Electronic Supplementary Information (ESI) for Chemical Science

Anticancer Dirhodium(II,II) Carboxylates as Potent Inhibitors of Ubiquitin- Proteasome System

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Materials and Methods

Synthetic procedures and characterization of the dirhodium(II,II) carboxylates

$Rh_2(\mu_2\text{-}OOCCH_3)_4$ (RhA)

RhA was prepared according to literature procedures.[1] Rhodium trichloride trihydrate (1.00 g) and sodium acetate trihydrate (1.20 g) were dissolved in a mixture of 1:1 glacial acetic acid and absolute ethanol (40 mL). The resulting mixture was refluxed under argon for one hour. After cooling to room temperature, the green solid was collected and re-dissolved in boiling methanol and filtered. The solution was kept in a refrigerator overnight. The recrystallized product was collected and dried *in vacuo*. Yield: 0.53 g (63.2 %). MS-FAB: 441.8 $[M]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.78 (s, 4H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 191.1, 23.6.

$Rh_2(\mu_2\text{-}OOCC_3H_7)_4$ (RhB)

RhB was synthesized according to literature procedures.[2] **RhA** (1.00 g) was refluxed in a solution of butyric acid (25 mL) and butyric anhydride (5 mL) for 3 hours under argon atmosphere. The solvent was removed under vacuum. The solid product was extracted with hot toluene. Green **RhB** was crystallized out of the cooled solution, filtered and heated under vacuum at about 100 °C to remove excess butyric acid. Yield: 0.44 g (80.0 %). Anal. Calcd. for **RhB**: C, 34.68; H, 5.09. Found: C, 34.89; H, 5.08. MS-FAB: 554.0 $[M]^+$. ^{1}H NMR (400 MHz, CD_3OD): δ 2.04 (t, J = 7.1 Hz, 8H), 1.44 (qt, J_1 = 7.1 Hz, J_2 = 7.4 Hz, 8H), 0.73 (t, J = 7.4 Hz, 12H). ^{13}C NMR (100 MHz, CD_3OD): δ 194.3, 39.6, 20.3, 13.7. X-ray power diffraction (XRD) patterns were recorded on a Bruker D8 advance powder X-ray diffractometer using CuKα radiation (λ=1.54146 Å). The identity of **RhB** was confirmed by comparison with literature results.[3] We thank Dr. Sin-Yin Stephen Chui of the Department of Chemistry of the University of Hong Kong to carry out X-ray powder diffraction analysis.

Tetrakis[μ-(4-oxo-4-phenylbutanoato-O1:O1')]dirhodium(II,II) $Rh_2(\mu_2\text{-}OOCC_2H_4COPh)_4$ (RhPCOPh)

The dirhodium(II,II) carboxylate compounds were synthesized by ligand exchange method. $Rh_2(OAc)_4$ (0.144 g, 0.284 mmol) and 3-benzoylpropionic acid (0.400 g, 2.23 mmol) was dissolved and refluxed in chlorobenzene (30 mL) for 7 hours. Distillation was set up throughout the reaction to distill out acetic acid with chlorobenzene. Light green precipitate was collected, washed by hot boiling chlorobenzene followed by diethyl ether and dried *in vacuo*. Yield: 0.25 g (96.0 %). The light green solid product could be further purified by recrystallization in CH_3CN. Anal. Calcd. for $Rh_2(\mu_2\text{-}OOCC_2H_4COPh)_4$: C, 52.53; H, 3.97. Found: C, 52.31; H, 3.96. MS-FAB: 915.2 $[M\text{-}H]^+$. ^{1}H NMR (400 MHz, CD_3CN): δ 7.90 (d, J = 7.1 Hz, 8H), 7.59 (tt, J_1 = 7.4 Hz, J_2= 1.2 Hz, 4H), 7.45 (d, J = 7.7 Hz, 8H), 3.12 (t, J = 6.7 Hz, 8H), 2.45 (t, J = 6.7 Hz, 8H). ^{13}C NMR (100 MHz, CD_3CN): δ 199.7, 193.2, 137.9, 133.9, 129.6, 128.8, 35.0, 31.8.

Tetrakis[μ-(3-methylbutanoato-O:O')]dirhodium(II,II) $Rh_2(\mu_2\text{-}OOCCH_2CH(CH_3)_2)_4$ (RhIsoVal)

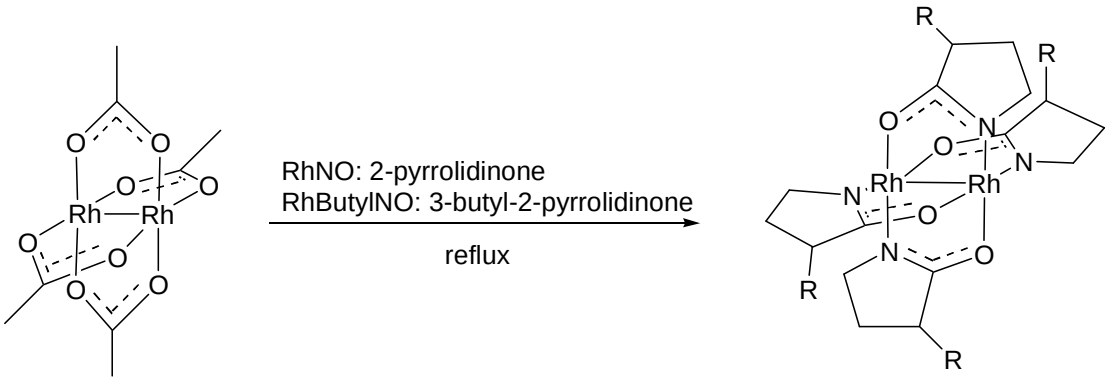
RhIsoVal was prepared from the same synthetic method as **RhPCOPh** using $Rh_2(OAc)_4$ (0.210 g, 0.415 mmol) and isovaleric acid (0.4 mL, 3.62 mmol). The resulting green

