Cytosolic Delivery of Cytochrome C Conjugates Induces Apoptosis at Nanomolar Levels through a Caspase-3-Dependent Pathway

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Materials. Cytochrome c (CytC), phosphate buffered saline (PBS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) staining kit, micro BCA protein assay kit, anti-ACTB mouse mAb antibody, HRP-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, high sensitive ECL luminescence reagent, Alexa Fluor 488-conjugated goat anti-rabbit IgG, and other reagents unless otherwise mentioned were purchased from Sangon Biotechnology (Shanghai, China). Traut's reagent (2-iminothiolane) was purchased from Sigma-Aldrich (MO, USA). Anti-mitochondria mouse mAb, Alexa Fluor Plus 555-conjugated goat anti-mouse IgG, and ProLong glass antifade mountant with NucBlue stain were obtained from Invitrogen (CA, USA). Ac-DEVD-CHO was purchased from Selleck China (Jiangsu, China). Mitochondrial membrane potential

assay kit with JC-1 and cell mitochondria isolation kit were obtained from Beyotime Biotechnology (Jiangsu, China). Anti-cytochrome c rabbit mAb was purchased from Bimake Biotechnology (Shanghai, China). Anti-Tom 20 rabbit mAb and anti-caspase 3 rabbit mAb were obtained from Cell Signaling Technology (MA, USA). All the other chemicals and reagents were obtained from Energy Chemical (Shanghai, China).

