Electronic Supporting Information

Osmium(VI) complexes as a new class of potential anti-cancer agents

Wen-Xiu Ni,[a] Wai-Lun Man,[a] Myra Ting-Wai Cheung,[a] Raymond Wai-Yin Sun,[b] Yuan-Lan Shu,[c] Yun-Wah Lam,*[a] Chi-Ming Che*[b] and Tai-Chu Lau*[a]

[a] *Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong, China.*

[b] *Department of Chemistry and Open Laboratory of Chemical Biology of the Institute of Molecular Technology for Drug Discovery and Synthesis, The University of Hong Kong, Pokfulam Road, Hong Kong, China.*

[c] *State Key Laboratory Incubation Base of Chinese Medicine and Molecular Pharmacology, Shenzhen, China.*

E-mail: bhtclau@cityu.edu.hk; cmche@hku.hk.

Experimental Section

Materials

All starting chemicals were purchased from Sigma-Aldrich and used as received unless specified otherwise. Other reagents and materials include cell culture medium constituents and phosphate-buffered saline (PBS) (Gibco BRL), cell proliferation kit I (MTT) (Roche), calf thymus DNA (ctDNA) (purified by phenol/chloroform extractions).

Cell lines used in this work including cervical epithelioid carcinoma (HeLa), hepatocellular carcinoma (HepG2), leukemia (HL-60), myelogenous leukemia (K562), and human

non-small cell lung carcinoma (NCI-H460) were purchased from American-Type Culture Collection (ATCC), and were maintained in tissue culture at 37 °C in a humidified chamber containing 5% CO_2. HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) and the other three were cultured in Roswell Park Memorial Institute (RPMI 1640; Gibco), supplemented with 10% fetal bovine serum (FBS) in the presence of antibiotic-antimycotic solution (Gibco).

SPF grade BALB / c-nu male nude mice were purchased from Medical Laboratory Animal Center, Guangdong Province, and were kept in SPF grade animal laboratories equipped with independent ventilated cages (qualified number: 2006C021), Laboratory animal permission number: SCXK (Guangdong) 2008-0002, at 22–26℃, 40–70 % humidity, 12 h light-dark cycles. Independent ventilation cage with maximum wind speed of 35 m^3/ h and actual wind speed of 20 m^3/ h. Each nude mouse was kept in separated breeding cage.

Primary antibodies used were cyclin B1, cyclin D3, cyclin A (mouse, dilution 1:1000, BD Biosciences), Ki67 (Mouse, dilution 1:1000, ab**cam**), PARP 1 (rabbit, dilution 1:1000, Santa Cruz), *β*-actin (mouse, dilution 1:3000, Sigma-aldrich), And secondary antibodies used were ECL mouse lgG and ECL rabbit lgG (dilution 1:2000, GE Healthcare).

Instrumentation

^{1}H NMR spectra were obtained on a Varian (300MHz) FT NMR spectrometer. UV-vis absorption was recorded on a Perkin-Elmer Lambda 900 UV-visible spectrophotometer. ESI mass spectra were recorded on a PE-SCIEX API 300 triple quadrupole mass spectrometer. Elemental analysis was done on an Elementar Vario EL Analyzer. Infrared spectra were obtained from KBr plates using a Nicolet 360 FT-IR spectrophotometer. Cytotoxicity of the osmium complexes was determined by reading the absorbance at 550 nm on a multiplate reader (Perkin-Elmer FusionTM α-FP). Cell cycle analysis was analyzed by flow cytometry FACS Calibur, BD. Immunofluorescence images were recorded using Leica TCS SPE confocal laser scanning microscope. The nitrocellulose blotting membrane for western blot

analysis was visualized using high performance laser ge and bolt imager, Fujifilm LAS-4000. The gel for DNA migration study was stained with Gel Red and photographed with UV illumination, Bio-Rad Gel Doc.