precipitate was washed by hexane and dried *in vacuo*. Yield: 0.25 g (99.3 %). The light green solid product could be further purified by recrystallization in diethyl ether. Anal. Calcd. for $Rh_2(\mu_2\text{-}OOCCH_2CH(CH_3)_2)_4$: C, 39.36; H, 5.95. Found: C, 39.67; H, 5.95. FAB-MS: 610.1 $[M]^+$. ^{1}H NMR (400 MHz, CD_3OD): δ 1.84-1.91 (m, 12H), 0.75 (d, J = 6.4 Hz, 24H). ^{13}C NMR (125 MHz, CD_3OD): δ 194.0, 47.2, 27.5, 22.7.

Tetrakis[μ-(isobutyrato-O:O')]dirhodium(II,II)
$Rh_2(\mu_2\text{-}OOCCH(CH_3)_2)_4$ (RhIsoButyl)
RhIsoButyl was prepared from the same synthetic method as **RhPCOPh** using $Rh_2(OAc)_4$ (0.190 g, 0.384 mmol) and isobutyric acid (0.5 mL, 5.39 mmol). The resulting green precipitate was washed by hexane and dried *in vacuo*. Yield: 0.21 g (98.7 %). The product was further purified by slightly dissolving in diethyl ether and MeOH was added dropwise to dissolve the green solid completely in the recrystallization process. Anal. Calcd. for $Rh_2(\mu_2\text{-}OOCCH(CH_3)_2)_4$: C, 34.68; H, 5.09. Found: C, 34.52; H, 4.99. FAB-MS: 554.0 $[M]^+$. ^{1}H NMR (400 MHz, CD_3OD): δ 2.21-2.33 (m, 4H), 0.90 (d, J = 5.6 Hz, 24H). ^{13}C NMR (125 MHz, CD_3OD): δ 198.2, 37.7, 20.2.

Tetrakis[μ-(5-oxo-5-phenylpentanoato-O1:O1')]dirhodium(II,II)
$Rh_2(\mu_2\text{-}OOCC_3H_6COPh)_4$ (RhBCOPh)
RhBCOPh was prepared from the same synthetic method as **RhPCOPh** using $Rh_2(OAc)_4$ (0.200 g, 0.395 mmol) and 4-benzyolbutyric acid (0.600 g, 3.13 mmol). The green solid product was recrystallized in CH_3CN and dried *in vacuo*. Yield: 0.32 g (83.5 %). Anal. Calcd. for $Rh_2(\mu_2\text{-}OOCC_3H_6COPh)_4 \cdot 0.5CH_3CN$: C, 54.45; H, 4.57. Found: C, 54.53; H, 4.48. FAB-MS: 970.2 $[M]^+$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.82 (d, J = 7.6 Hz, 8H), 7.57 (t, J = 7.3 Hz, 4H), 7.46 (d, J = 7.6 Hz, 8H), 2.74 (t, J = 7.1 Hz, 8H), 2.11 (t, J = 7.1 Hz, 8H), 1.60 (tt, J_1 = 7.1 Hz, J_2 = 7.1 Hz, 8H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 199.3, 193.6, 136.5, 133.2, 128.7, 127.7, 36.6, 35.9, 20.0.

Synthetic procedures and characterization of dirhodium(II,II) carboxamidates



RhA

RhNO: R=H
RhButylNO: R=*n*-butyl

Tetrakis[μ-(2-pyrrolidinonato-N1:O2)]dirhodium(II,II) (RhNO)

RhNO was prepared according to literature procedures.[4] **RhA** (0.100 g, 0.2 mmol) and 2-Pyrrolindinone (2.30 g, 27 mmol) was mixed in chlorobenzene (200 mL) in a round-bottomed flask under argon. The flask was equipped with a Soxhlet extractor with a thimble containing a mixture of $MgSO_4$/$NaHCO_3$/sand (2:2:3, 7 g). The solution was reflux under argon with stirring overnight. Chlorobenzene was evaporated under reduced pressure and the purple residue was washed by diethyl ether and pentane to obtain purple solid. The resulting purple solid was recrystallized from an acetonitrile/methanol mixture at room temperature and dried *in vacuo*. Yield: 0.11 g (77.6 %). Anal. Calcd. for $Rh_2C_{16}H_{24}N_4O_4{\cdot}H_2O$: C, 34.30; H, 4.68; N, 10.00. Found: C, 34.11; H, 4.87; N, 9.79. FAB-MS: 542.1 $[M]^+$. ^{1}H NMR (400 MHz, CD_3CN + CD_3OD (10:1)): δ 3.44 (br, 8H), 2.13 (t, J = 8.1 Hz, 8H), 1.78-1.88 (m, 8H). ^{13}C NMR (125 MHz, CD_3CN + CD_3OD): δ 185.4, 54.4, 32.8, 21.8. Single crystal of **RhNO** was obtained by slow diffusion of diethyl ether to an acetonitrile solution. Detailed results are given in Table S1 and S2.