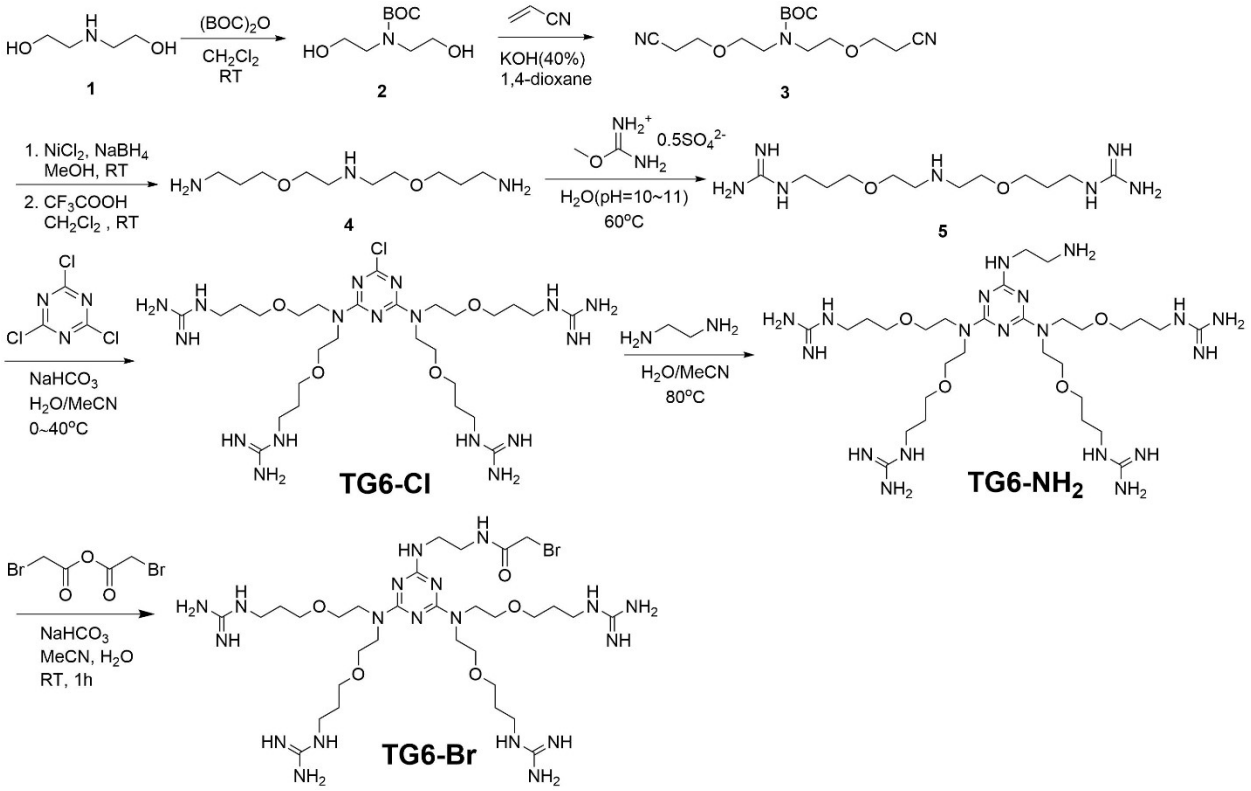

Figure S1. Synthesis of TG6-Br

Synthesis of TG6-Br. As shown in scheme 1, the new TG6 with a reactive $BrCH_2CONH$- group was synthesized and named as TG6-Br.

Compound 1 (diethanolamine, 0.45 g, 4.0 mmol, 1 eq) and $(BOC)_2O$ (1.79 g, 8.0 mmol, 2 eq) was dissolved in 20 mL CH_2Cl_2 and sitrred at room temperature for 4 h. The solvent was removed under vacuum to give compound 2 (tert-butyl bis(2-hydroxyethyl)carbamate) as a colorless oil (0.82 g, 100% yield). Then, 1,4-dioxane (15 mL) and acrylonitrile (1.1 g, 20 mmol) was added to mix well. Potassium hydroxide (0.23 g, 2 mmol) was dissolved in 0.5 mL water and slowly added. The mixture was stirred at room temperature for 12 hours. Most of the solvent was removed under

vacuum. 20 ml water and 20 mL ethyl acetate were added to the residue and stirred. The organic layer was washed with 10 mL water for 2 times. Then the organic layer was dried with Na_2SO_4 and evaporated, and purified on a flash silica column with hexane/ethyl acetate to give compound 3 (0.95 g, 75% yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.18 (s, 4 H), 3.76 (s, 4 H), 3.50 (s, 4 H), 3.46 (s, 4 H), 1.47 (s, 9 H).

