Supplementary Material (ESI) for Chemical Science

An anti-cancer trinuclear ruthenium(III) complex with 2-thiosalicylate ligands attenuates Wnt-β-catenin signaling

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Experimental Section

All starting materials were used as received from commercial sources. Acetonitrile (CH_3CN) was HPLC grade and distilled over CaH_2 prior to use. All other solvents were of analytical grade.

Elemental analyses were performed on a Perkin-Elmer 240 element analyzer at the Chinese Academy of Science, Beijing. Electronic spectra of **1** were recorded on Perkin-Elmer Lambda 900 diode array spectrophotometer. The mass spectra of **1** were acquired with a Q-TOF premier mass spectrometer with nanoAcquity LC system (Waters). Magnetic susceptibility data of crystalline solids of **1** (30–50 mg, sealed in Teflon tape) was obtained using Quantum Design MPMS XL-S SQUID Magnetometer in the temperature range of 2–300 K and at magnetic field strength of 1–50 kOe.

Synthesis

$[Ru^{III}_3(TSA\text{-}H)_2(TSA)_4][NEt_4]$ (**1**) was prepared as follow: A mixture of $Ru(acac)_3$ (50 mg, 0.13 mmol), H_2TSA (40 mg, 0.26 mmol), and $NEt_4(OAc).6H_2O$ (250 mg, 0.85 mmol) in acetic acid/water (1:1 v/v, 1 mL) was added into a Teflon-lined stainless steel autoclave (23 mL). The reaction mixture was kept at 150°C for 12 h. Purple needle-like crystals were formed upon cooling the solution to room temperature. These crystals were collected by filtration, washed with ice-cold acetic acid/acetone (1:1 v/v, 20 mL), then with diethyl ether, and finally air-dried. Yield: ~45%. The isolated yield of purple needle-like crystals could be increased to 70% (45 mg) by using a sealed Pyrex glass tube that was kept at 150°C fro 24 h.

UV/Vis (DMSO): λ_{max} (ε) 315 nm (31975 $dm^3mol^{-1}cm^{-1}$), 504 nm (7924 $dm^3mol^{-1}cm^{-1}$) and 638 nm (4136 $dm^3mol^{-1}cm^{-1}$); ESI-MS (m/*z*): 1218.7 $[M–H]^-$_and 609.6 $[M–2H]^{2-}$; Anal. Calcd (%) for $C_{42}H_{27}O_{12}S_6Ru_3\cdot 3H_2O$: C, 39.62; H, 2.61; Found (%): C, 39.63, H, 2.60.

X-ray crystallography. The X-ray data were collected with Mo Kα radiation (λ = 0.71073 Å) using Agilent Technologies (Oxford Diffraction) Gemini Ultra diffractometer with CCD Area Detector. The power of X-ray was rated at 50 kV and 40 mA. Structure solution and refinement were performed using SHELX-97 suite program on PC platform. The crystal structure was solved by the direct method. All non-hydrogen atoms were initially located and later refined anisotropically and the

positions of the aromatic hydrogen atoms were refined as riding models with restrained C–H distances of 0.96 Å and fixed thermal parameters (1.2 times of that of attached carbon atom). Some of carboxylic protons in **1** were found in the vicinity of carboxylate oxygen atoms with O–H distance of 0.75–0.90 Å and these protons were chemically sensible to make intermolecular hydrogen bonds with surrounding water/anions moieties (See Table S2 Supporting Information). For those missing carboxylic protons for **1**, hydrogen atoms were added based on the difference in bond length (*ca.* 0.05–0.10Å) of the two C–O bonds of the carboxylate groups. Nevertheless the positions of all carboxylic protons were not refined and were restrained as 0.82–0.86 Å with a fixed thermal parameter (1.5 times of that of attached atom).

Cellular-uptake experiment. Cellular-uptake experiment was conducted according to literature method. In general, HeLa cells (5×10^4 cells) were seeded in 60 mm tissue-culture dishes with culture medium (2 mL) and incubated at 37ºC in a humidified atmosphere of 5% CO_2/95% air for 24 h. The culture medium was removed and replaced with a medium containing the ruthenium complexes. After exposure to the ruthenium complexes for 4 h, the medium was removed and the cell monolayer was washed four times with ice-cold PBS. Milli-Q water (0.5 mL) was added and the cell monolayer was scraped off from the culture dish. Samples (0.3 mL) were digested in 70% HNO_3 (0.5 mL) at 70ºC for 2 h, and then were diluted with water (1:100) for inductively-coupled plasma-mass spectrometry (ICP-MS) analysis.

Cell Lines and Cell Culture. Human normal lung fibroblast (CCD-19Lu), cervical epithelial carcinoma (HeLa), breast carcinoma (MDA-MB-231) and hepatocellular carcinoma (HepG2) cell lines were commercially obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Human nasopharyngeal carcinoma cells (SUNE1) were generously provided by Prof. S. W. Tsao (Department of Anatomy, The University of Hong Kong). Cell-culture flasks and 96-well microtitre plates were purchased from Nalge Nunc. Culture medium, other medium constituents, and phosphate-buffered saline (PBS) were purchased from Gibco BRL.