General synthesis and characterization of complexes 1-5

The complexes, $[^{n}Bu_4N][Os^{VI}(N)(Cl)_4]$ and ^{15}N-labelled $[^{n}Bu_4N][Os^{VI}(^{15}N)(Cl)_4]$, were prepared by literature procedures.[11*d*] The Schiff base ligands, 5-R-H_2sap (R = H, Cl, Br, MeO, Me) were synthesized by condensation of corresponding salicylaldehyde and amine with 1:1 ratio in refluxing ethanol. Solvents and reagents were of reagent grade and use without further purification.

A stoichiometric amount of 5-R-H_2sap (1.0 mmol) (R = H, Cl, Br, MeO, Me) was added to a solution of $[^{n}Bu_4N][Os^{VI}(N)(Cl)_4]$ (588 mg, 1.0 mmol) in methanol (30 mL) and the mixture was refluxed for 1 d. The resulting red precipitate was filtered, washed with cold methanol (5 mL) and then air-dried.

$[Os^{VI}(N)(sap)(Cl)(OH_2)]$(1) Yield: 69%. IR (KBr, cm^{-1}) : $\nu(Os\equiv^{14}N)$1098, $\nu(Os\equiv^{15}N)$1063. Anal. calcd. (found) for $C_{13}H_{11}N_2O_3ClOs$: C, 33.30 (33.27), H, 2.36 (2.47), N, 5.97 (5.90). ^{1}H NMR (300MHz, CD_3CN): δ 7.02-7.06 (t, 1H), 7.05-7.10 (t, 1H), 7.22-7.26 (d, 1H), 7.28-7.34 (t, 1H), 7.65-7.70 (t, 1H), 7.80 (d, 1H), 7.91 (d, 2H), 9.12 (s, 1H, N=CH). UV/Vis (CH_3CN): $\lambda_{max[}$nm](ε[$mol^{-1}dm^3cm^{-1}$]) 320 (9590), 457 (4490).

$[Os^{VI}(N)(5\text{-}Cl\text{-}sap)(Cl)(OH_2)]$ (2) Yield: 55%. IR (KBr, cm^{-1}): $\nu(Os\equiv^{14}N)$ 1097. Anal. calcd. (found) for $C_{13}H_{10}N_2O_3Cl_2Os$: C, 31.02 (31.06), H, 2.00 (2.26), N, 5.57 (5.75). ^{1}H NMR (300 MHz, CD_3CN): δ 6.98-7.04 (t, 1H), 7.27-7.30 (d, 2H), 7.33-7.38 (t, 1H), 7.63-7.67 (d, 1H), 7.80-7.81 (s, 1H), 7.92-7.95 (d, 1H), 9.08 (s, 1H, N=CH). UV/Vis (CH_3CN): λ_{max} [nm] (ε [$mol^{-1}dm^3cm^{-1}$]) 320 (16000), 469 (7750).

$[Os^{VI}(N)(5\text{-}Br\text{-}sap)(Cl)(OH_2)]$ (3) Yield: 62%. IR (KBr, cm^{-1}): $\nu(Os\equiv^{14}N)$ 1097. Anal. calcd.

(found) for $C_{13}H_{10}N_2O_3ClBrOs$: C, 28.50 (28.77), H, 1.84 (2.01), N, 5.11 (5.15). ^{1}H NMR (300 MHz, CD_3CN): 6.98-7.03 (t, 1H), 7.27-7.29 (t, 2H), 7.30-7.38 (t, 1H), 7.74-7.78 (d, 1H), 7.92-7.96 (d, 2H), 9.07 (s, 1H, N=CH). UV/Vis (CH_3CN): λ_{max} [nm] (ε [$mol^{-1}dm^3cm^{-1}$]) 320 (16000), 470 (7680).

[Os^{VI}(N)(5-MeO-sap)(Cl)(OH_2)] (**4**) Yield: 45%. IR (KBr, cm^{-1}): ν(Os≡^{14}N) 1092. Anal. calcd. (found) for $C_{14}H_{12}N_2O_4ClOs$: C, 33.77 (33.67), H, 2.43 (2.55), N, 5.63 (5.39). ^{1}H NMR (300 MHz, CD_3CN): δ 3.86-3.87 (s, 3H, OCH_3), δ 6.97-7.02 (t, 1H), 7.25 (d, 2H), 7.28-7.32 (t, 1H), 7.34-7.38 (d, 2H), 7.91-7.94 (d, 2H), 9.12 (s, 1H, N=CH). UV/Vis (CH_3CN): λ_{max} [nm] (ε [$mol^{-1}dm^3cm^{-1}$]) 323 (15700), 502 (6300).

