Electronic Supplementary Information

A mitochondrion-targeting copper complex exhibits potent cytotoxicity against cisplatin-resistant tumor cells through a multiple mechanism of action

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Experimental

1. Materials and methods

Reagents such as $CuBr_2$, 4-methylbenzaldehyde, 2-acetylpyridine, ammonium acetate, *N*-bromosuccinimide (NBS), benzoyl peroxide (BPO),triphenylphosphine (TPP) and 1-octanol were of analytical grade and used without further purification. Supercoiled pBR322 DNA and 6X loading buffer (0.05% bromophenol blue, 0.035% xylene cyanol FF, 36% glycerol and30 mM EDTA)were purchased from TaKaRa Biotechnology (Dalian, China).Calf thymus DNA (CT-DNA), Tris, and ethidium bromide (EB) were purchased from Sunshine Bio (Nanjing, China).ROS assay kit, cellular mitochondrion isolation kit, genomic DNA mini preparation kit with spin column and mitochondrial membrane potential assay kit with JC-1 dyeing buffer (5X) were purchased from Beyotime Institute of Biotechnology (Haimen, China). Milli-Q water was used in all aqueous solutions.

The human breast carcinoma (MCF-7), the human cervical cancer (HeLa), the human ovarian cancer (Skov-3), the human lung adenocarcinoma (A549) and its cisplatin-resistant (A549R) cell lines were originated from Cancer Institute & Hospital, Chinese Academy of Medical Sciences.A549R cells were recovered and cultured in RPMI-1640 medium (containing 10 % FBS) with continuous selection pressure of cisplatin (2 μg/L).

The electrospray ionization mass spectrometry (ESI-MS) spectra were recorded using an LCQ fleet ESI-MS spectrometer (Thermo Scientific) in the positive mode, and the isotopic distribution patterns of the observed species were simulated using the Isopro 3.0 program.UV-vis spectra were determined on a Shimadzu UV 3600 (UV-vis-near-IR) spectrophotometer.Circular dichroism (CD) spectra were measured by a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Japan). Fluorescence spectra were recorded on an LS-50B spectrofluorimeter (Perkin-Elmer, U.S.A.).The images of agarose gel electrophoresis were obtained by using a Bio-Rad Gel-Doc XR imaging system. The optical density (OD) of formazan was tested on Thermo Scientific Varioskan Flash. The inductively coupled plasma mass spectrometry (ICP-MS) data were obtained on ELAN9000 ICP-MS (PerkinElmer Inc., U. S. A.). The mitochondrial membrane potential ($\Delta\Psi_m$)of HeLa cells was tested on a flow cytometer BD LSRFortessa^{TM} (Amersham Biosciences Corp., U. S. A.).

The electrochemical property of CTBwas tested on CHI660C electrochemical workstation. A three-electrode cell containing a 2.0-mm-diameter glassy carbon working electrode (polished with Al_2O_3), a platinum auxiliary electrode, and a non-water Ag/Ag^+ reference electrode (revised by 1 mM $K_3Fe(CN)_6$) were used. The potential of CTB was measured in PBS (pH 7.4) under the protectionof nitrogenat room temperature using a 660C potentiostat/glavanostat, with KNO_3 (0.1 M) as supporting electrolyte.[1]

2. Synthesis of the ligand and complex

Ligand ttpy-tpp was prepared according to the reported procedures.[2] CTB was prepared by mixing aqueous solution of $CuBr_2$ (100 mM, 10 mL) and methanol solution of ttpy-tpp (100 mM, 10 mL) and stirring for 3 h at room temperature. After the removal of solvent, the resulting solid was washed with acetone and diethyl ether, and dried *in vacuo*. Green crystals suitable for single crystal X-ray diffraction analysiswere obtaineddirectly by slow evaporation of the complex solution. Yield: 0.613 g, 69.1%. ESI-MS (m/z): 806.42, $[Cu(ttpy\text{-}tpp)Br_2]^+$ ($C_{40}H_{31}Br_2CuN_3P$); 364.17, $[Cu(ttpy\text{-}tpp)Br]^{2+}$ ($C_{40}H_{31}BrCuN_3P$). Elemental analysis found (calcd) for $C_{40}H_{31}Br_3CuN_3P$ (%): C 53.82 (54.11),H 3.85 (3.52),N 4.59 (4.73).