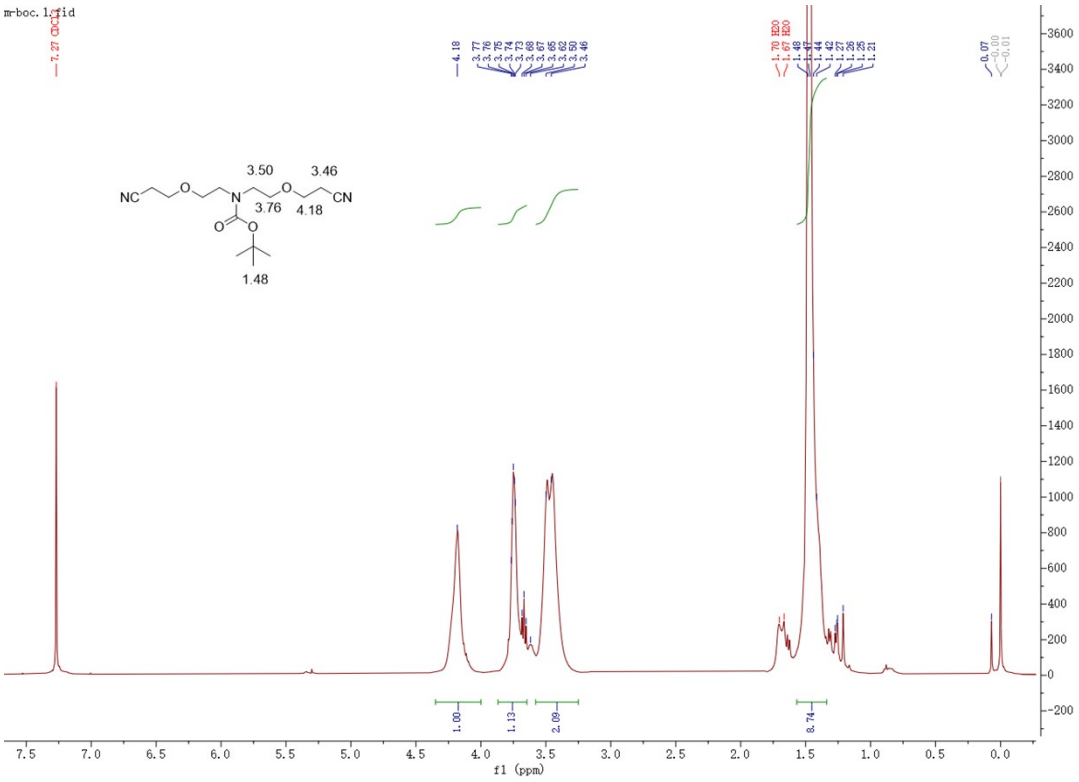

Figure S2. ^{1}H NMR spectrum of compound 3. The peak around 1.70 ppm is water peak. The peak around 0 ppm is grease. The splitting of some peaks is absent, probably because of the restricted rotation of the two alkyl chains attached to the amide, causing the broadening of peaks.

$CoCl_2 \cdot 6H_2O$ (1.43 g, 6.0 mmol) was dissolved in 50 mL MeOH and added to compound 3 (0.95 g, 3 mmol). $NaBH_4$ powder (0.74 g, 20 mmol) was slowly added within 10 min. The mixture turns into black immediately and H_2 bubbles form. The black mixture was stirred at room temperature for 12 hours. Then, most of the solvent was removed under vacuum. 30 ml water and 30 mL ethyl acetate were added to the residue and stirred for 1 hour. The mixture was filtered to remove black solid residues.

The water layer was extracted with 10 mL ethyl acetate for 3 times. Then the organic layer was combined and dried with Na_2SO_4 and evaporated to get an oil. 2 mL TFA was added to the oil and stirred at room temperature for 2 hours. Then, TFA was removed under vacuum to give a solid residue as the TFA salt of compound 4. O-Methylisourea hemisulfate (0.78 g, 6.6 mmol) was dissolved in 15 mL water and added to compound 4. The mixture was stirred at 50°C for 10 h. Then, the clear solution was cooled down to 4°C in a fridge and stored for overnight. The sulfate of compound 5 was precipitated as a white solid from the solution and filtered, washed with cool water and ethanol. The crude product was re-dissolved in 10 mL water at 50°C and cooled down to 4°C in a fridge to yield a precipitate. After the re-crystallization, the purified compound 5 was obtained as sulfate salt (white solid, 0.68 g, 56% yield). ^{1}H NMR (400 MHz, D_2O): δ 3.67 (m, 4 H), 3.55 (m, 4 H), 3.21 (m, 4 H), 3.11 (m, 4 H), 1.81 (m, 4 H).

