Supporting Information

Self-Delivering THPC-Modified Cytochrome c Induces Cancer Cell Apoptosis

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1. Materials and instruments

All chemicals, biological reagents, and proteins were purchased from OriLeaf, Energy, Servicebio, Proteintech, and Beyotime.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on an Autoflex Speed MALDI-TOF system (Bruker, Karlsruhe, Germany). Protein size and zeta potential were determined using a Zetasizer Nano-ZS90 (Malvern Instruments, Malvern, UK). Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan). Fluorescence-activated cell sorting (FACS) was carried out on a CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, IN, USA). Fluorescence microscopy images were acquired with an LSM 710 confocal microscope (Zeiss, Oberkochen, Germany). Cell viability (CCK-8 assay) was measured using an iMark microplate reader (Bio-Rad Laboratories, Richmond, CA, USA). Other microplate-based assays were conducted with a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2. Synthesis and characterization of CytC-THPC

2.1 Synthesis of CytC-THPC

Cytochrome c (CytC, 1 μmol) and THPC (20 μmol) were dissolved in 20 mL of PBS buffer (pH 7.4). The reaction mixture was stirred at 37 °C for 12 h in the dark. The mixture was then concentrated to ~2 mL by ultrafiltration at 8000 rpm using a 3 kDa MWCO centrifugal filter (Millipore). The retained product was diluted with ultrapure water and concentrated again to remove excess THPC and salts. Finally, the product was lyophilized to obtain CytC-THPC as a solid powder.

2.2 Characterization of CytC-THPC

2.2.1 MALDI-TOF MS

CytC and CytC-THPC were each dissolved in deionized water at 1 mg/mL and analyzed by MALDI-TOF MS. The singly charged molecular ion peak was used to determine the most probable molecular weight. The degree of modification (average number of THPC residues attached per CytC molecule) was calculated from the molecular weight shift using the following formula:

$$\text{Modification Degree} = \frac{\text{Molecular weight of CytC - THPC} - \text{Molecular weight of CytC}}{\text{Molecular weight of THPC residue}}$$

Due to the known instability of THPC in aqueous solution (partial loss of formaldehyde to form THP), the exact molecular weight of the attached moiety is uncertain. Therefore, the modification degree is reported as a range that encompasses both possibilities.

2.2.2 Dynamic light scattering (DLS)

CytC and CytC-THPC were prepared at 1 mg/mL in PBS (pH 7.4) or acetate buffer (pH 5.5), filtered through a 0.22 μm membrane, and analyzed in quartz cuvettes using a Zetasizer. All measurements were performed in triplicate. Particle size and zeta potential were recorded.

2.2.3 UV-Vis spectroscopy

CytC and CytC-THPC were dissolved in PBS (pH 7.4) at 0.3 mg/mL. UV-Vis absorption spectra were recorded from 300 to 600 nm in quartz cuvettes using a UV-visible spectrophotometer.

2.2.4 Circular dichroism (CD) spectroscopy

CytC and CytC-THPC were prepared at 0.3 mg/mL in PBS (pH 7.4). CD spectra were recorded over 190-250 nm in a 1 mm quartz cuvette using a CD spectrometer. Three consecutive scans were accumulated and averaged for each sample. Spectra were smoothed with Spectra Analysis software. Secondary structure composition was quantified using the DichroWeb platform with the CONTIN/LL algorithm and the SP175 reference dataset. Percentages of α-helix, β-sheet, β-turn, and random coil were obtained, and the values were compared between CytC and CytC-THPC.

2.3 Cell culture and bioassay of CytC-THPC

2.3.1 Cell culture

Four human cancer cell lines (HeLa, A375, MCF-7, and Huh-7) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. For MCF-7 cells, the medium was additionally supplemented with 1% ITS-G. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2.

2.3.2 CCK-8 cytotoxicity assay

Cells were seeded in 96-well plates at 5×10^3 cells per well and allowed to adhere for 12 h. The medium was then replaced with 100 μL of fresh medium containing increasing concentrations (20-100 μM) of CytC-THPC, and the cells were incubated for 24 h. Control groups (HeLa and MCF-7) were treated with equivalent concentrations of unmodified CytC or free THPC under identical conditions. After treatment, the medium was replaced with 100 μL of CCK-8 working solution, and the plates were incubated for 1.5 h at 37 °C. Absorbance at 450 nm was measured using a microplate reader (mean ± SD, n = 5). Cell viability curves and IC_{50} values were generated using GraphPad Prism.