3. X-ray crystallography

X-ray crystallographic data were collected on a Bruker SMART APEX CCD area-detector diffractometer operating in the φ-ω scan mode with graphite-monochromated Mo-Ka radiation (λ = 0.71069 Å) at 298 K. Empirical absorption corrections were carried out using a multiscan program. The SMART software was used for data acquisition and the SAINT software for data extraction.[3,4] The structure of CTB was solved by direct methods and refined on F^2 by full matrix least-squares methods using the SHELXTL program.[5] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included in calculated positions and refined with isotropic thermal parameters.

4. DNA binding and cleavage

The UV absorption titration was carried out by adding increasing concentrations of CT-DNA to CTB solution and subsequent measuring of the UV spectrum. CD spectra were recorded after each increase of the [CTB]/[DNA] ratio. Fluorescence changes of the EB−DNA system with addition of CTB were determined with the method we adopted in previous studies.[6,7] The apparent binding constant (K_{app}) of CTB was calculated using the following equation:

$$K_{EB}[EB] = K_{app}[complex]$$

whereK_{EB} = 1.0 $\times 10^7$ M^{-1}, [EB] = 1.0 μM, [complex] = 2.47 μM, which is the concentration of CTB when a 50% reduction of the fluorescence occurred. The electrostatic interaction between CTB and DNA was studied by UV spectroscopy in a [DNA]/[CTB] ratio of 0.4 while [NaCl] of the buffer solutions (5 mM Tris-HCl/NaCl, pH 7.4) increased from 5 to 200 mM.

The DNA cleavage activity and ROS responsible for the cleavage were investigated using agarose gel electrophoresis as we described previously.[6] Cellular DNA was isolated from HeLa cells after cultivation in RPMI-1640 medium with or without CTB (2 μM) for 48 h by a genomic DNA mini preparation kit. Briefly, about 2 million HeLa cells were collected from a petri dish and resuspended in the mixture of PBS (200μL), proteinase K (20 μL) and cell lysis solution (200 μL). Ethanol (200 μL) was added into the mixture after it was incubated at 70°C for 10 min. The mixed solution was then added in a DNA spin column and centrifuged at 8000 rpm for 1 min. After centrifuging and washing for three times, eluent (50μL) was added into the column to collect the purified DNA. The concentration of DNA was tested with Nanodrop 1000 spectrophotometer (Thermo Scientific), which was then diluted to 20 ng μL^{-1} for agarose gel electrophoresis.

ROS in HeLa cells were detected by a ROS assay kit. Specifically, HeLa cells were seeded in 6-well plates and cultivated for 20 h, and then were exposed to fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA, 10 μM) at 37 °C for 20 min.CTB (10 μM, 20 μM, 30 μM), $CuCl_2$ (10 μM, 20 μM, 30 μM),cisplatin (10 μM) and positive control rosup (10 μM) were added into each well, respectively, and cultivated at 37 °C for 1 h. Cells were washed twice and resuspended with RPMI-1640 medium for fluorescence testing.

5. Cellularand mitochondrial uptake

Tumor cells were cultivated with CTBor its analogue [Cu(ttpy)Br_2](2 μM) in RPMI-1640 medium for 48 h and then were digested by water (50 μL), concentrated nitric acid (70 μL) at 95 °C for 2 h, hydrogen peroxide (30%, 20 μL) at 95 °C for 1.5 h, and concentrated hydrochloric acid (35 μL) at 37 °C for 0.5 h, successively. The solution was diluted to 1 mL with water and cellular Cu content was determined directly by ICP-MS.

Mitochondria of HeLa cells (10^6) were isolated by a cell mitochondria isolation kit.Briefly, cells were collected from a petri dish and washed with PBS for three times, and then were suspended in the mitochondria isolation solution (1 mL)and cooled in icy water for 10 min. The cell solution was homogenized and the homogenates were centrifuged at 600 g (*ca* 800 rpm) for 10 min.The supernatant was transferred to another tube andcentrifuged at 11000 g for 10 min. The precipitate was collected as cellular mitochondria, which was dissolved in water (1 mL).The content of mitochondrial Cu was tested by ICP-MS.Untreated tumor cells were used as controls in these determinations.In an alternative assay, Skov-3 cells were cultivated with CTB(2 μM) in RPMI-1640 medium for 48 h.The mitochondria (3μL)were isolated as described above and cracked by a mitochondrial cracking liquid. Meanwhile, the same volume of Skov-3 cells cultivated in the same condition were digested by water (50 μL), concentrated nitric acid (70 μL) at 95 °C for 2 h, hydrogen peroxide (30 %, 20 μL) at 95 °C for 1.5 h, and concentrated hydrochloric acid (35 μL) at 37 °C for 0.5 h, successively. Both the mitochondrial and digested cellular solutions were diluted to 0.7 mL with water and subjected to fluorescence determination.

