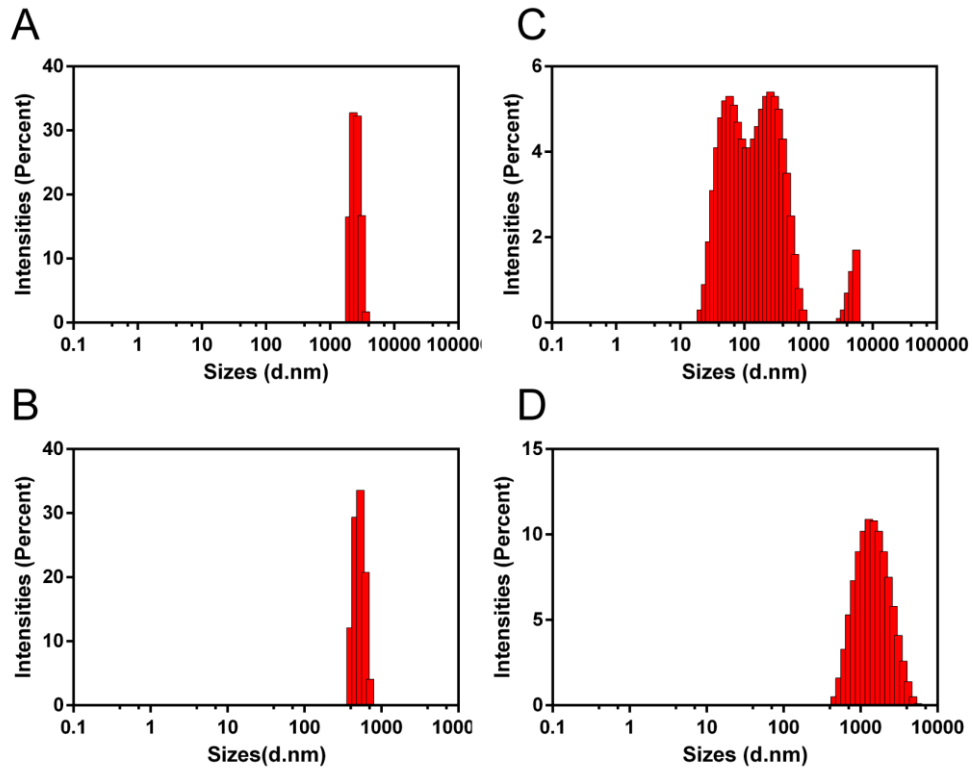



**Figure S12.** The hydrodynamic size distributions of the nanoassemblies with the feed ratio of Ce6 and GVS at (A)1:5 ,(B) 5:1,(C)1:10 and (D)10:1.

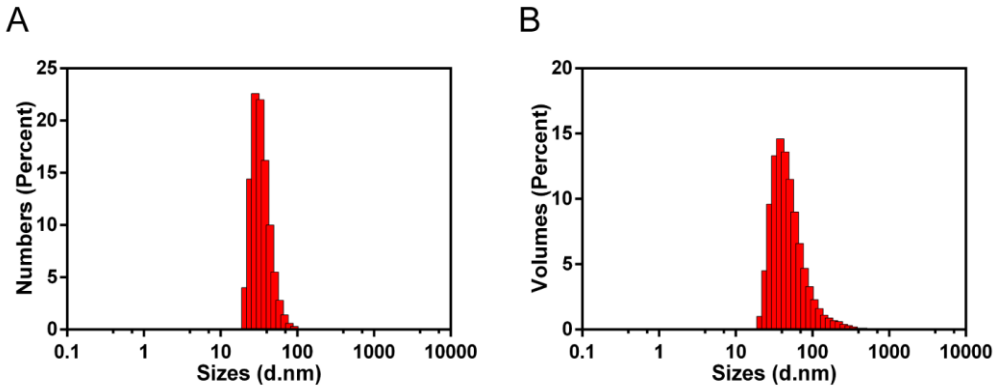



**Figure S13.** The DLS diagrams based on (A) number% and (B) volume% for the Ce6-GVS NPs (1:1 molar ratio of GVS and Ce6).