The CCD-19Lu and HeLa cells were maintained in a minimum essential medium (MEM) with Earle's balanced salts. The HepG2 cells were maintained in a minimum essential medium (DMEM) with *D*-glucose content of 4,500 mg/L. The SUNE1 cells were maintained in RPMI 1640 medium. The MDA-MB-231 cells were maintained in ATCC-formulated Leibovitz's L-15 Medium. All the medium were supplemented with L-glutamine (2 mM) and fetal bovine serum (10%). Penicillin (100 UmL^{-1}) and streptomycin (100 μgmL^{-1}) were added to all media.

Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air, and were sub-cultured trice weekly.

Cytotoxicity Evaluation. Assays of cytotoxicity were conducted in 96-well, flat-bottomed microtitre plates. The supplemented culture medium (90 μL) with cells (1×10^5 cells per mL) was added to the wells. The ruthenium complex dissolved in the culture medium with 1% DMSO to concentrations of $0.5 - 1$ μM, and aliquots of the solutions were subsequently added to a set of wells. Cells for control experiments were treated with supplemented media with 1% DMSO (100 μL). The microtitre plates were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air for a further 3 days. All the assays were run in parallel with a negative control (i.e., vehicle control) and a positive control, in which cisplatin was used as a cytotoxic agent.

Assessment of cytotoxicity was carried out by using a modified method of the Mosmann-based 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [T. Mosmann. *J. Immunol. Methods*, 1983, **65,** 55]. At the end of each incubation period, MTT solution (10 μL, Cell Proliferation Kit I, Roche) was added into each well and the cultures were incubated further for 4 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. A solubilizing solution (100 μL) was added into wells to lyse the cells and to solubilize the formazan complex formed. The microtitre plates were maintained in a dark, humidified chamber overnight. The formation of formazan was measured by using a microtitre plate reader at 550 nm and the percentages of cell survival were determined. The cytotoxicity was evaluated based on the percentage cell survival in a dose-dependent manner relative to the negative control.

Cell Cycle Arrest. HeLa cells treated with **1** were collected, fixed with ice-cold 70% ethanol in PBS and incubated with 0.1 mL RNase I (1 mg/ mL) and 0.1 mL propidium iodide (0.4 mg/mL) at 37ºC for 30 minutes. Samples were analyzed by flow cytometry (Coulter, Luton, United Kingdom). The cell-cycle phase distribution was determined from a resultant DNA histogram using Multicycle AV software.

Absorption Titration. A solution of **1** (25 µM) in PBS-DMSO (19:1) solution was placed in a thermostatic cuvette in a UV-vis spectrophotometer and absorption spectrum was recorded. An aliquots of a millimolar stock ctDNA solution was added to the solution and the absorption spectrum was recorded.

Gel-Mobility-Shift Assay. Circular plasmid DNA was incubated with ethidium bromide or the ruthenium complex in a 1:1 ratio of DNA base pair to the complex for 30 min. The mixtures were analyzed by gel electrophoresis using a 2% (w/v) agarose

gel and tris-acetate-EDTA (TAE) buffer. The gel was immersed ethidium bromide solution after electrophoresis, and visualized using UV transillumination.

Comet Assay. The OxiSelect™ Comet Assay Kit was purchased from Cell Biolabs, Inc. HeLa cells treated with **1** at 30 μM for 3 and 24 h were collected and the assay was conducted according to the manufacturer's instruction.

BrdU Assay. The APO-BrdU™ TUNEL Assay Kit was purchased from Invitrogen. HeLa cells treated with **1** at 30 μM for 3 and 24 h were collected and the assay was conducted according to the manufacturer's instruction. HeLa cells treated with DNase I (300 U/mL in 50 mM Tris-HCl, pH7.5, 1mg/mL BSA in PBS) for 10 min at 15-25°C to induce DNA strand breaks were used as positive controls.

Microarray hybridization and data analysis. Total RNA was extracted from cultured HeLa cells treated with **1** (30 mM) or vehicle control for 24 h using RNeasy® Mini Kit (Qiagen, Valencia, CA). Five micrograms of total RNA was used for microarray hybridization. The fragmented complementary RNA was hybridized with the Human Genome using the Hybridization Oven 640 (Affymetrix). The washing and labeling procedures were performed using the Fluidics Station 400 (Affymetrix) according to the manufacturer's instructions. The arrays were then scanned using the GeneChip Scanner 3000 (Affymetrix), and the signal intensity for each transcript was determined using Microarray Suite Software 5.0.