1-(Trimethylsilyl)-2-pyrrolidinone

1-(Trimethylsilyl)-2-pyrrolidinone was prepared according to literature procedures.[5] 2-Pyrrolidinone (7.84 g, 9 mL, 92.1 mmol) and triethylamine (14.52 g, 20 mL, 143.5 mmol) were dissolved in toluene (80 mL) under N_2 at room temperature. Trimethylsilyl chloride (14.12 g, 16.5 mL, 130 mmol) was added to the reaction mixture. The resulting solution was stirred overnight at 40 ^{o}C. The product together with toluene was distilled out at controlled pressure from the reaction flask. Distilled hexane was added to the product mixture and the resulting solution was filtered under N_2. Hexane was distilled away under N_2 to obtain 1-(trimethylsilyl)-2-pyrrolidinone. Yield: 2.51 g (17.3 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.19-3.32 (m, 2H), 2.12-2.26 (m, 2H), 1.98-2.07 (m, 2H), 0.15 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 182.8, 46.0, 32.2, 21.1, -1.41.

3-Butyl-2-pyrrolidinone

3-Butyl-2-pyrrolidinone was synthesized according to literature procedures.[6] Lithium diisopropylamide (LDA) solution was prepared by adding 7.6 mL of 2.5 M *n*-BuLi (1.2 equiv, 19.1 mmol) in hexane to a pre-cooled (–78 ^{o}C) solution of *i*-Pr_2NH (2.67 mL, 1.2 equiv, 19.08 mmol) in THF (25 mL). After 15 mins, the LDA solution was warmed and stirred at room

temperature. The reaction mixture was cooled again to –78 ^{o}C for another 15 mins. Solution of 1-(Trimethylsilyl)-2-pyrrolidinone (2.50 g, 15.9 mmol) in THF (5 mL) was pre-cooled and added to the LDA solution dropwise via a cannula. After 15 mins, 1-iodobutane (2 mL, 17.49 mmol, 1.1 equiv) was added dropwise. The reaction mixture was stirred overnight at room temperature. The reaction was quenched by adding 2N HCl (40 mL). The aqueous layer was extracted with CH_2Cl_2 (2 x 70mL). The organic fractions were collected, combined and washed with water and brine. The resulting solution was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The product was purified by chromatography (SiO_2, EA: hexane = 8:1) to afford 0.81 g (36.1 %) of white solid (R_f: 0.46, EA: hexane = 8:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.70 (NH, 1H), 3.21-3.38 (m, 2H), 2.27-2.34 (m, 2H), 1.76-1.87 (m, 2H), 1.22-1.42 (m, 5H), 0.91 (t, *J* = 4.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 180.5, 40.5, 39.9, 29.8, 28.7, 26.7, 21.9, 13.2.

Tetrakis[μ-(3-Butyl-2-pyrrolidinonato-N1:O2)]dirhodium(II,II) (RhButylNO)

RhButylNO was prepared from the same synthetic method as **RhNO**.[4] **RhA** (0.100 g, 0.2 mmol) and 3-Butyl-2-pyrrolidinone (0.500 g, 3.54 mmol) was mixed in chlorobenzene (200 mL) in a round-bottomed flask under argon. The flask was equipped with a Soxhlet extractor with a thimble containing a mixture of $MgSO_4$/$NaHCO_3$/sand (2:2:3, 7 g). The solution was reflux under argon with stirring for 3 days. The excess ligand was removed by sublimation by heating the product mixture at 100 ^{o}C. The purple red product was recrystallized from a CH_2Cl_2/hexane mixture at room temperature and was obtained as a mixture of diastereomers. Yield: 0.051 g (28.7 %). Anal. Calcd. for $Rh_2C_{32}H_{56}N_4O_4{\cdot}CH_2Cl_2{\cdot}H_2O$: C, 45.58; H, 6.95; N, 6.44. Found: C, 45.45; H, 6.92; N, 6.18. FAB-MS: 766.3 $[M]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.16 (br, 8H), 2.49 (br, 4H), 2.07 (br, 8H), 1.56 (br, 8H), 1.24 (br, 16H), 0.86 (br, 12H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 187.0, 51.3, 43.3, 29.3, 26.9-28.7 (2C), 22.8, 14.1. Single crystal of **RhButylNO** was obtained by slow diffusion of hexane to CH_2Cl_2. Detailed results are given in Table S1 and S2.

Chemicals and antibodies.

Stock solutions of dirhodium(II,II) carboxylates and dirhodium(II,II) carboxamidates were made up with ethanol (**RhA**, **RhB**, **RhIsoVal**, **RhIsoButyl** and **RhButylNO**), acetonitrile (**RhPCOPh**), acetonitrile/methanol (10:1 mixture) (**RhNO**) and dimethyl sulfoxide (**RhBCOPh**) to concentrations of 5-10 mM. MG132, purified human erythrocyte 20S proteasome, UCH-L5, N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (Suc-LLVY-AMC), ubiquitin-AMC and anti-ubiquitin conjugates antibody (clone FK2) were from Enzo Life Sciences. Antibodies against caspase-3, casapse-7, caspase-8, caspase-9, α-tubulin, glyceraldehyde-3-phosphate dehydrogenase and γ-H2AX were from Cell Signaling. Proteasome sensor vector (GFP-ODC) was from Clontech. Ub^{G76V}YFP and CD3δ-YFP were from Dr. N. P. Dantuma through Addgene.