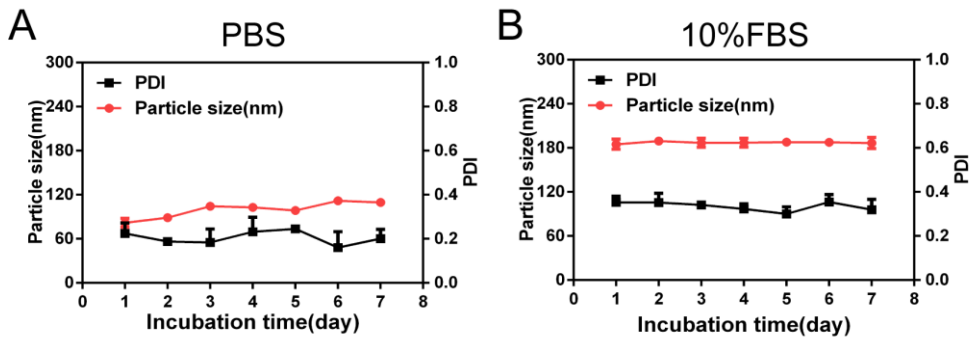



**Figure S14.** Stability of Ce6-GVS NPs (A) in PBS and (B) in PBS containing 10 % (*v*/*v*) FBS.

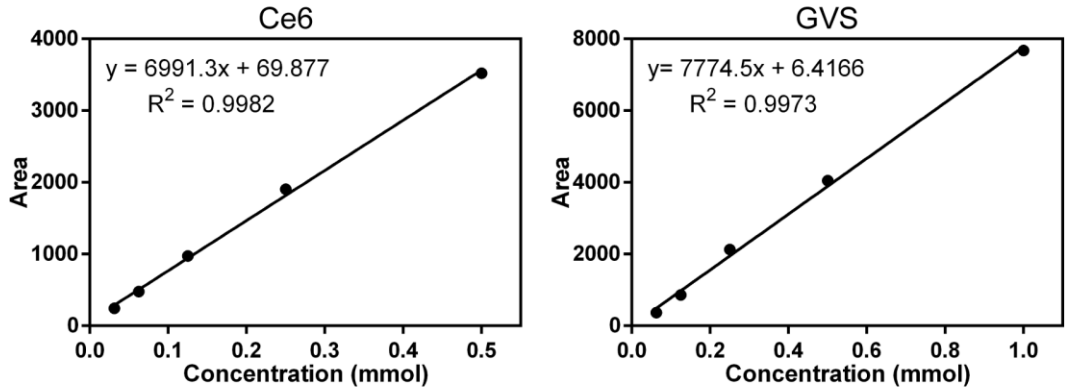



**Figure S15.** The calibration curves of GVS and Ce6 quantified by HPLC.

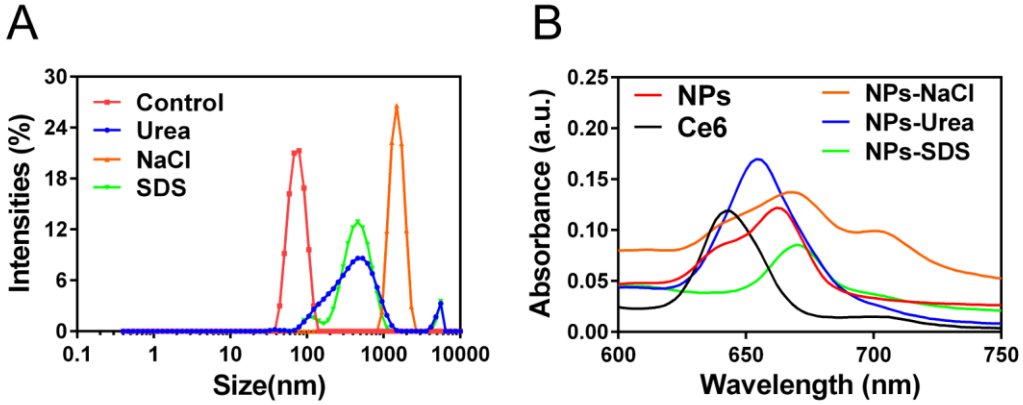



**Figure S16.** (A) Size distribution of Ce6-GVS NPs measured by DLS in the presence of different agents. (B) UV–*vis* spectra of Ce6-GVS NPs in the presence of different agents.