Reverse transcriptase –polymerase chain reaction. HeLa cells were treated with **1** at 30 μM for 24 h. The cells were harvested and total mRNA was isolated according to the manufacturer's instruction. RT-PCR was carried out by incubating mRNA (10 ng), forward and reverse primers (0.1 μM) and Superscript III RT/Platinum Taq Mix (invitrogen, 1 μl) at 55 ^{o}C for 30 min, 94 ^{o}C for 5 min, followed by 29 cycles (94 ^{o}C for 30 s, 55 ^{o}C for 1 min and 68 ^{o}C for 45 s) and further 5 min at 68 ^{o}C for product extension. PCR products were separated with 1% agarose gel.

Western Blotting. HeLa cells were treated with **1** at 30 μM for 24 h. After washing, 2 × 10^6 cells were re-suspended in 100 μL of RIPA buffer (1% Trition X-100, 10% deoxycholate, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin) on ice. After centrifugation, the supernatants were harvested. The cellular protein content was quantified by the DC Protein Assay (Bio-Rad). For detection, samples (15 μg/lane) were fractionated on a 12.5% SDS-PAGE in a Tris-Glycine running buffer and blotted on polyvinylidene fluoride membranes. Staining the membrane with red Ponceau and the gel with Coomassie blue checked the loading homogeneity and transfer efficiency. The PVDF membranes

were pre-blocked overnight at room temperature in PBS containing 5% non-fat milk powder. Afterwards, the blots were incubated at room temperature for an hour with the primary antibody diluted in PBS containing 0.5% non-fat milk powder. Mouse polyclonal of anti-β-actin, anti-Wnt-5α and anti-β-catenin were used as primary antibodies at 1:500 dilutions. After washing with PBS twice, the membranes were then incubated with the respective peroxidase-conjugated secondary antibody for an hour. Detection was performed using the chemiluminescence procedure (ECL, Amersham) according to the manufacturer's recommendations.

ROS formation. HeLa cells (8×10^3 per well) were cultured in 96-well black plates for 24 h. Complex **1** was added for the final concentrations of 2.5 and 10 μM. At each of the indicated time points, cells were washed once with 1X HBSS and incubated with CM-H_2DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester, final concentration of 5 μM) in HBSS for 20 minutes. After washing with HBSS for three times, the cell-associated fluorescence was measured with the microplate analyzer with the excitation and emission wavelengths of 488 and 530 nm, respectively.

Wound-Healing Assay. HeLa cells were cultured on 60-mm culture dishes at a density of 2×10^6 cells/dish. After 24 h, a single wound was created in the middle of the cell monolayer by gently removing the attached cells with a sterile plastic pipette tip. The cells were washed twice with PBS and solutions of different concentrations of **1** (0.3 and 1 μM) in 5 mL of DMEM were added. After incubation at 37°C for 8 and 24 h, migration of the cells into the wound was observed under an inverted microscope.

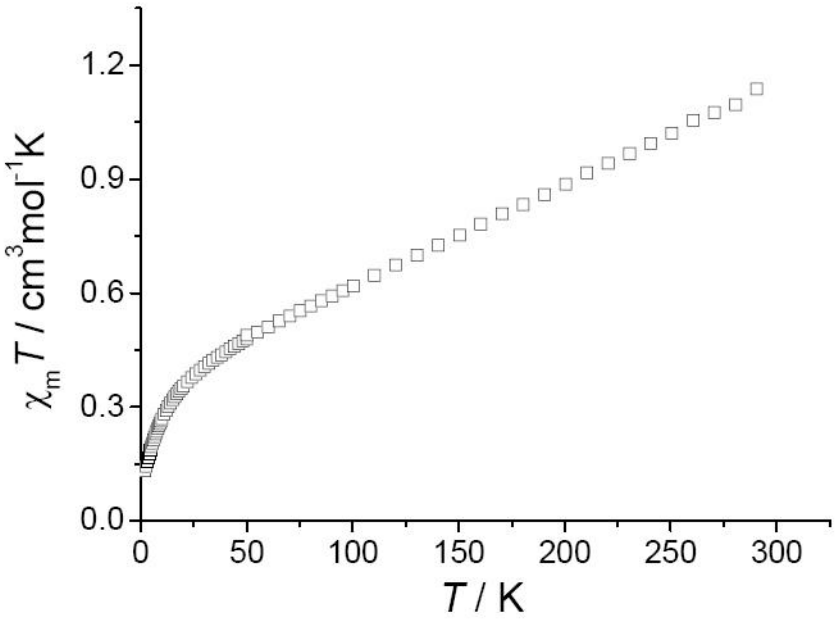

Figure S1. The $\chi_m T$ vs T plot of **1** before subtraction of its TIP contribution.