[Os^{VI}(N)(5-Me-sap)(Cl)(OH_2)] (**5**) Yield: 43%. IR (KBr, cm^{-1}): ν(Os≡^{14}N) 1092. Anal. calcd. (found) for $C_{14}H_{12}N_2O_3ClOs$: C, 34.89 (34.62) H, 2.51 (2.88) N, 5.81 (5.69). Found: C, 34.62; H, 2.88; N, 5.69%. ^{1}H NMR (300 MHz, CD_3CN): δ 2.39 (s, 3H, CH_3), δ 6.96-7.02 (t, 1H), 7.26-7.28 (d, 2H), 7.30-7.35 (t, 1H), 7.54-7.59 (d, 2H), 7.94-7.97 (d, 1H), 9.09 (s, 1H, N=CH). UV/Vis (CH_3CN): λ_{max} [nm] (ε [$mol^{-1}dm^3cm^{-1}$]) 323 (16200), 468 (7470).

Cytotoxicity Tests in Cancer Cell Lines

Cytotoxicity was determined by the colorimetric MTT assay (MTT = 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma). HeLa, HepG2, HL-60, K562 and CCD-19Lu cells were seeded into 96-well microculture plates at a cell density of about 8×10^3 cells/well (for HeLa and HepG2) or about 1.5×10^4 cells/well (for CCD-19Lu, HL-60 and K562), in order to ensure exponential growth throughout drug exposure. After an overnight incubation to allow cells attachment (HeLa and HepG2), cells were exposed for 24h to solutions (100μL/well) of the test compound, which were first dissolved in DMSO and then diluted in culture medium to the desired concentrations (final DMSO concentration < 1%in all cases). At the end of exposure, drug solutions were replaced by 100μL/well medium with 10% MTT solution in phosphate-buffered saline (5 mg/ml PBS). After incubation for 1 h at 37º C, medium was removed (HeLa and HepG2), and the reduced formazan product formed by the mitochondrial dehydrogenase activity of vital cells were

dissolved in 100 μL (HeLa and HepG2) / 150 μL (CCD-19Lu, HL-60 and K562) DMSO per well. Optical densities at a wavelength of 570 nm were measured with a microplate reader (BioTek, Power Wave XS). The quantity of vital cells was expressed in terms of T/C values by comparison to untreated control microcultures, and IC_{50} values were calculated from concentration-effect curves by logarithmic interpolation. Evaluation is based on means from at least three independent experiments, each comprising four replicates per concentration level.

Cell Cycle Analysis by Flow Cytometry

HeLa cells were treated with 6 μM **1** for 6, 12, 24, 30, 36 and 48 h at 37 $^{\circ}$C in six-well plates. Appropriate controls, in which cells were mock-treated by DMSO for the same durations, were also set up. After being treated, all cells were trypsinized and collected, washed with PBS twice, fixed with -20 $^{\circ}$C, 70% ethanol overnight at 4 $^{\circ}$C. After this, cells were spun down at 1500 rpm for 5 min, washed once with PBS, and then treated with the mixture of 10 μL RNase (10 mg/mL), 50 μL propidium iodide (1 mg/mL) and 940 μL 1×PBS, incubated at room temperature for 30 min in the dark. The stained cells were analyzed by flow cytometry (BD FACS Calibur).

Immunofluorescence

HeLa cells were grown on coverslips, treated with 6 μM **1** for 12 h, washed with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, rinsed with PBS, and permeabilized with 0.1% Triton X-100 solution for 5 min on ice. The cells were blocked with 1% BSA in PBS for 30 min at room temperature and incubated with 0.1 ug/ml 4',6-diamidino-2-phenylindole (DAPI) (invitrogen) for 10 min and afterwards mounted. Images were recorded using confocal laser scanning microscope (Leica TCS SPE).