6. Detection of $\Delta\Psi_m$

HeLa cells cultivated with RPMI-1640 (containing 10% FBS) were seeded in 6-well plates and exposed to different concentrations (1, 2, 4 μM) of CTB and cisplatin for 48 h, respectively. The cells were collected in tubes and incubated with lipophilic cationfluorochromeJC-1 at 37 °C for 20 min. The cells were then washed twice with diluted JC-1 dyeing buffer (1X), resuspended in the same buffer and examined by flow cytometry. Carbonylcyanide*m*-chlorophenylhydrazone (CCCP) was used as a positive contrast.

7. Measurement of lipophilicity

Lipophilicity was measured in a 1-octanol/buffer system using the shake-flask method.[8] Solutions ofCTB (50, 100, 200μM, respectively) were prepared in the phosphate buffer (pH 7.4) presaturated with 1-octanol.Equal volumes (2.0 mL) of the solution and 1-octanol presaturated with the phosphate buffer were mixed and placed in a thermostatic (25.0±0.1 °C)air bath orbital shakerat 200 rpm for 4 h.The samples were separated into two phases after centrifugation at 2500 rpm for 15 min.The concentration of the solute in the aqueous phase was determined by

spectrophotometry (λ_{max}= 288 nm). According to the law of mass conservation, the drug concentration of corresponding 1-octanol phase and the lipo-hydro partition coefficient $P_{o/w}$ ($P_{o/w} = C_o/C_w = A_o/A_w$, A stands for absorbance) were calculated. The average of three parallel experimental data was reported as the final result.

8. Cytotoxicity assay

Tumor cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS;Gibco) and 100 U mL^{-1} penicillin in 5% CO_2atmosphere at 37 °C. The cells were seeded in the 96-well plates with 4000 cells per well. Solutions of CTB (50, 20, 10, 5, 2, 1 μM) were added into the wells and incubated for 48 h. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (20 μL, 5 mg mL^{-1}, PBS buffer) was added to each well and incubated for 4 h.[9] Final samples were dissolved in DMSO (150 μL) and the absorbance of the purple formazanwas recorded on an ELISA plate reader. Inhibition rate (%) and IC_{50} were calculated based on the data of three parallel tests.

Supplementary results and figures

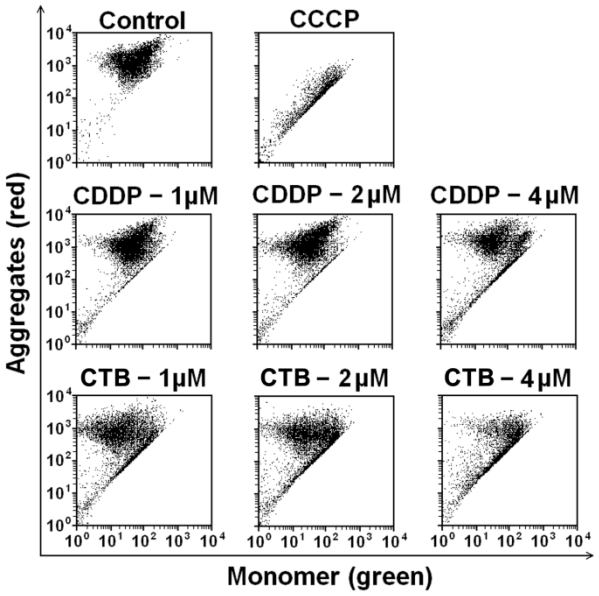

Fig. S1 A representative flow cytometric plot reflecting the effect of CTB or cisplatin on HeLa cells after incubation for 48 h with JC-1.

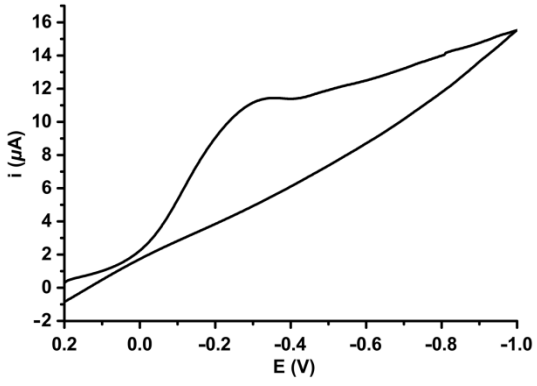

Fig. S2 Cyclic voltammogram of CTB under nitrogen in KNO_3 (0.1 M) at room temperature (v = 200 mV/s). CTB displayed an irreversible Cu(II)/Cu(I) reduction

peak at −0.33 V vs. the normal hydrogen electrode (NHE) at 200 mV s^{-1} scan rate, which is comparable to the redox potentialof the copper-thiosemicarbazone complexes.