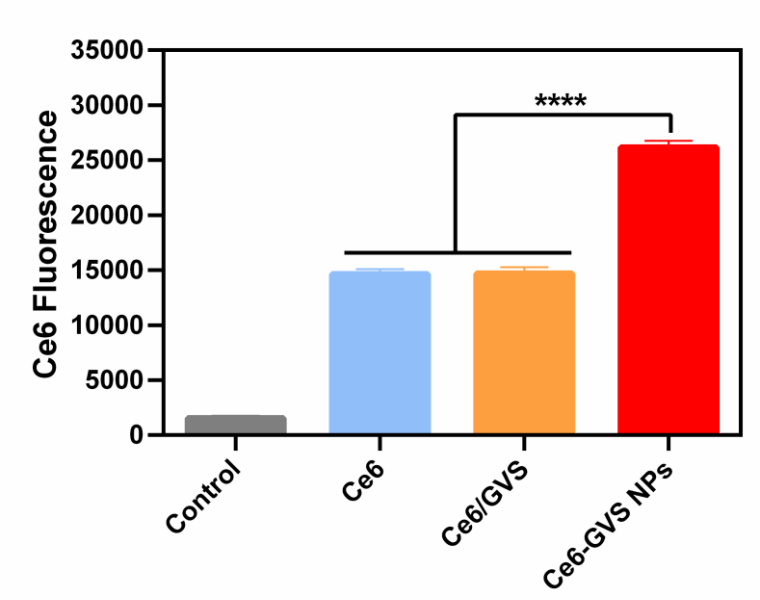



**Figure S17.** Quantitative analysis of fluorescence intensities (Ce6) in different treatment groups.

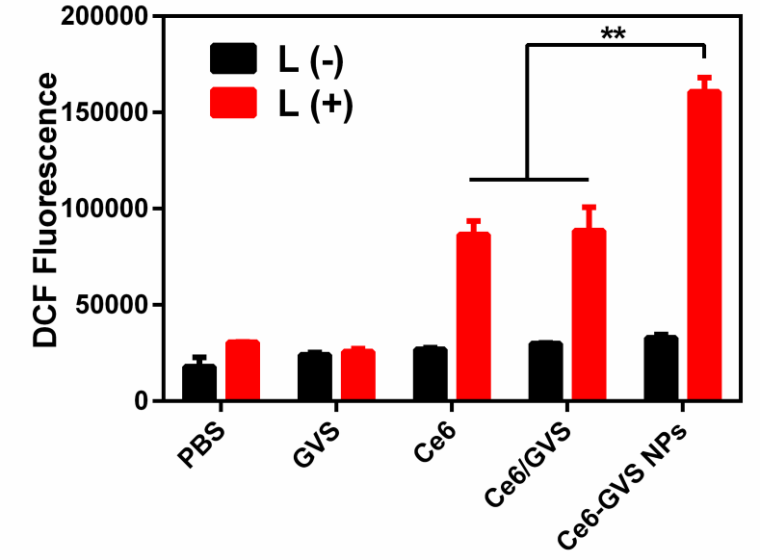



**Figure S18.** The numerical statistics for DCF fluorescence cytometry analysis.

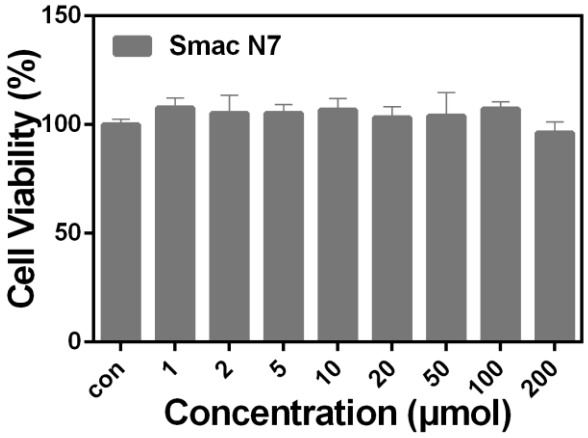



**Figure S19.** Cell viability against Smac N7 using a CCK-8 assay

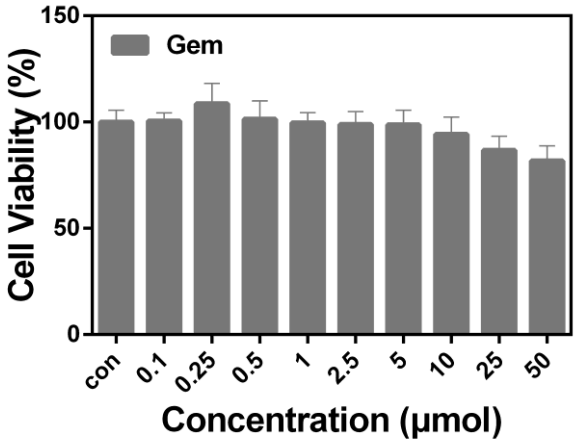



**Figure S20** Cell viability against Gem using a CCK-8 assay.

**Table S1.** The IC50 of Gem, Smac N7 and Ce6 against PANC-1 cells

| | Gem | Smac N7 | Ce6 | Ce6/GVS | Ce6-GVS NPs |
|---|---|---|---|---|---|
| IC50(μM) | 223.1 | ＞5000 | 3.2 | 2.4 | 1.7 |
| CI | - | - | - | 0.76 | 0.53 |