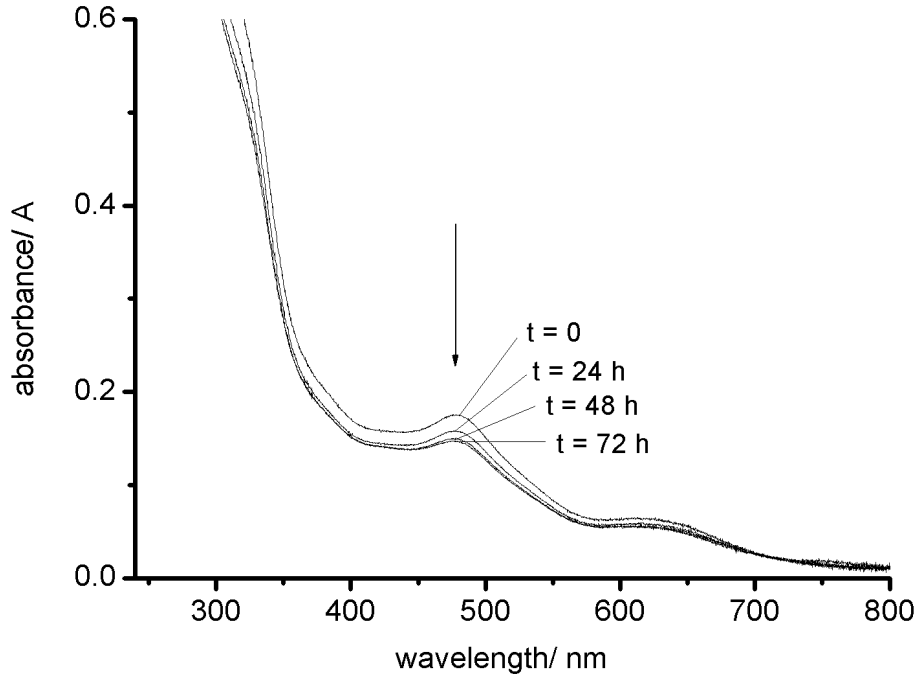

Figure S2. UV-vis absorption spectral changes of **1** in PBS (pH 7.4)-DMSO solution (99:1, v/v) in 72 h.

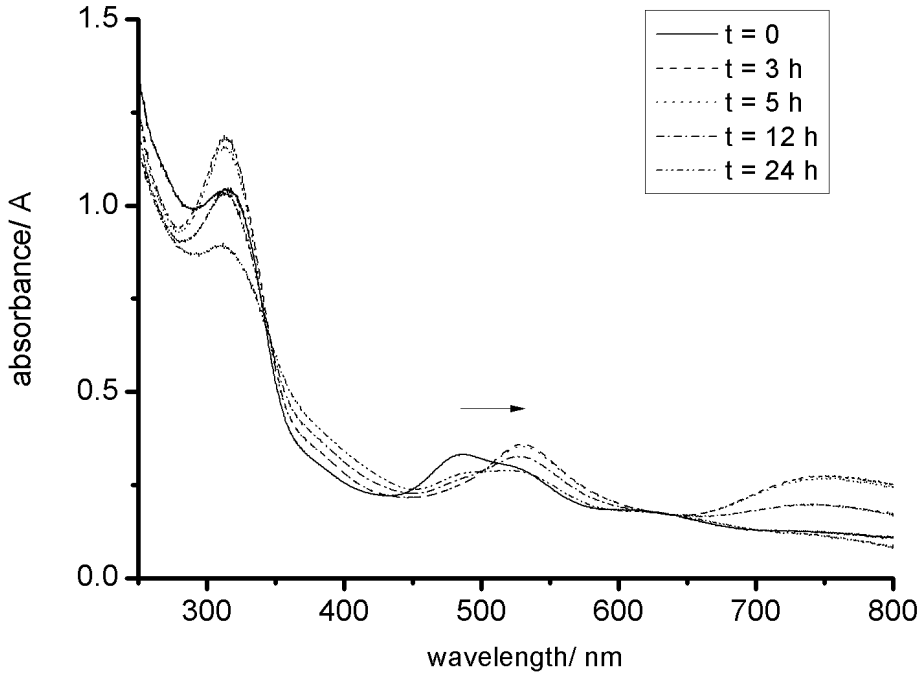

Figure S3. UV-vis absorption spectral changes of **1** in PBS (pH 7.4)-DMSO solution (99:1, v/v) in the presence of glutathione (GSH, 2 mM) in 24 h.