Cell culture.

The human cervix epithelial carcinoma cells (HeLa), human hepatocellular carcinoma cells (HepG2), human lung epithelioid carcinoma cells (NCI-H460), human nasopharyngeal carcinoma cells (SUNE1) and human normal lung fibroblast cells (CCD-19Lu) were maintained in minimal essential medium supplemented with 10% fetal bovine serum. HeLa cells stably expressing the UPS reporters were generated according to the published procedures.[7,8] The reporters used were yellow fluorescent protein (YFP)/green fluorescence protein (GFP)-based fusion proteins of ubiquitin-independent proteasome substrate ornithine decarboxylase (GFP-ODC), ubiquitin fusion degradation substrate (Ub^{G76V}-YFP).

Cytotoxicity assay

The cytotoxicities of dirhodium(II,II) carboxylates and dirhodium(II,II) carboxamidates were evaluated by standard dimethyl thiazolyl diphenyl tetrazolium (MTT) assays. The IC_{50} values were determined as the concentration at which 50% of cell survival was inhibited.

Flow cytometry analysis

Evaluation of apoptosis was performed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences PharMingen, San Jose, CA) according to the supplier's instructions. Briefly, drug-treated cells grown in 6-well plate were harvested with trypsin and washed in PBS. Cells were then resuspended in binding buffer [10 mmol/L HEPES/NaOH (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$]. Next, cell suspension was stained with Annexin V-FITC and propidium iodide (PI) provided with the kit at room temperature for at least 15 minutes in the dark. Cells were then analyzed in a FACSDiva flow cytometer (BD Biosciences PharMingen, San Jose, CA) within 1 hour after staining. Control cells stained with annexin V-FITC or PI alone was used to compensate for the flow cytometric analysis. Annexin V and PI double-negative cells are defined as live cells; annexin V-positive, PI-negative cells are defined as early apoptotic cells; and annexin V and PI double-positive cells are defined as late apoptotic and necrotic cells.

Evaluation of apoptosis was performed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) according to the supplier's instructions. Stained cells were analyzed with FACSDiva flow cytometer (BD Biosciences). Annexin V and propidium iodide (PI) double-negative cells are defined as live cells; annexin V-positive, PI-negative cells as early apoptotic cells; and annexin V and PI double-positive cells as late apoptotic and necrotic cells.

Microarray and Connectivity Map analysis.
The total RNA was isolated by Trizol reagent (Invitrogen) followed by clean-up with RNeasy kit (Qiagen). Human genome-wide gene expression was examined with the Affymetrix Human Genome U133 Plus 2.0 GeneChip, composed of more than 54,000 probe sets that analyzes over 47,000 human transcripts and variants. The quality of total RNA was checked by the Agilent 2100 bioanalyzer. The RNA was amplified and labeled with MessageAmp II-Biotin Enhanced Single Round aRNA Amplification kit (Ambion Inc., Texas). The GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix Inc.) The hybridization data was analyzed with RMA (Robust Multichip Average) algorithm using the Affymetrix Expression Console software. Regulated genes[9] with 2-fold or more differences in expression between groups were listed in Table S4. Upon publication, complete microarray data for each sample will be made available through National Cancer for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). Profiles for up-regulated and down-regulated genes were analyzed with the Connectivity Map resources. [10]

RT-PCR analysis.
RT-PCR was performed with 30 ng of RNA using SuperScript III one-step RT-PCR system (Invitrogen). The cDNA was synthesized at 55 °C for 30 min. The PCR profile was 94 °C denatured for 1 min, 1 min annealing at 55 °C, and 68 °C extension for 1.5 min for 28 cycles (PTC-100 Peltier Thermal Cycler, MJ Research). Primer sequences are stated in Supplementary Data (Table S5).

Western blotting analysis.
Cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate) supplemented with protease inhibitor. Equal amount of proteins (30 μg) was resolved by SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 and 3% BSA, and then incubated with the primary antibodies at 4 ^{o}C overnight, followed with appropriate horseradish peroxidase conjugated secondary antibodies for 2 h. The immunoreactivities were detected using enhanced chemiluminescence detection kit (GE Healthcare).

UPS reporter assays.
HeLa cells stably expressing the UPS reporters were treated with compounds for 24 h. Cells were lysed with buffer containing 0.5% Triton X-100, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA. The levels of fluorescence proteins in the cell lysates were measured with a fluorescence plate reader, and normalized against total protein concentrations determined by BioRad protein assays.

Immunofluorescence staining.

HeLa cells pretreated with the compounds were fixed with 3% formaldehyde, permeabilized with 0.2% Triton X-100 and blocked with 1% BSA in PBS. The fixed cells were incubated with primary antibodies followed by AlexaFluor 488 secondary antibodies and then examined by fluorescence microscopy.

Proteasome and deubiquitinating enzymes activity assays.

Purified 20S proteasome was used at 1 μg /mL in the presence of 0.03% SDS to activate the proteolysis. Purified UCH-L5 was used at 50 nM. The enzyme was preincubated with the test compounds for 10 min. The chymotrypsin-like activity of proteasome was measured with fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (SUC-LLVY-AMC, 50 μM). The deubiquitinating enzyme activity was measured with ubiquitin-C-terminal 7-amido-4-methylcoumarin (Ub-AMC, 500 nM). The initial rates of substrate hydrolysis were measured with a fluorescence plate reader using excitation and emission wavelengths of 360 nm and 480 nm, respectively.