Western Blot Analysis

After 6μM **1** treatment, HeLa cells were washed (PBS) and collected at different time points. Cells were lysed in 2% sodium dodecyl sulfate (SDS) in PBS supplemented with protease inhibitor cocktail (Roche). Protein concentrations were measured using the Coomassie protein

assay reagent according to manufacturer's instruction (Thermo Scientific). Equal amounts of cellular proteins were mixed with sample buffer containing 2% SDS, 50 mM Tris-HCl (pH 6.8), 0.2 mg/ml bromophenol blue, 0.1 M dithiothreitol (DTT), 0.1% glycerol, boiled at 95 $^{\circ}$C for 5 min, and run on 12% Bis-Tris Novex precast gels (invitrogen). Protein was transferred to nitrocellulose blotting membrane (Pall Corporation), which was incubated with 5% nonfat milk (Nestle) in PBS-T buffer (1×PBS, 0.05% Tween-20) for 1 h to block non-specific binding, and then probed with primary antibody followed by secondary antibody. Next, the ECL detection kit (GE Healthcare) was added to catalyze oxidation of luminol to emit chemiluminescence, and the membrane was visualized using high performance laser gel and bolt imager (Fujifilm LAS-4000).

Reaction of complex with plasmid DNA VSFP2.4

The reaction of **1** with DNA was conducted using plasmid DNA (VSFP2.4, 7492 base pair (bp) and 52.5% GC) and analyzed by gel electrophoresis. The plasmid DNA concentration per nucleotide was determined by absorption spectroscopy, using the molar extinction coefficient 6600 $M^{-1}cm^{-1}$ at 260nm wavelength. Briefly, 0.55 *u*g of plasmid DNA was incubated with varying concentrations of the nitridoosmium compound at room temperature for 24 h. Following incubation, samples were analyzed by agarose gel electrophoresis (1% agarose, 1× TAE), and then the gel was stained with Gel Red and photographed with UV illumination.

***In Vivo* Study**

All *in vivo* experiments were tested by PearL Materia Medica Development (Shenzhen) Ltd. A total of 15 male BALB/c-nu nude mice weighing between 16 and 18 g were inoculated subcutaneously 0.2 mL of the NCI-H460 cell suspension (1.5 x10^6 cells/ rats) at the beginning of the experiment. The day after inoculation (administration day 1), the nude mice were randomly divided into three groups containing five mice each. One group of mice were injected intraperitoneally with 30.0 mg/kg cyclophosphamide as positive group, one group was injected 20% PET (Polyethylene glycol 400, 12%; ethanol 6%; Tween 80, 2% and PBS 80%) as negative group. The remaining three groups of mice were treated with 1 mg/kg nitridoosmium compound **1**. Mice in each group were received intraperitoneal injection twice

a week, with a total number of eight injections. Tumor sizes were measured every 3 days. The treated mice were scarified at the end of the studied period (30 days) with anatomical separation of tumor nodules. The tumors were weighed and photographed.

The inhibition rate of tumor growth and tumor volume were determined using the following formulas:

Tumor growth inhibition rate (%) = (1 - mean tumor weight of the treatment / mean tumor weight of the negative group) × 100% ;

Tumor volume = longest dimension (mm) × [shortest dimension of the tumor (mm)]^2/ 2 .

All measurements were expressed in Mean ± SD, using SPSS 10.0 for statistical analysis.