2.3.3 Flow cytometry (FACS) apoptosis assay

HeLa cells were seeded in 6-well plates at 3×10^5 cells per well and cultured overnight. Cells were treated with CytC-THPC at a final concentration of 75 μM for 0, 6, 12, or 24 h. After treatment, the culture medium was collected, and the cells were washed twice with PBS, detached with trypsin, and harvested. Cells were centrifuged, washed twice with PBS, and resuspended in 100 μL of 1×Binding Buffer. Then, 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were added, and the cells were incubated in the dark at room temperature for 10 min. Finally, 400 μL of 1×Binding Buffer was added to adjust the cell density to $\sim 1 \times 10^6$ cells/mL. Fluorescence was analyzed immediately by flow cytometry, and data were processed using FlowJo software.

2.3.4 Western blotting (WB)

HeLa cells were seeded in 100 mm dishes and cultured overnight. The medium was replaced with fresh medium containing 75 μM CytC-THPC, and the cells were incubated for 6, 12, or 24 h. After treatment, cells were lysed on ice for 30 min using RIPA lysis buffer. Lysates were centrifuged, and the supernatant was collected. Protein concentration was quantified by BCA assay. Equal amounts of protein (35 μg per lane) were mixed with SDS-PAGE loading buffer, denatured at 95 °C for 5 min, and separated on SWE rapid high-resolution gels at 200 V for 45 min. Proteins were transferred onto a 0.45 μm PVDF membrane at 300 mA for 35 min using ice-bath-free rapid transfer buffer. The membrane was blocked with 5% skim milk in TBST for 2 h and incubated overnight at 4 °C with primary antibodies against caspase-3, caspase-9, and β-tubulin. After washing with TBST, the membrane was incubated with HRP-conjugated secondary antibodies for 2 h. Protein bands were visualized using an ECL chemiluminescence kit and imaged with a multi-function imaging system.

2.3.5 Fluorescence imaging (FLI) of caspase activation

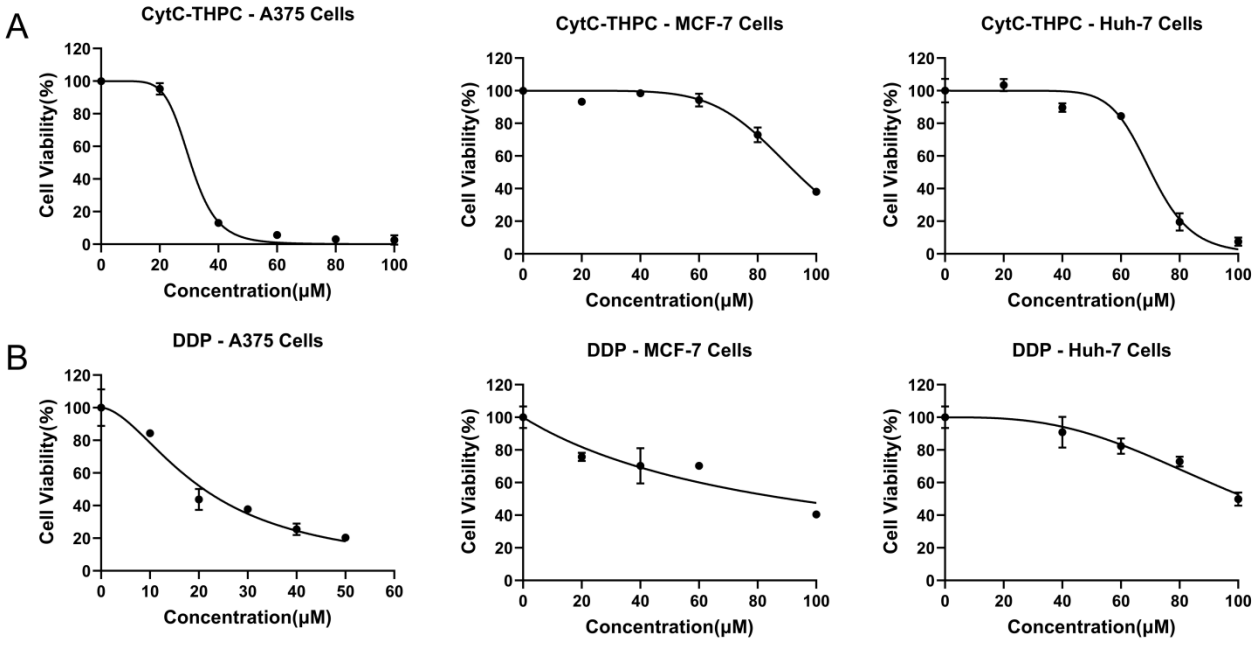
HeLa cells were seeded in 24-well plates and cultured overnight. The medium was replaced with 1 mL of fresh medium containing: (1) no treatment (control); (2) 75 μM CytC-THPC alone; or (3) 75 μM CytC-THPC plus either 10 μM Ac-DEVD-CHO (caspase-3 inhibitor) or 10 μM Ac-LEHD-CMK (caspase-9 inhibitor). After 12 h of treatment, the medium was replaced with a staining solution containing 5 μM of the appropriate caspase fluorescent substrate (DEVD or LEHD conjugate) and 2 μg/mL Hoechst 33342. After 30 min incubation at room temperature in the dark, the staining solution was removed. Cells were observed under a fluorescence microscope: Hoechst 33342 (blue) was excited at ~350 nm, and caspase activity (green) was detected at ~490 nm excitation.