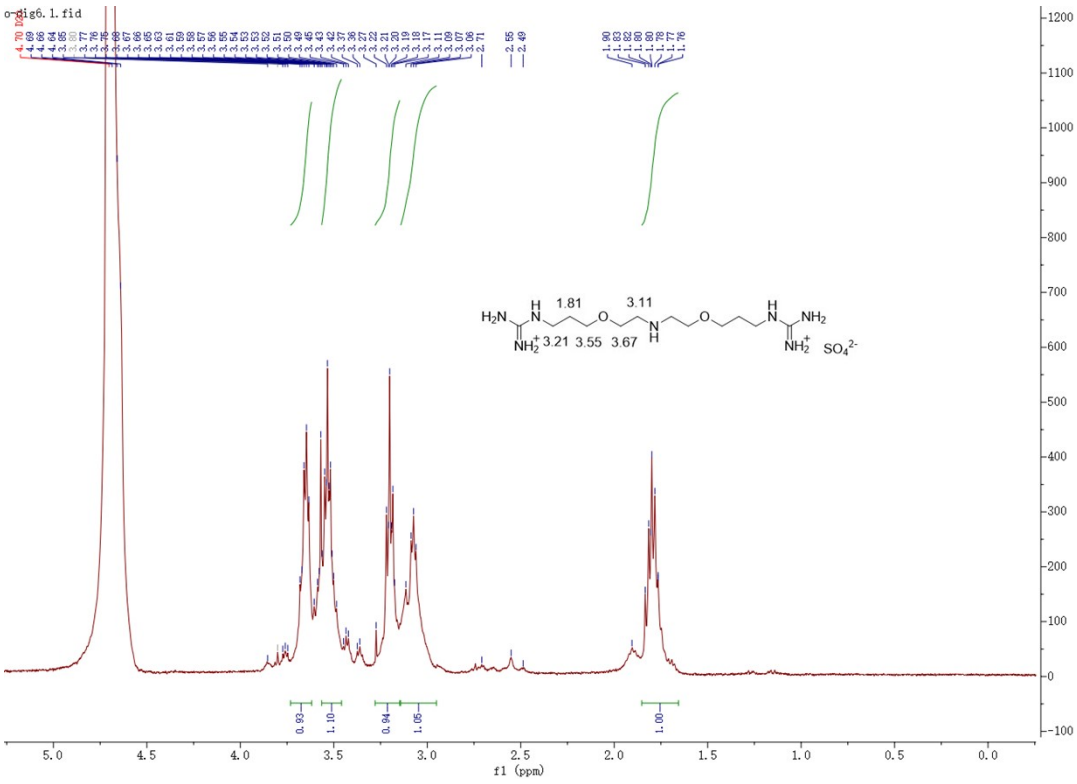

Figure S3. ^{1}H NMR spectrum of compound 5. The large broad peak at 4.7 ppm is water peak.

Cyanuric chloride (0.095 g, 0.5 mmol) was dissolved in 5 mL acetonitrile and cooled to 0°C in an ice-water bath. Compound 5 as a sulfate salt (0.45 g, 1.1 mmol) and $NaHCO_3$ (0.35 g, 4 mmol) was dissolved in 15 mL water and added. The mixture was stirred at 0°C for 1 hour and then warmed to 40°C and stirred for 1.5 h. Then, ethylenediamine (180 mg, 3.0 mmol) was added to the solution, and the solution was stirred at 80°C for 2 h. Most of the solvent was removed under vacuum. The white solid was rinsed by a small amount of water to remove excess ethylenediamine and salt. The remaining white solid was dissolved in 10 mL H_2O/MeCN (v/v = 3/1), and 2-Bromoacetic anhydride (390 mg, 1.5 mmol) was dissolved in 2 mL MeCN and added to the solution. $NaHCO_3$ (126 mg, 1.5 mmol) was also added. The solution was stirred at room temperature for 1 h. Most of the solvent was removed under vacuum, and the solid residue was dissolved in minimum amount of water and purified by flash chromatography on a C-18 flash column. The product solution was concentrated and lyophilized. TG6-Br (as a chloride salt, 230 mg, 46%) was obtained as a white solid. ^1H NMR (400 MHz, D_2O): δ, 4.40 (s, 8 H), 4.23 (s, 8 H), 4.03 (s, 8 H), 3.78 (s, 8 H), 3.73 (s, 2 H), 3.69 (m, 2 H), 3.54 (m, 2 H), 1.75 (m, 8 H). ESI-HRMS m/z: $C_{31}H_{64}BrN_{19}O_5$ MW 861.4521. $[M+3H+H_2O]^{3+}$ calcd for 294.1615, found: 294.1635.