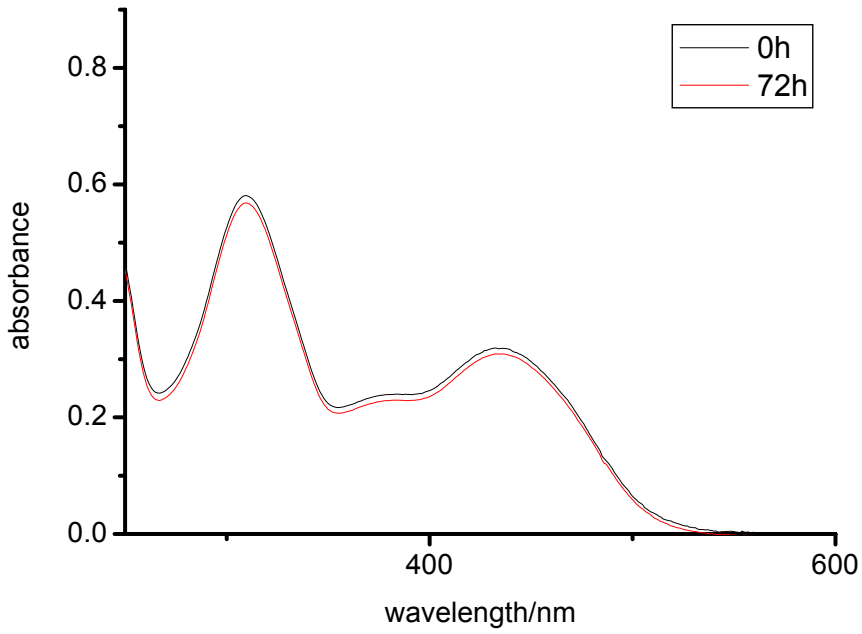

Fig. S1 UV-visible spectral change of **1** in DMSO/PBS (1:19) from 0 to 72 h

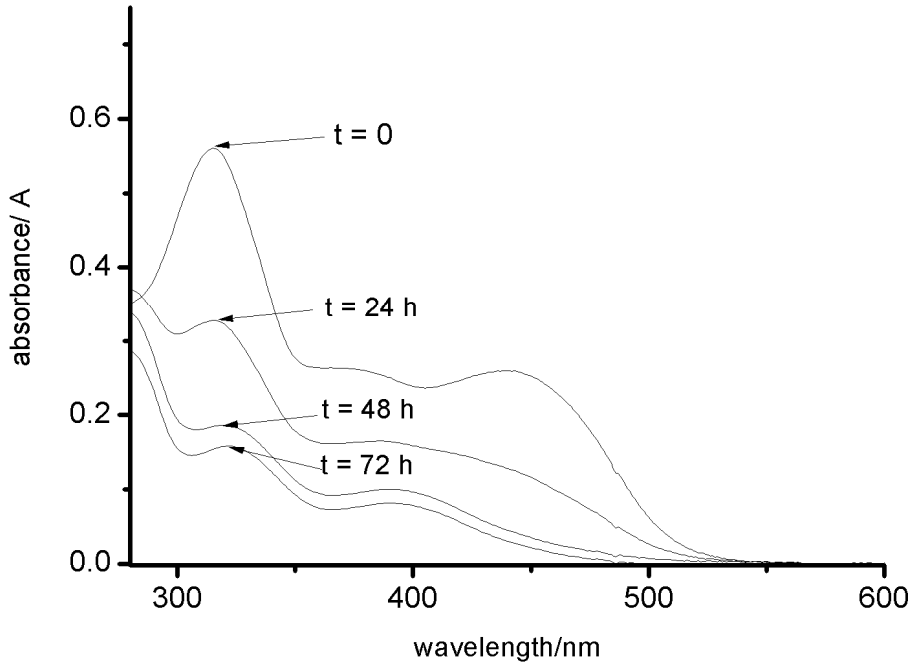

Fig. S2 UV-vis spetral changes during the reaction of complex **1** with GSH from 0 to 72 h.