Alkaline comet assays.

The alkaline comet assay was performed according to published procedures with some modifications.[11] Cells were trypsinized and suspended at 1x 10^6/ mL in 0.5% molten low melting point agarose. A 75 μL cells–agarose mixture was layered onto microscope slides, which were pre-coated with 1% normal agarose. After solidification at room temperature for 30 min, the embedded cells were lysed at 4 °C for 1 h in buffer containing 2.5 M NaCl, 10 mM Tris, 100 mM EDTA, and 1% Triton X-100, pH 10. The slides were incubated in an alkaline electrophoresis solution (1 mM EDTA, 300mM NaOH, pH >13) at 4°C for 15 min; followed by electrophoresis (2V/cm) for 30 min. The slides were washed with neutralization buffer (0.4 M Tris, pH 8), air dried at room temperature and then stained with ethidium bromide. The comet moment was examined with a fluorescence microscope.

NCI-60 cytotoxicity profiling.

The NCI screening procedures were described as stated previously.[12] GI_{50} measures the growth inhibitory activity of the test agent. The NCI renamed the IC_{50} value to GI_{50} value, the concentration that causes 50% growth inhibition, to emphasize the correction for the cell count at time zero. The GI_{50} value is the concentration of the test drug :

$GI_{50} = 100 \times (T-T_0)/(C-T_0) = 50$

Where T = optical density of the test well after a 48-h period of exposure to test drug

T_0 = the optical density at time zero;

C = the control optical density.

Mean Graphs are constructed with bars depicting the deviation of individual tumor cell lines from the overall mean value of all the cells tested.

Metal uptake and the cellular distribution

Isolation of Nuclear fraction

HeLa cells were grown in 10 cm culture dishes and treated with the dirhodium(II,II) compounds for 2h. The cells were washed twice by PBS and trypsinized. Cell pellets were collected and lysed with 0.5 mL lysis buffer containing 10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 % Triton X-100. The total cellular proteins in the cell lysates were determined by Bio-Rad protein assays. The nuclear pellets (N) were collected by centrifugation at 1,000 g for 10 min following by washing once with 1 mL of lysis buffer and resuspended in 0.5 mL lysis buffer. The nuclear pellets and postnuclear supernatant (S, as cytoplasmic fraction) were digested by addition of 0.5 mL concentrated nitric acid and incubation at 70^{o}C overnight. The metal contents of the digests were determined by inductively coupled plasma mass spectrometry (ICP-MS).

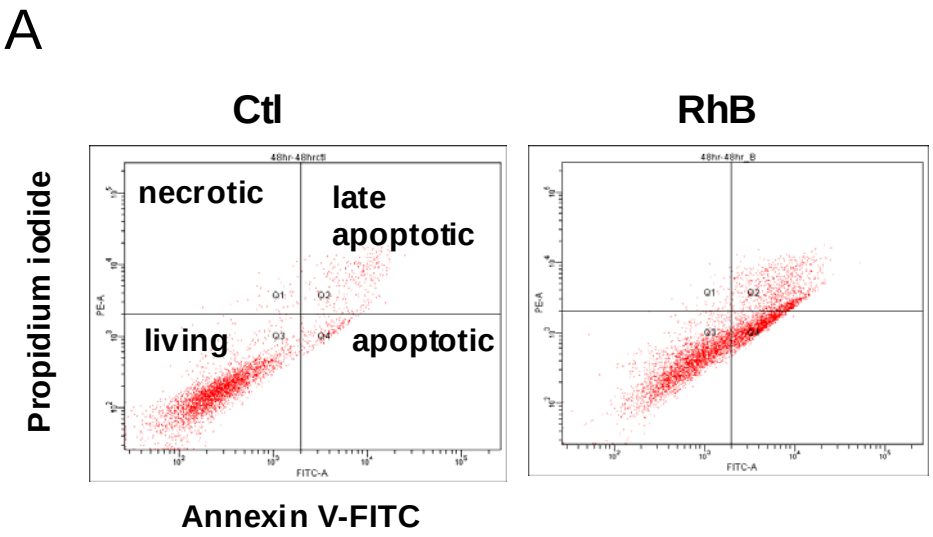
DNA isolation

Isolation of DNA was performed according published procedures with some modifications[13, 14]. HeLa cells were grown in 10 cm culture dishes and treated with the Rh compounds for 2 h. The treated cells were washed twice by PBS, trypsinized and digested in 0.3 mL digestion buffer containing 10 mM Tris, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5 % SDS, 0.1 mg/mL proteinase K at 50^{o}C for 18 h. The digested lysates were extracted twice with 1 vol of phenol/chloroform mixture (1:1) and aqueous layer (top) was collected. 1 μg/mL DNase-free RNase were added to the collected fraction and incubated at 37^{o}C for 1 h. 0.5 vol of 7.5 M ammonium acetate and 2 vol of absolute ethanol were added for DNA precipitation. Stringy DNA precipitates appeared after incubation at -80^{o}C for 30 min and was collected by centrifugation at 10,000 g for 5 min. The pellet was washed twice with 70 % ethanol, air-dried and re-dissolved in 50 μl TE buffer. The DNA concentrations were determined as absorbance at 260 nm. The DNA was digested by addition of 100 μl of concentrated nitric acid and incubation at 70^{o}C overnight. The metal contents of the digests were determined by ICP-MS.