**Figure S21.** Raw image of the western blot image for the β-actin expression without light illumination.

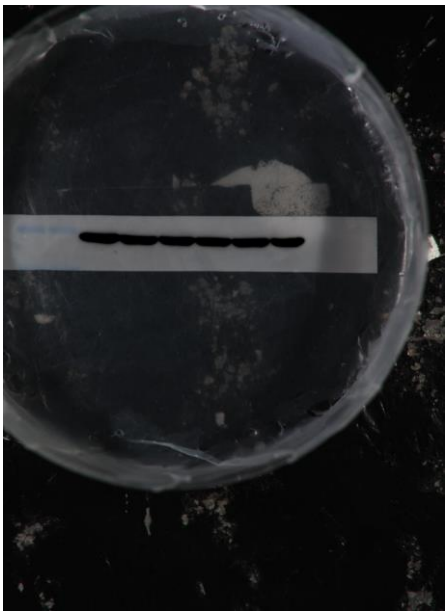


**Figure S22.** Raw image of the western blot image for the β-actin expression with light illumination.

**Figure S23.** Raw image of the western blot image for the c-IAP1 expression without light illumination.

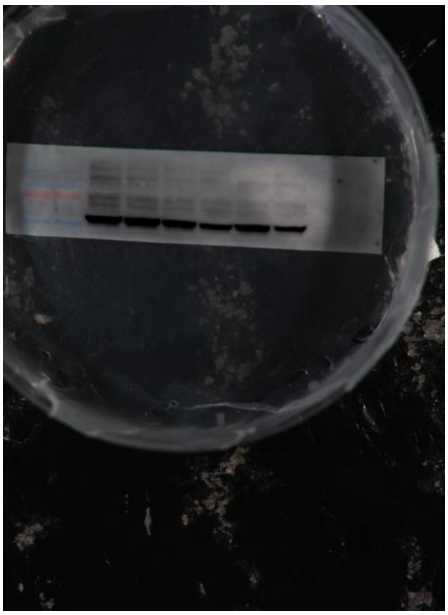


**Figure S24.** Raw image of the western blot image for the c-IAP1 expression with light illumination.

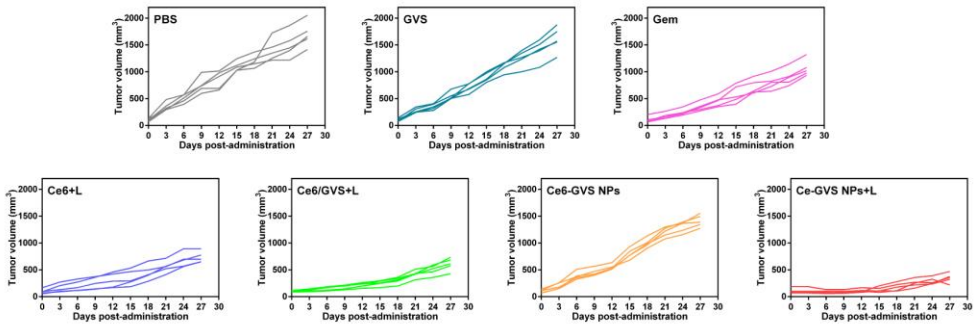