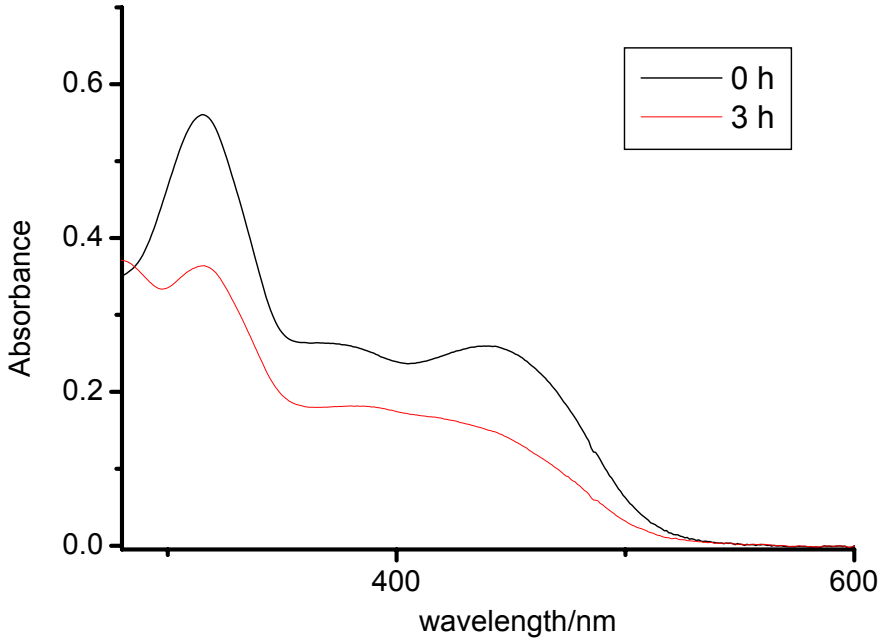

Fig. S3 UV-visible spectral change of **1** in DMSO/PBS (1:19) in the presence of 5'-GMP from 0 to 3 h

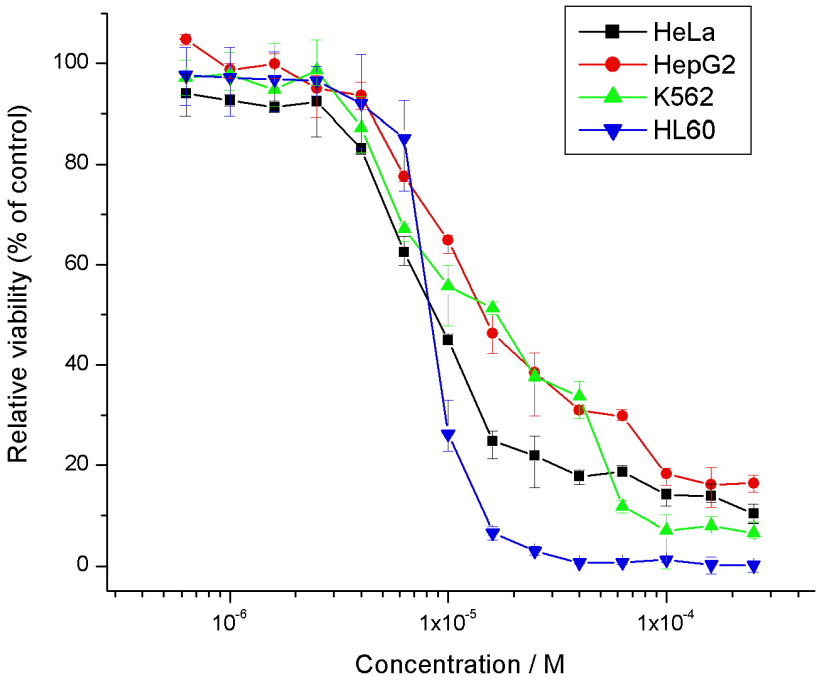

Fig. S4 Cytotoxic profiles of **1** towards cancerous cell lines, values are means ± standard deviations from four replicates per concentration.