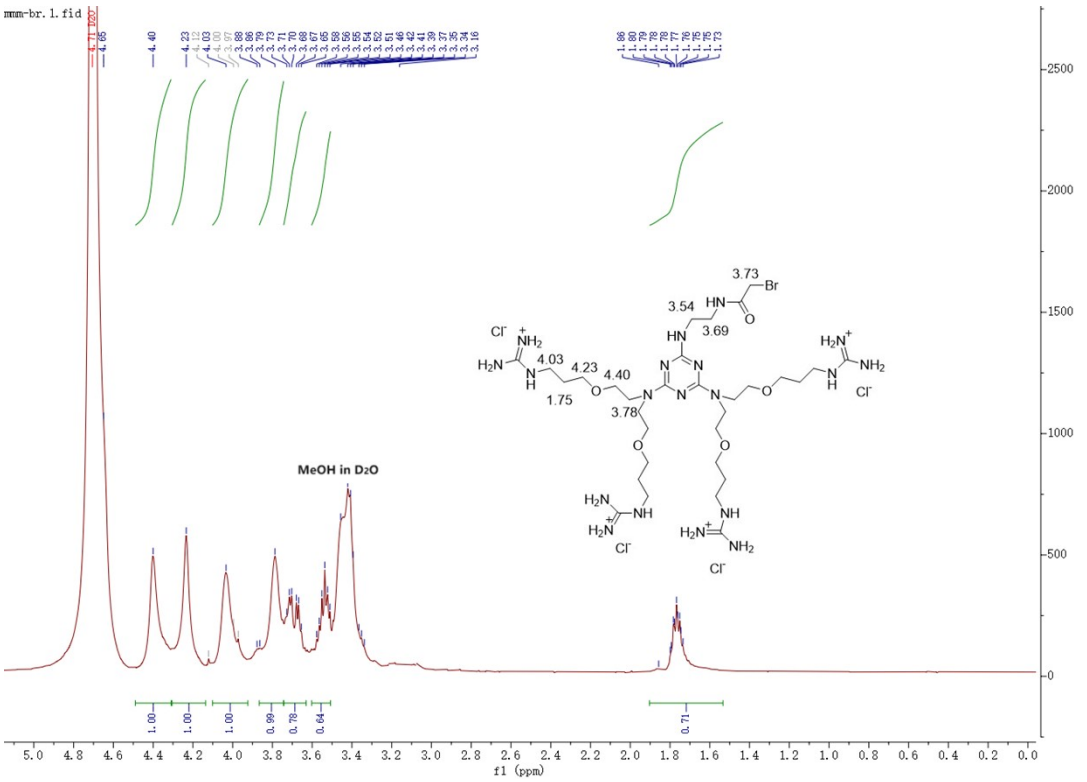

Figure S4. ^{1}H NMR spectrum of TG6-Br. The large broad peak at 4.7 ppm is water peak.