**Figure S25.** The individual tumor growth curves of Panc-1 tumor-bearing mice after various treatments.

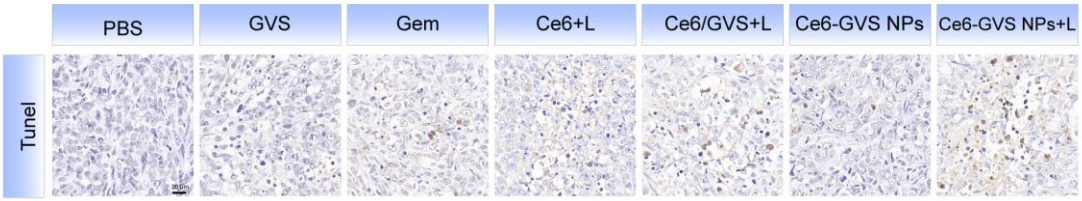



**Figure S26.** Representative TUNEL staining of tumor slice images of mice after various treatments. Scale bar = 20 μm.

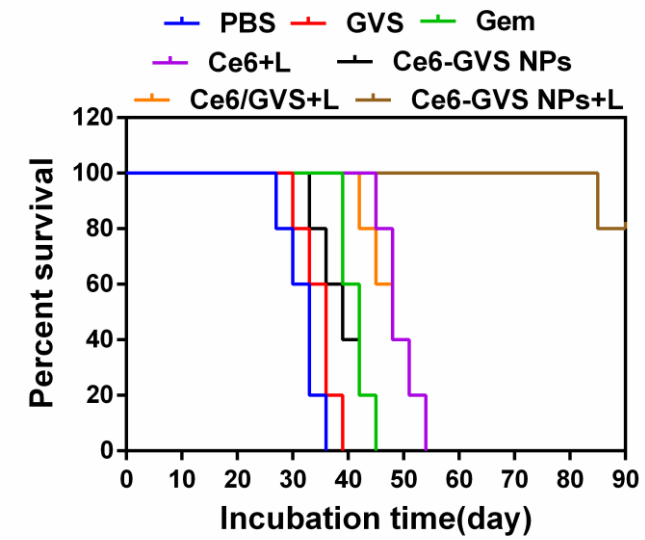



**Figure S27.** Survival curves of PANC-1 tumor bearing BALB/c nude mice with various treatments (n = 5).

**Supplementary References**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