Table S1. Secondary structure proportions of CytC and CytC–THPC determined by CD spectroscopy.

Compound	α-helix（%）	β-sheet（%）	β-turn（%）	Random Coil（%）
CytC	9.2	36.5	13.1	41.3
CytC-THPC	11.3	34.8	12.8	40.0

Table S2. Cytotoxicity of CytC–THPC against human cancer cell lines.

Cell line	Tissue	IC_{50} (μM)
HeLa	Cervix	75.7
A375	Skin	30.5
MCF-7	Breast	92.9
Huh-7	Liver	70.3



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Cell	IC_{50}(μM)	
	CytC-THPC	DDP
A375	30.5	21.0
MCF-7	92.9	90.5
HUH-7	70.3	103.8

Figure S1. Cytotoxicity assessment of CytC-THPC and cisplatin (DDP) in A375, MCF-7, and Huh-7 cell lines. (A) Dose-response curves of CytC-THPC. (B) Dose-response curves of DDP. (C) Comparison of IC_{50} values for both compounds.

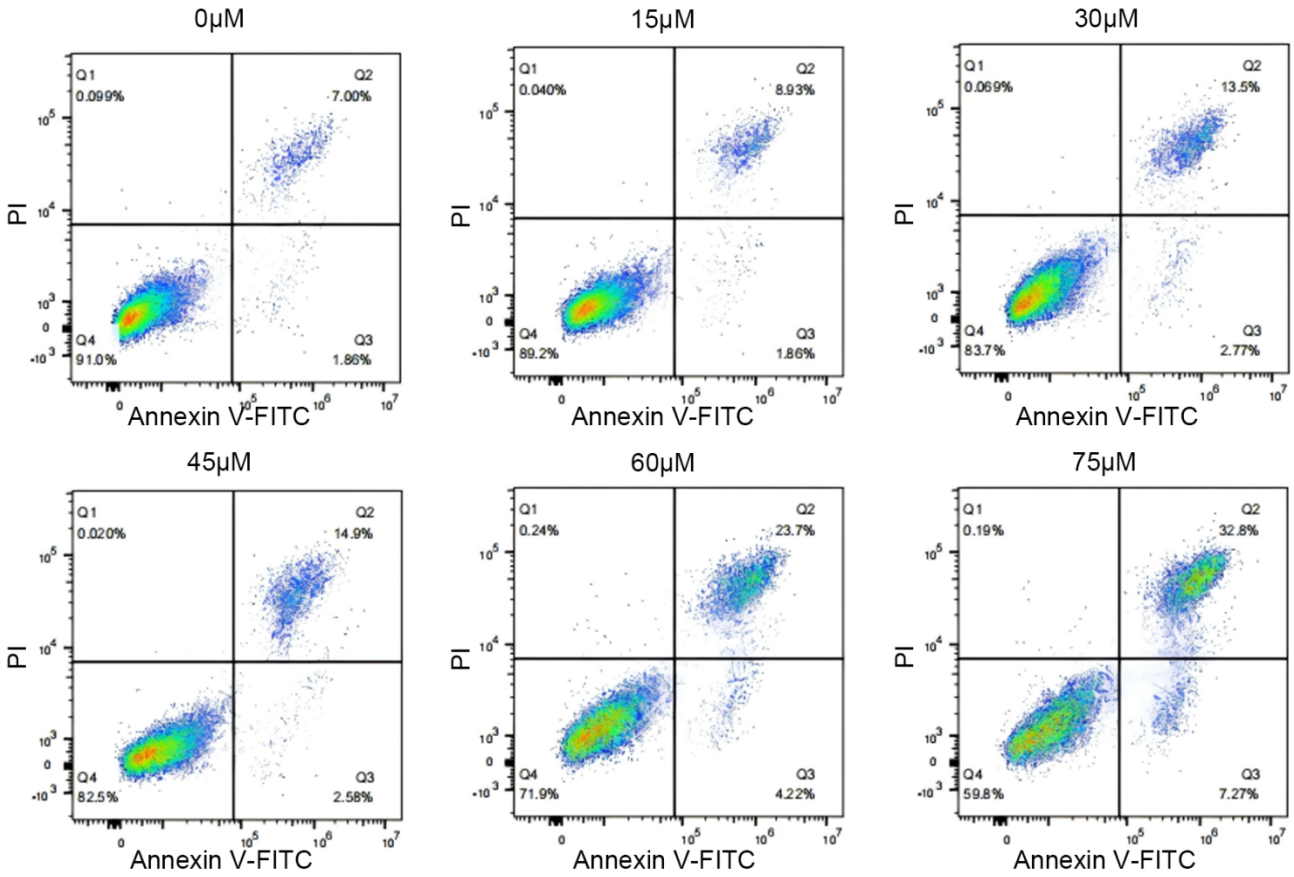

Figure S2. FACS assay of HeLa cells treated with 0, 15, 30, 45, 60, or 75 μM CytC–THPC for 24 h, using Annexin V-FITC and PI double staining.

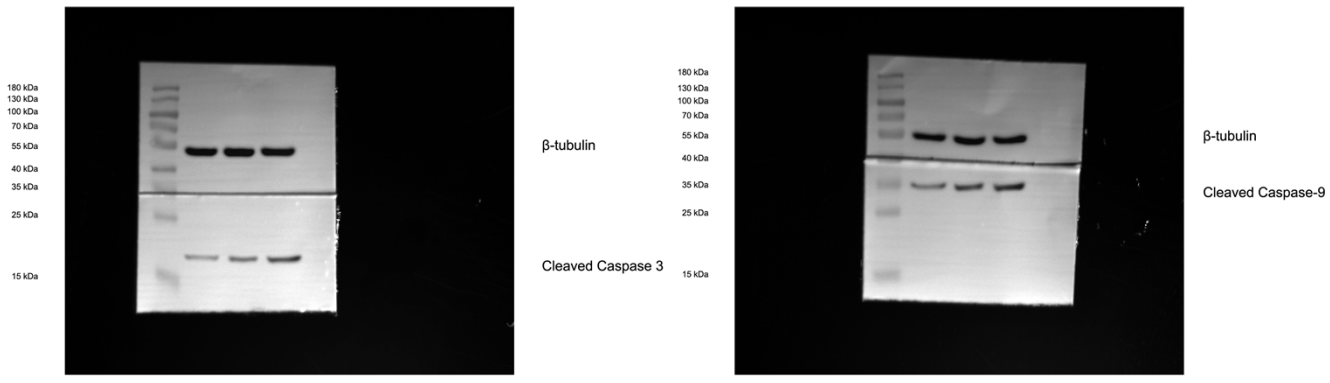

Figure S3 The original western blot images of cleaved caspase-3, cleaved caspase-9, and β-tubulin in HeLa cells after 6, 12, 24 hours treatments (from left lane to right lane) with 75 μM CytC-THPC.