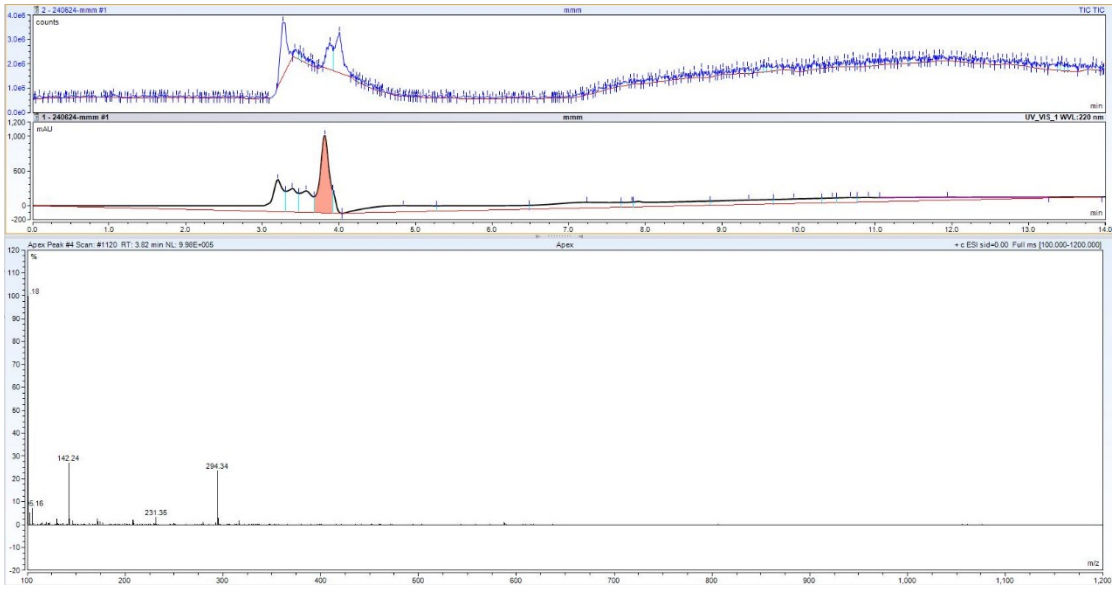

Figure S5. LC-MS of TG-Br. The major peak in MS spectrum shows 294.34 for the $[M+3H+H_2O]^{3+}$ ion. The TG-Br sample was injected into HPLC as a salt with Cl^- as the counter ion, while the CF_3COO^- ion in the HPLC mobile phase replaces the Cl^-.

Thus, the four peaks in the LC spectrum are TG-Br compounds (based on their MS spectrum) with different counter ions.