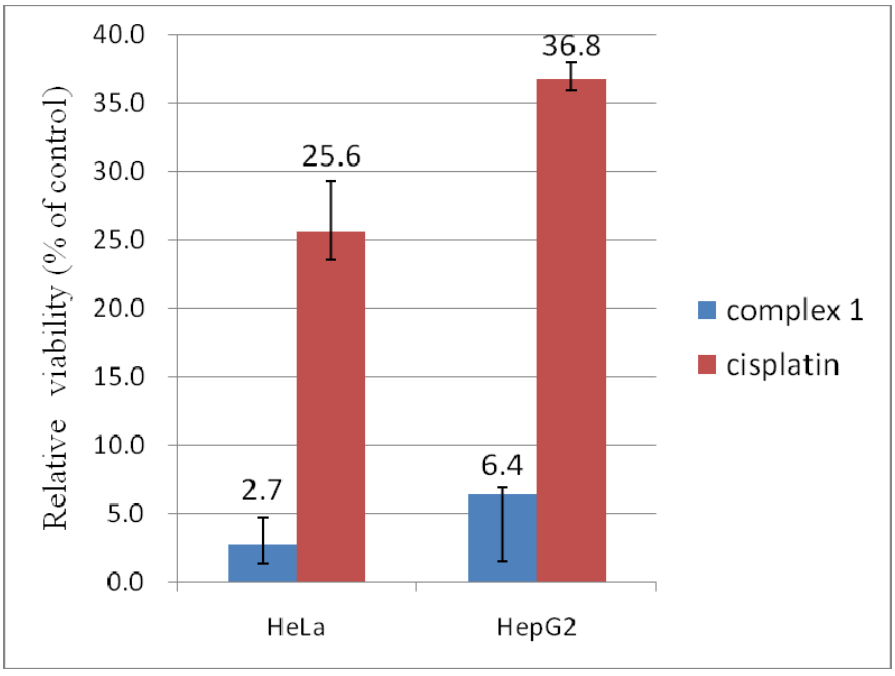

Fig. S5 Antiproliferative effects of complex **1** and cisplatin (6 μM) in HeLa and HepG2 human cancer cell lines for 72 h.

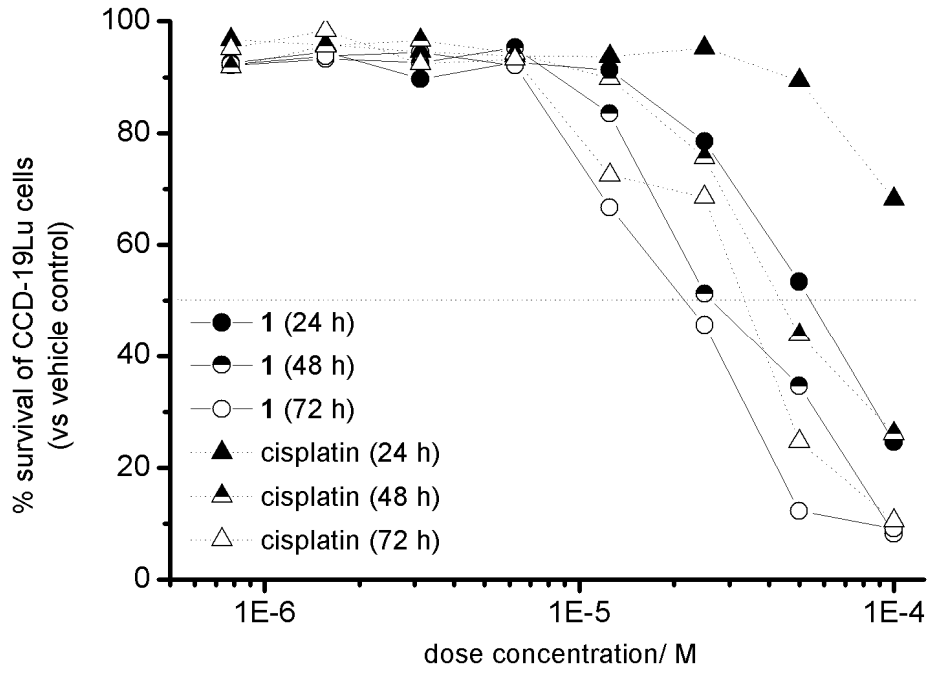

Fig. S6 Antiproliferative effects of complex **1** and cisplatin towards normal lung fibroblast CCD-19Lu.