Statistical analysis

Data were expressed as the mean ± SE of three determinants. Statistical analysis was done by Student's t test. $P \leq 0.05$ was considered statistically significant. Pearson's correlation coefficient is defined as the covariance of the two variables divided by the product of their standard deviations. The Pearson's correlation coefficients range from -1 to +1. A value of +1 implies the relationship between two samples correlates perfectly and one sample increases as the other sample increases. A value of -1 implies that one sample decreases as the other sample increases. A value of 0 implies that there is no correlation between the samples.

Fig. S1. Cytotoxicity of dirhodium(II,II) carboxylates. *A*. Induction of apoptosis by **RhB**. Cells were treated with 0.5 μM **RhB** for 48 h and then subjected to annexin V-FITC and propidium iodide staining and flow cytometry analysis. *B*. Activation of caspases by **RhB**. HeLa cells were treated with 0.5 μM **RhB** for the indicated period of times, and the expression of caspases was determined by immunoblot analysis.



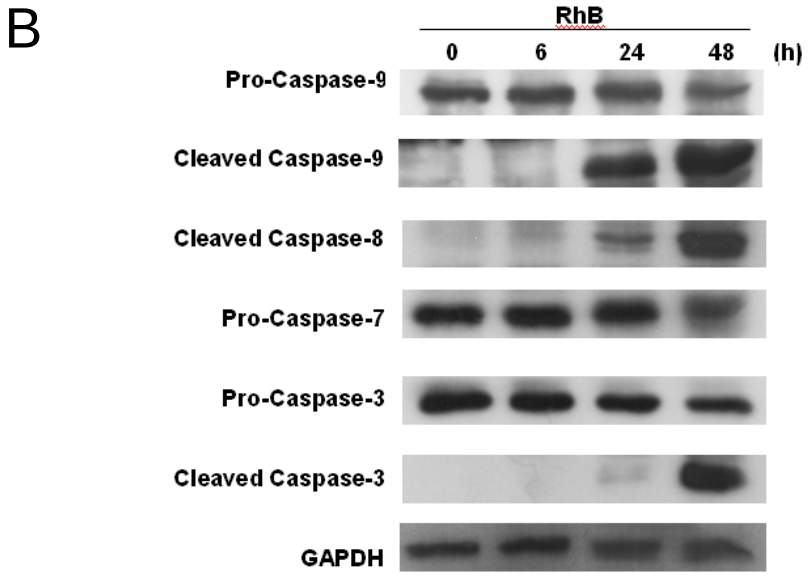


Fig. S2. Cluster analysis of the microarray data using average linkage and euclidean distance. Triplicate runs for each sample.

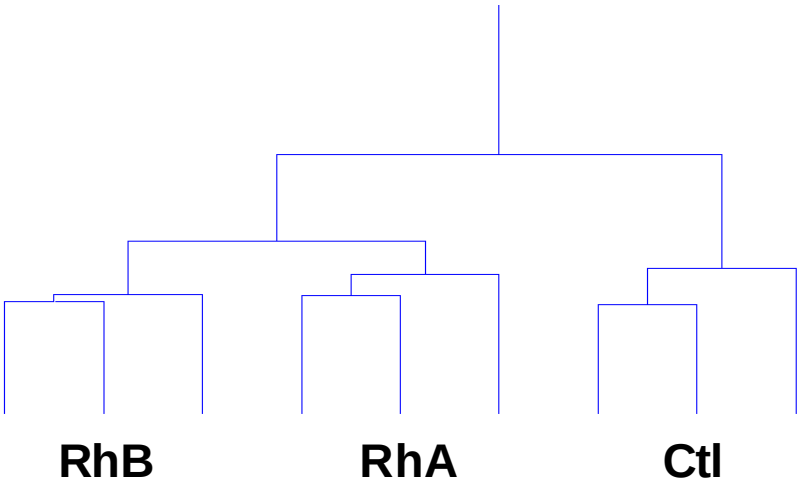


Fig. S3. Semiquantitative RT-PCR analysis of *gapdh* (internal control), *ho-1*, *chop*, *stc2*, *asns*, *atf3*, *xbp1*, *egr1* and *dkk1* expression in untreated control, **RhB**-treated and **RhA**-treated HeLa cells. Representative agarose gel electrophoresis images of three independent experiments are shown.