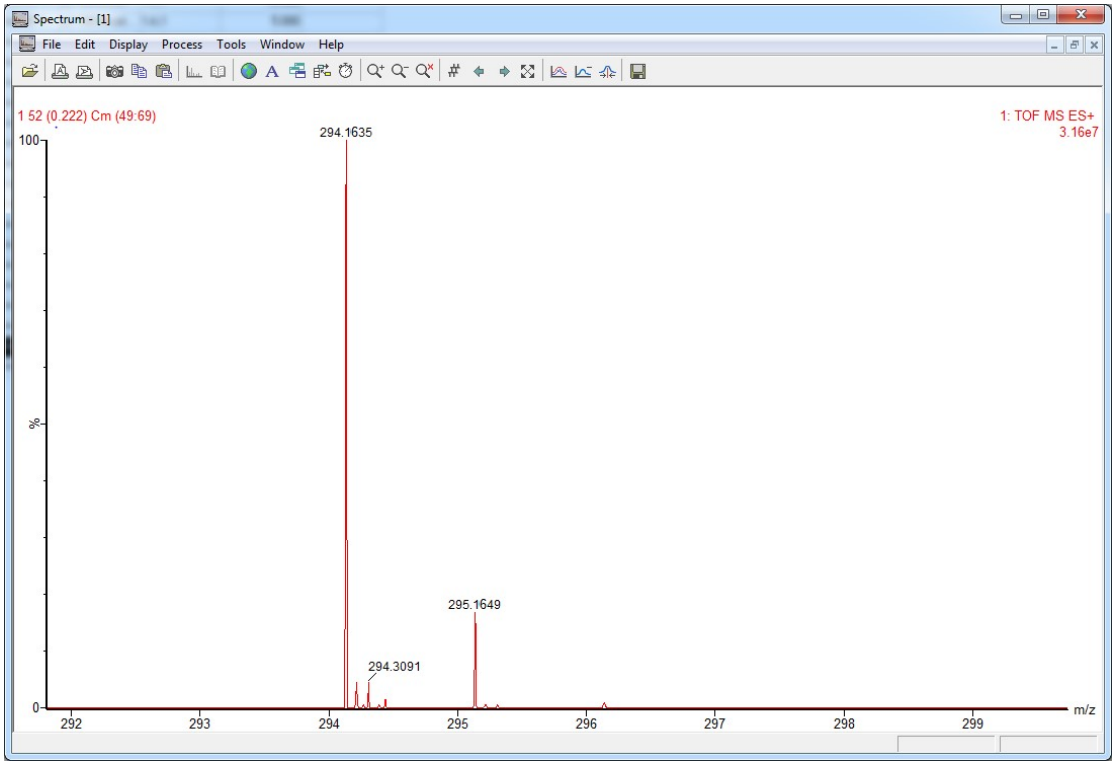

Figure S6. ESI-HRMS of TG6-Br.

Thiolation of cytochrome c and synthesis of TG6-CytC. Traut's reagent (4 mg/mL) in 0.1 M PBS buffer (pH = 8) was mixed with cytochrome c solution (50 mg/mL) in equal volumes for 90 minutes, which was purified by using Amicon Ultra-15 centrifugal filter unit (3 kDa cut-off) against PBS to obtain thiolated cytochrome c (Cyt-SH). TG6-Br was dissolved in 0.1 M PBS buffer (pH = 8) and mixed with Cyt-SH with a molar ratio of TG6-Br : Cyt-SH= 2:1. The solution was gently mixed at 4 ˚C in dark for 2 h, then purified by three times of ultrafiltration using Amicon Ultra-15 centrifugal filter units with 3 kDa cutoff against PBS (pH = 7.4) to give TG6-CytC.

Characterization of TG6-CytC. The mass spectra of proteins were determined by MALDI-TOF (Bruker) with synaptic acid as matrix. TG6-CytC was analyzed by 10% SDS-PAGE electrophoresis followed with Coomassie Blue staining. The UV-Vis

spectra of CytC and TG6-CytC were recorded on a Colibri Microvolume Spectrometer (Titertek-Berthold, Germany).

Cell culture. HeLa human cervical cancer cells, HepG2 human liver cancer cells, and A549 human lung cancer cells were purchased from Cobioer Biosciences (Jiangsu, China). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS, HyClone Lab, USA) and 1% penicillin and streptomycin at 37 ℃ under 5% CO_2, and subcultured when they reach 70% confluency until further study.

Cell viability test by MTT assay. Cells were seeded in a 96-well plate at 1×10^4 cells per well, and cultured overnight. After media change with FBS-free media, cells were then treated with CytC, CytC-SH, and TG6-CytC at indicated concentrations for 24 hours, and incubated with MTT for another 4 hours. Then the media were removed and DMSO was added to dissolve formazan. The absorbance at 570 nm of each well was measured by spectrophotometry on a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA). For the experiments carried out with caspase inhibitor, Ac-DEVD-CHO at the concentrations of 0 μM, 10 μM, 20 μM or 40 μM were added to wells for 2 hours, then removed and washed with PBS. Fresh FBS-free media with CytC or TG6-CytC at a concentration of 24 nM were then added to all wells and incubated for 24 hours before MTT assay. Data were represented as mean ± S.E.M. (n=3). Three biological repeats were performed for all subsequent experiments.

Apoptosis analysis by flow cytometry. HeLa cells were cultured in 3.5 cm dishes overnight at a concentration of 2×10^5 cells per dish. After treatment with 24 nM CytC, CytC-SH, and TG6-CytC in fresh FBS-free media for 24 hours, the cells were dissociated with trypsin, collected with centrifuge, and resuspended in binding buffer. Annexin V-FITC and PI staining were consecutively carried out with Annexin V-FITC/PI staining kit according to manufacturer's protocol. Doubled stained cells were analyzed on a CytoFLEX Flow Cytometer (Beckman, USA).