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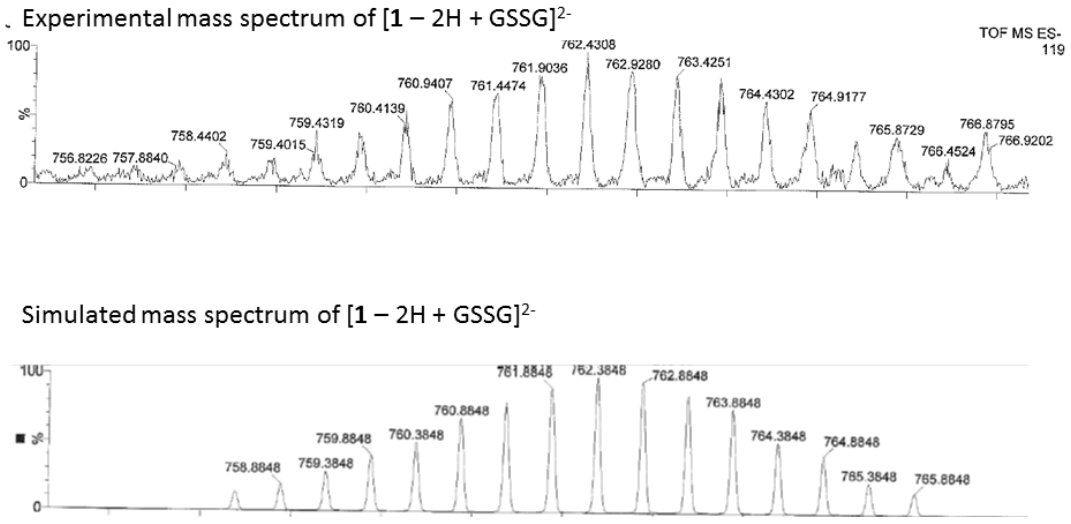

Figure S4. MALDI-TOF-MS (negative mode) of **1** incubated with glutathione (GSH, top) for 24 h. (Bottom) The simulated isotope patterns based on the [**1**-GSSG]$^{2-}$ adduct..

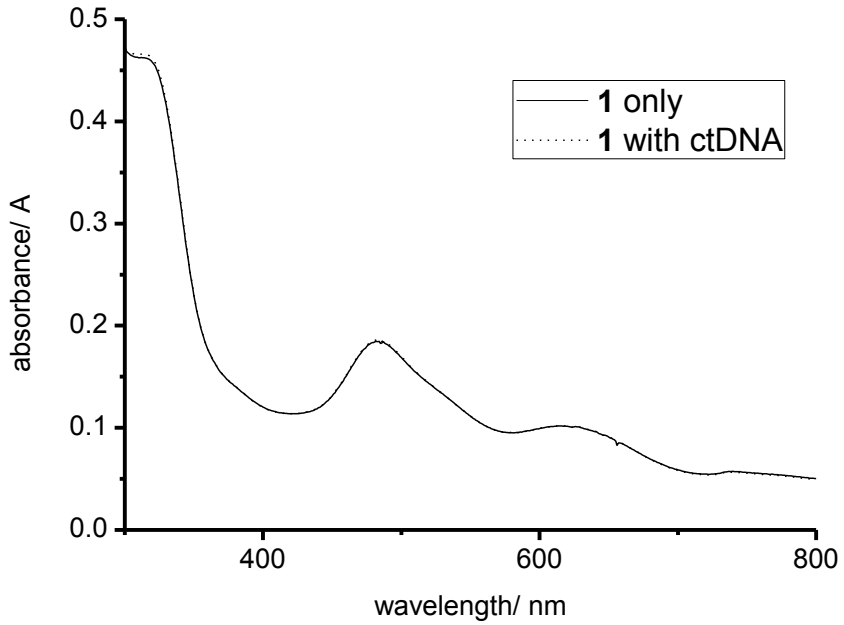

Figure S5. UV-visible spectral change of **1** (25 μM) in PBS-DMSO (19:1) with increasing [ctDNA] (ratio of [ctDNA, base pair]/[**1**] = 10) at 298 K.

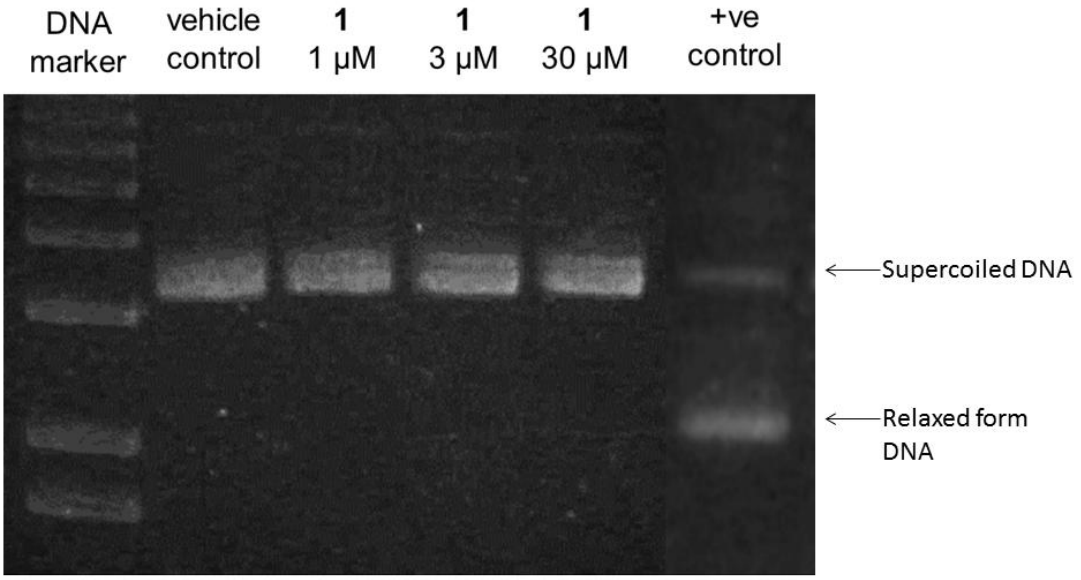

Figure S6. Gel-electrophoretic separation of the DNA mixture shows that **1** neither induced plasmid DNA relaxation nor DNA strand break of 24-h of incubation.