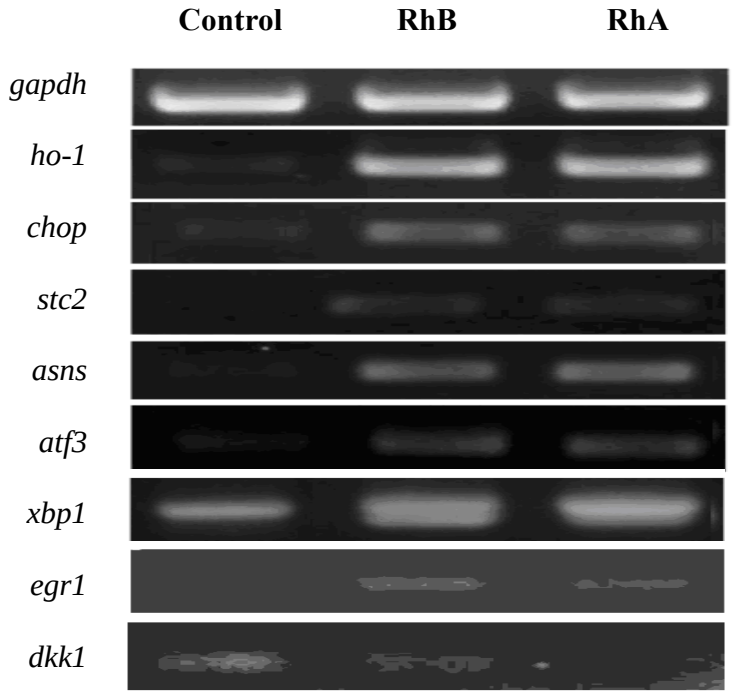


Fig. S4. γ-H2AX foci assay. (A) HeLa cells were treated with indicated concentration of **RhA**, **RhB**, **RhIsoButyl**, **RhPCOPh**, **RhButylNO** and cisplatin for 24 h. Magnification: 40x, oil. (B) γ-H2AX foci of cisplatin-, carboplatin-, oxaliplatin-treated cells compared with control and **RhB**-treated cells. Magnification: 100x, oil.

A

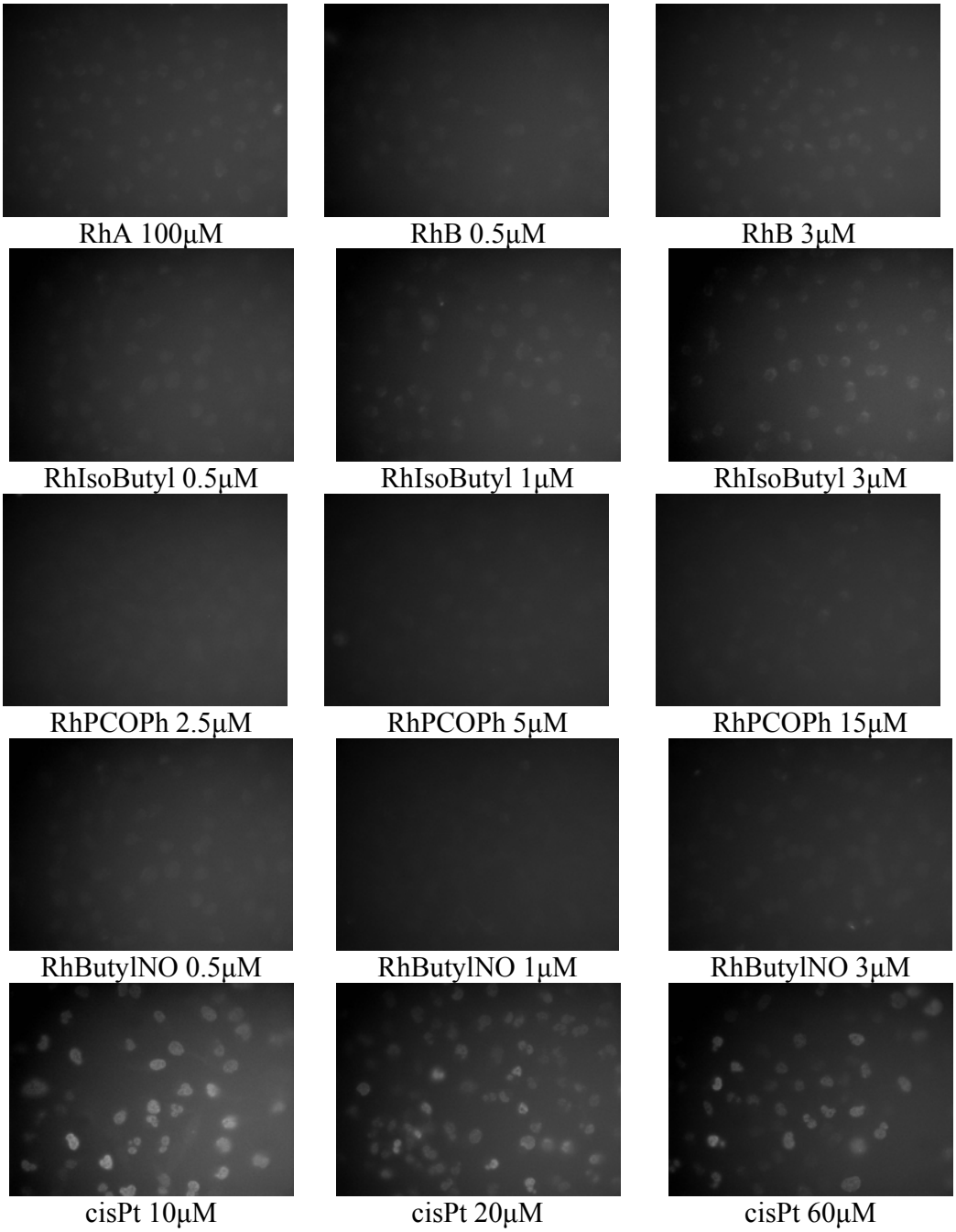


Fig. S4 (B)