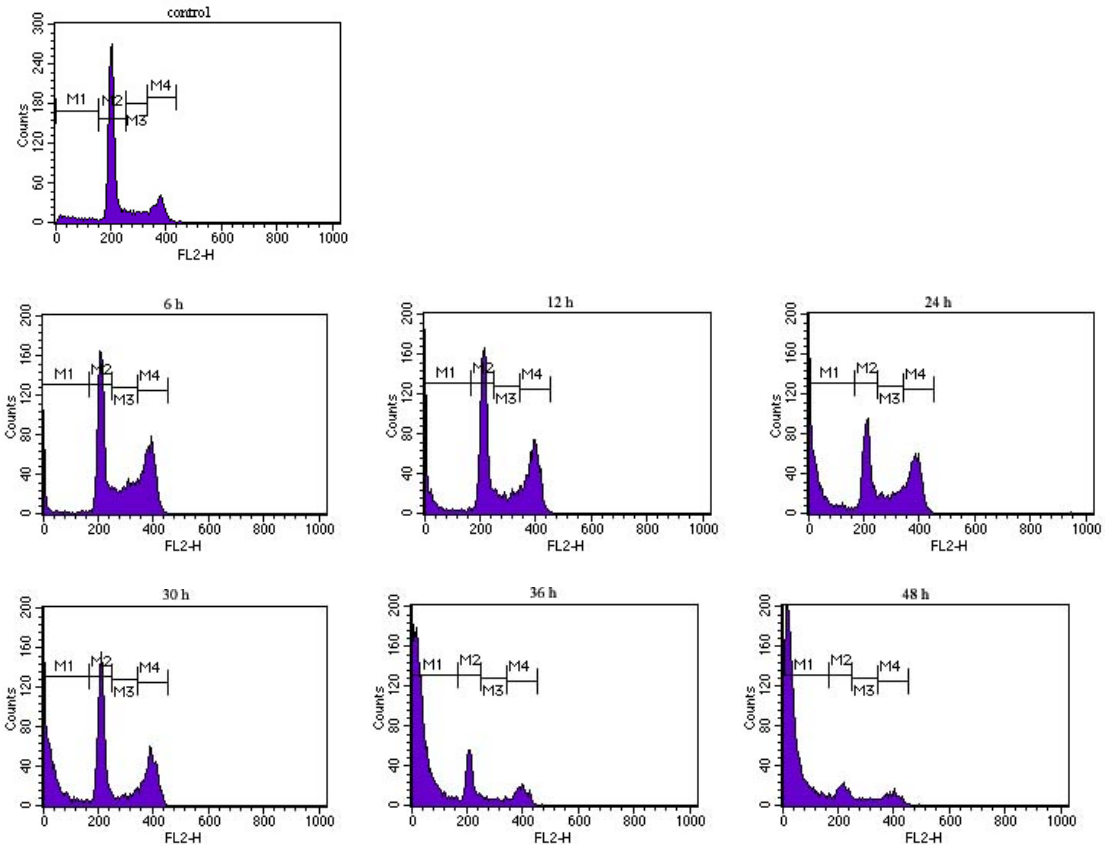

Fig. S7 6 μM compound **1** on HeLa cell cycle distribution determined by flow cytometry

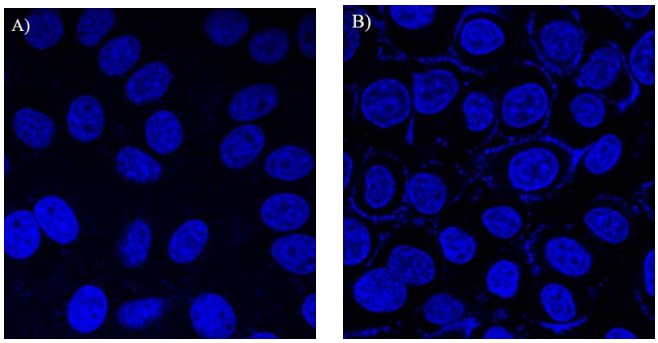

Fig. S8 A) Untreated HeLa cells stained with DAPI were viewed with laser-scanning confocal microscopy; B) HeLa cells treated with 6uM compound **1** for 12 h, then stained with DAPI were viewed. Scale bars = 50 μm.

Table S1: Tumor size measurement (Mean ± SD)

group	number of mice	dose /mg·kg^{-1}	tumor weight(g)	inhibition rate (%)
negative control	5	-	2.35±1.05	-
positive control	5	30.0	0.61±0.55 *	74.0
1 (1 mg/kg)	5	1	1.22±0.65	48.2

Compared with negative control： * P < 0.05