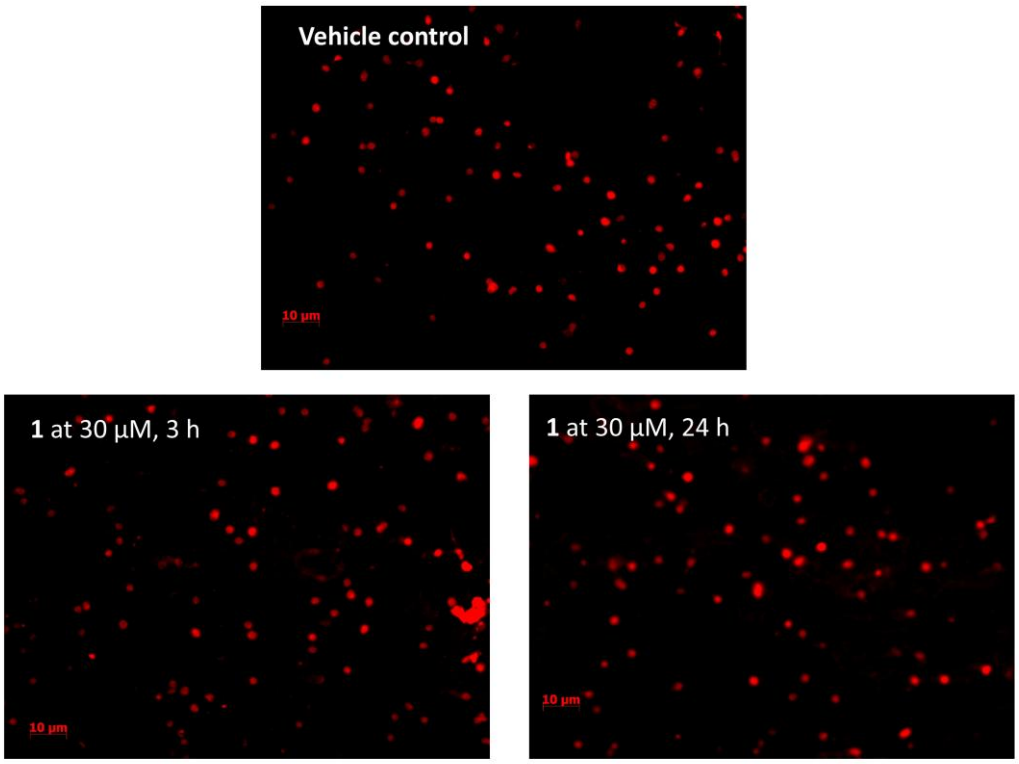

Figure S7. Comet assay revealed that treatment of **1** at 30 µM for 3 and 24 h in HeLa cells does not induce DNA strand break.