Mitochondrial membrane potential assay by JC-1. HeLa cells were seeded into glass-bottom dishes (Nest Biotech, China) and cultured overnight at a concentration of 2×10^5 cells per dish. The cells were untreated, pretreat with 20 μM Ac-DEVD-CHO when in need, and treated with 24 nM CytC or TG6-CytC for 1 hour, or 10 μM Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) for 20 minutes as positive control. JC-1 staining was carried out with the Mitochondrial membrane potential assay kit according to manufacturer's protocol. Images were taken on a fluorescent microscope (Micro-shot Tech, China), and analyzed by ImagJ. The red to green fluorescence intensity ratio (R/G ratio) was calculated by normalize the untreated group to 100%. Data were represented as mean ± S.E.M. (n=3). The other two sets of images for R/G ratio analysis are presented in Figure S7.

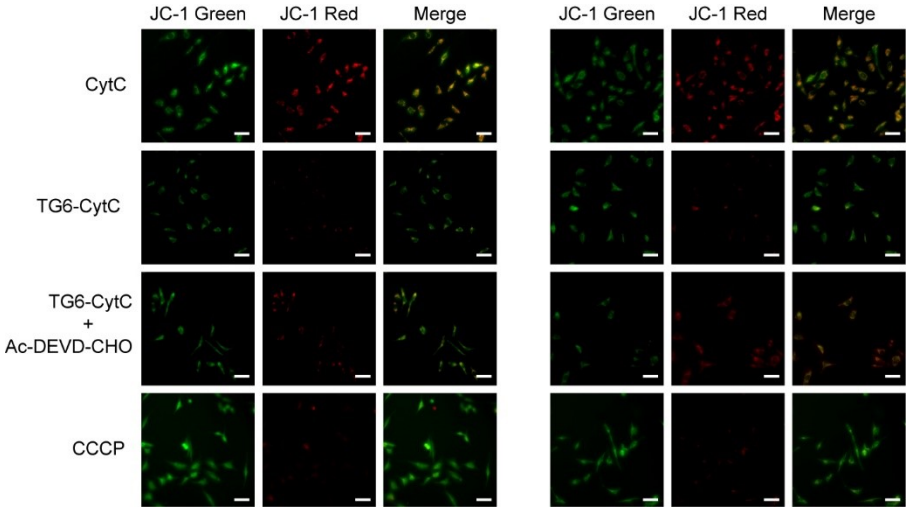

Figure S7. JC-1 assay.

Mitochondria isolation and western blot. HeLa cells were treated with CytC, TG6-CytC, or Ac-DEVD-CHO at the same conditions as above, and dissociated with trypsin. Their mitochondria and cytosol were separated and collected with cell mitochondria isolation kit according to manufacturer's protocol. The protein concentration of mitochondria, cytosolic, and total cell lysate were determined with a micro BCA protein assay kit. The samples were separated by denatured electrophoresis and transferred to polyvinylidene fluoride (PVDF) films (Millipore, Germany). Then the films were

blocked with 5% skim milk for 1 hour, blotted with the indicated antibodies at 4 ℃ overnight, incubated with HRP-conjugated secondary antibodies for 1 hour, and developed with ECL luminescence reagent. The images were taken on automatic chemiluminescence image analysis system (Tanon, China).

Immunofluorescence microscopy. HeLa cells were seeded onto coverslips and treated with CytC, TG6-CytC, or Ac-DEVD-CHO with the same conditions as above. The cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized and blocked by 0.1% Triton X-100, 1% BSA, and 5% goat serum in PBS for 1 hour, incubated with anti-cytochrome c rabbit mAb and anti-mitochondria mouse mAb at 4 ℃ overnight, and labeled with Alexa Fluor Plus 555-conjugated goat anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG for 1 hour. Samples were then cured in ProLong glass antifade mountant with NucBlue stain overnight and sealed with nail polish. The images were collected with a Ultraview VOX Spinning Disc Confocal Microscope (Perkin Elmer, USA), and analyzed by ImagJ.