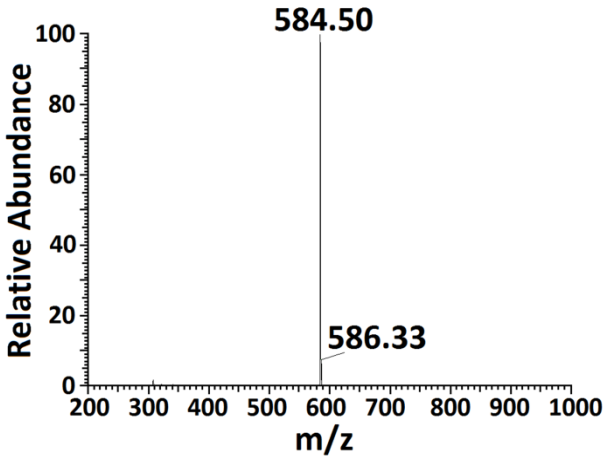

Fig. S3ESI-MS spectrum (positive mode)of the ligand ttpy-tppin water.

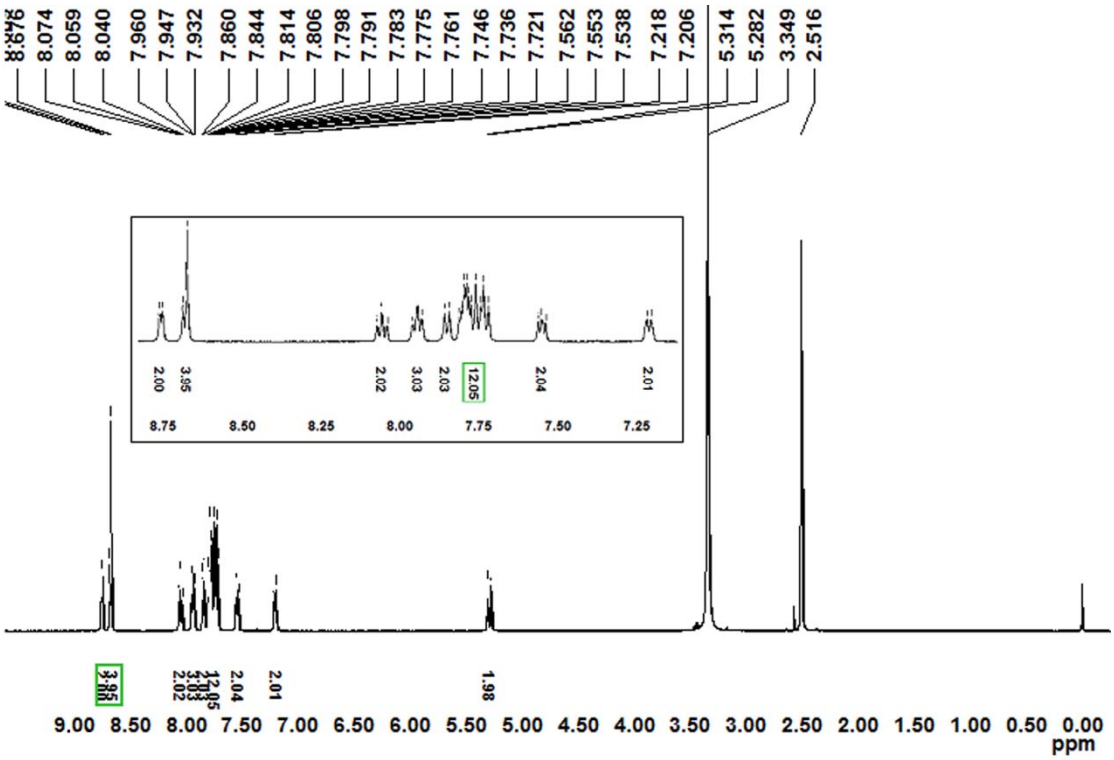

Fig.S4[1]H NMR spectrum (DMSO-d_6) of the ligand ttpy-tpp.

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