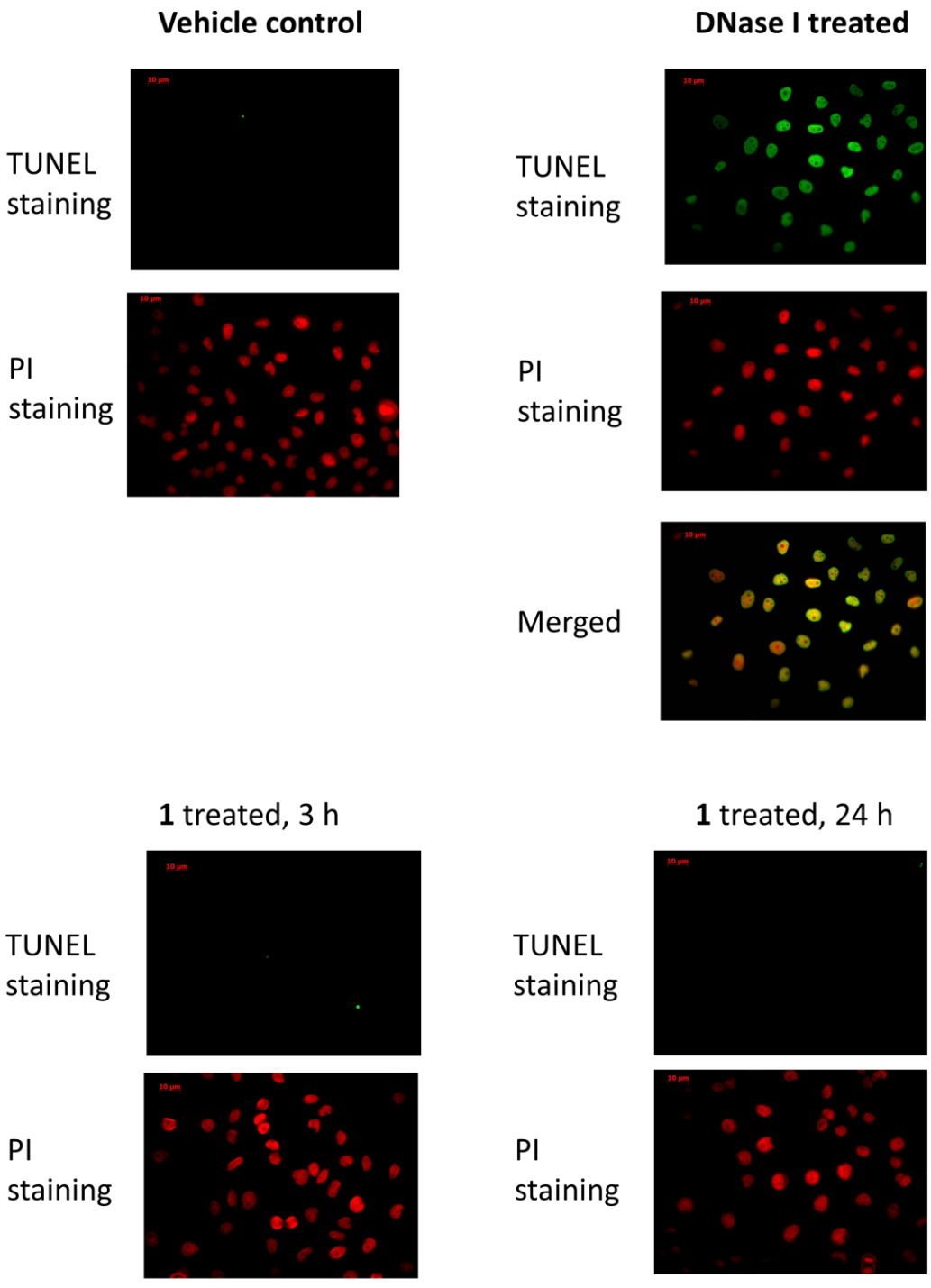

Figure S8. BrdU-TUNEL assay revealed that treatment of **1** at 30 μM for 3 and 24 h in HeLa cells does not induce DNA damage.

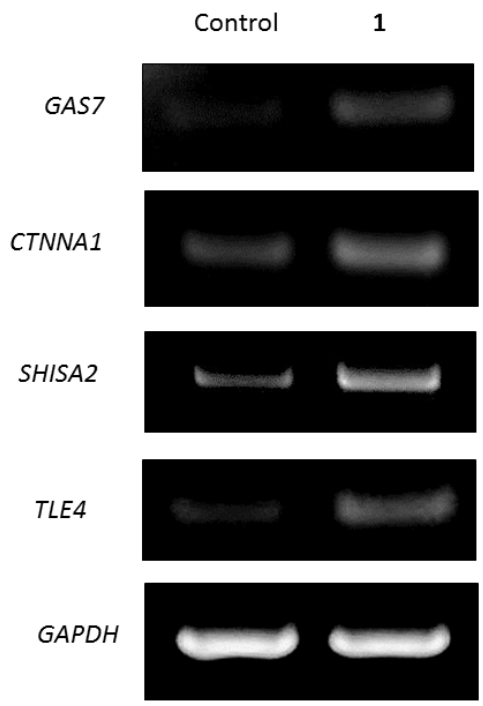

Figure S9. Expression profiles of *Gas7*, *Ctnna1*, *Shisa2* and *Tle4* in HeLa cells in response to **1** at 30 μM for 24 h by RT-PCR experiment.

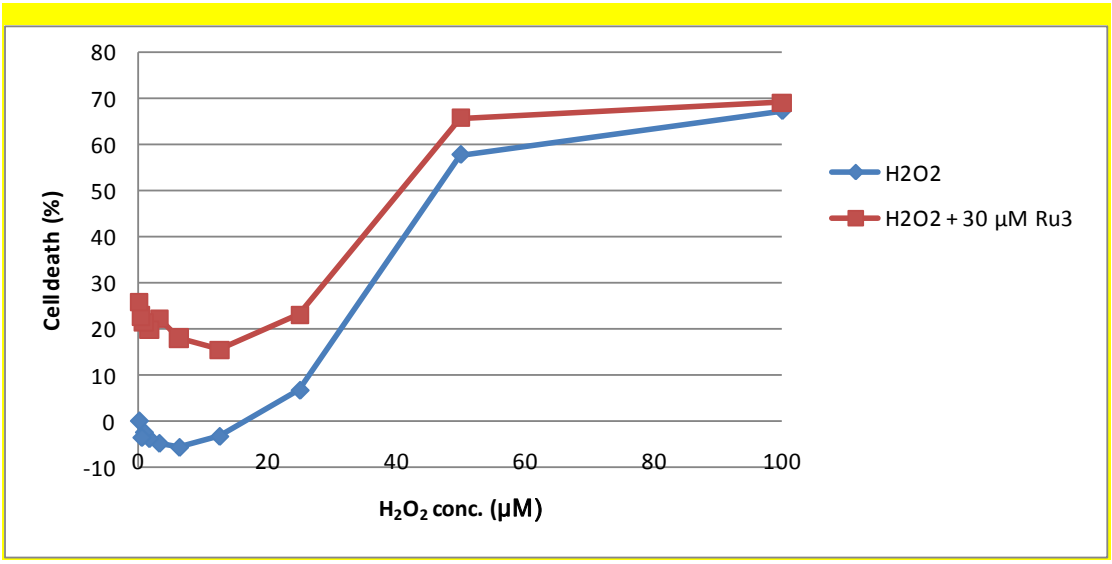

Figure S10. Synergistic effect on the cytotoxic property toward HeLa cells of **1** at 30 µM with increasing concentration of H_2O_2.

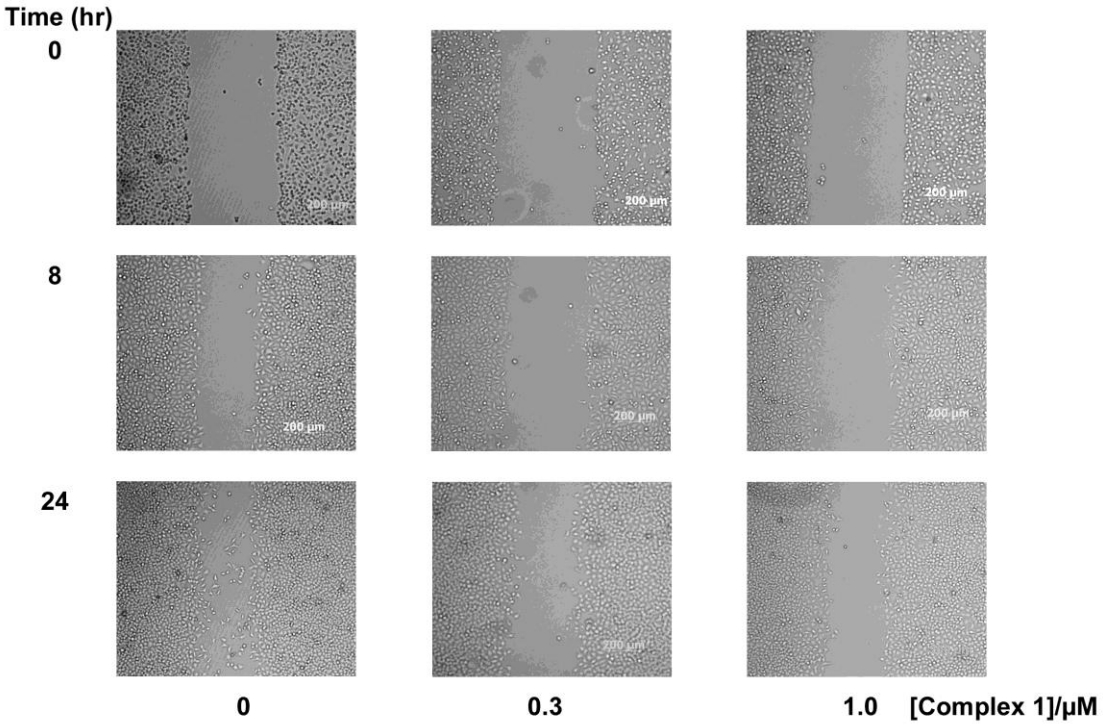

Figure S11. Wound-healing assay on HeLa cells shows that **1** inhibits cellular migration at 0.3 and 1 µM level in 8 and 24 h.

Table S1. Crystal data and structural refinement for **1**·6H_2O

	1·6H_2O
Formula	$C_{50}H_{58}NO_{18}S_6Ru_3$
Weight, g mol^{-1}	1456.60
Crystal system, space group	Triclinic, P-1
a, Å	10.577 (2)
b, Å	10.715 (2)
c, Å	13.367 (3)
α, β, γ, °	106.03(3), 90.59(4), 105.44(2)
V, $Å^3$	1397.7 (6)
μ,mm^{-1} (radiation)	1.097 (Mo)
Z, ρ_{calc}, g cm^{-3}	1, 1.730
T, K	298
F(000)	737
Reflection (R_{int})	7179 (0.021)
Data/parameters/restraints	4794/403/19
Goodness-of-fit	1.038
R1(I>2σ),[a] wR2 (all data),[b]	0.028, 0.078
Largest peak / hole, e$Å^{-3}$	1.078 / -0.682

[a] $R = \Sigma||F_o| - |F_c|| / \Sigma|F_o|$ [b] $wR = [\Sigma w(|F_o| - |F_c|)^2 / \Sigma w|F_o|^2]^{1/2}$