B

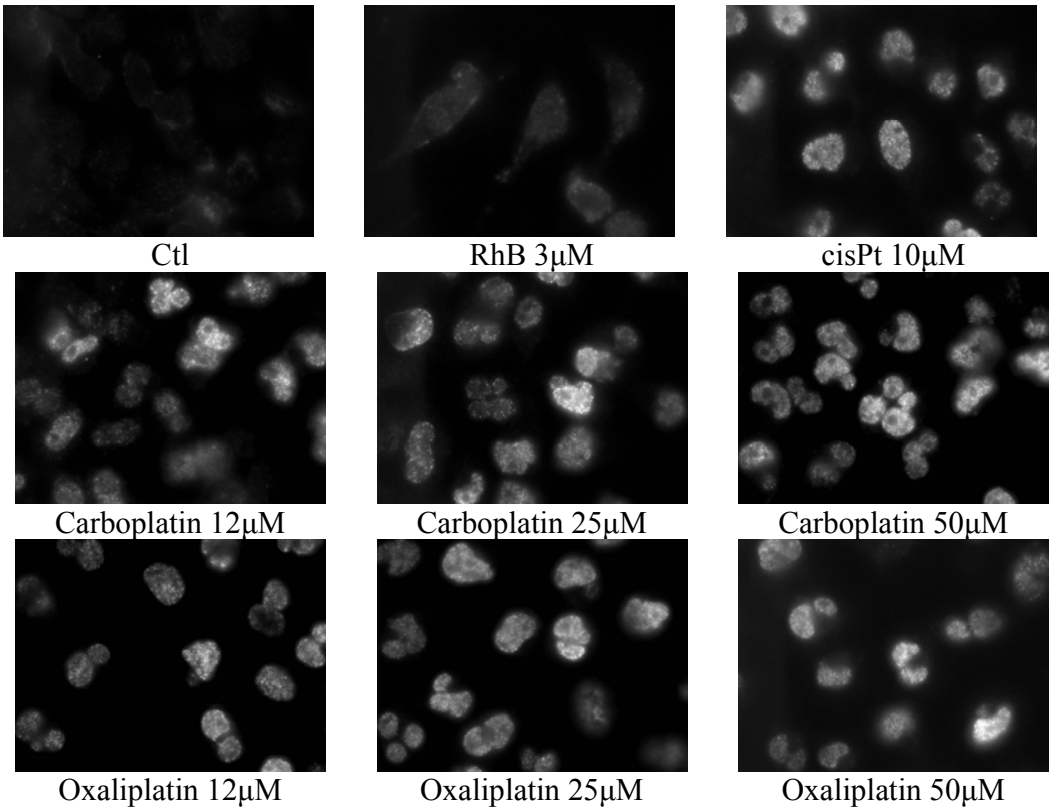


Fig. S5 Comet assay. HeLa cells were treated with indicated concentrations of **RhA**, **RhB**, **RhIsoButyl**, **RhPCOPh** and **RhButylNO** for 2 h.

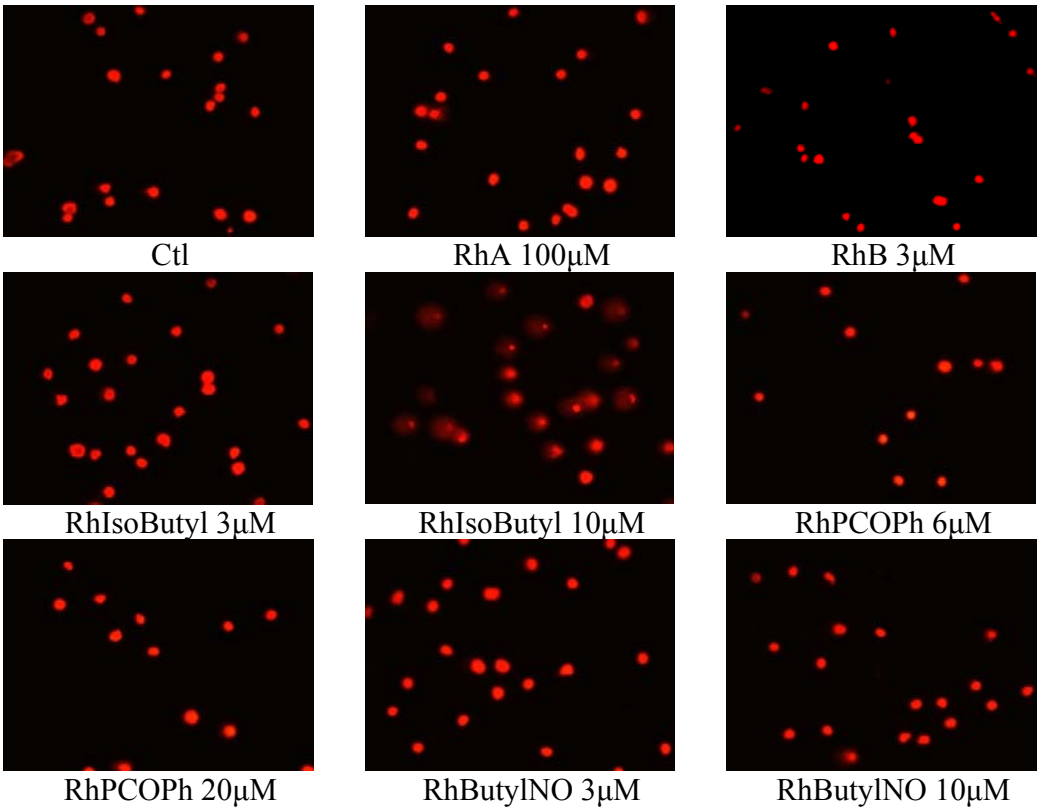
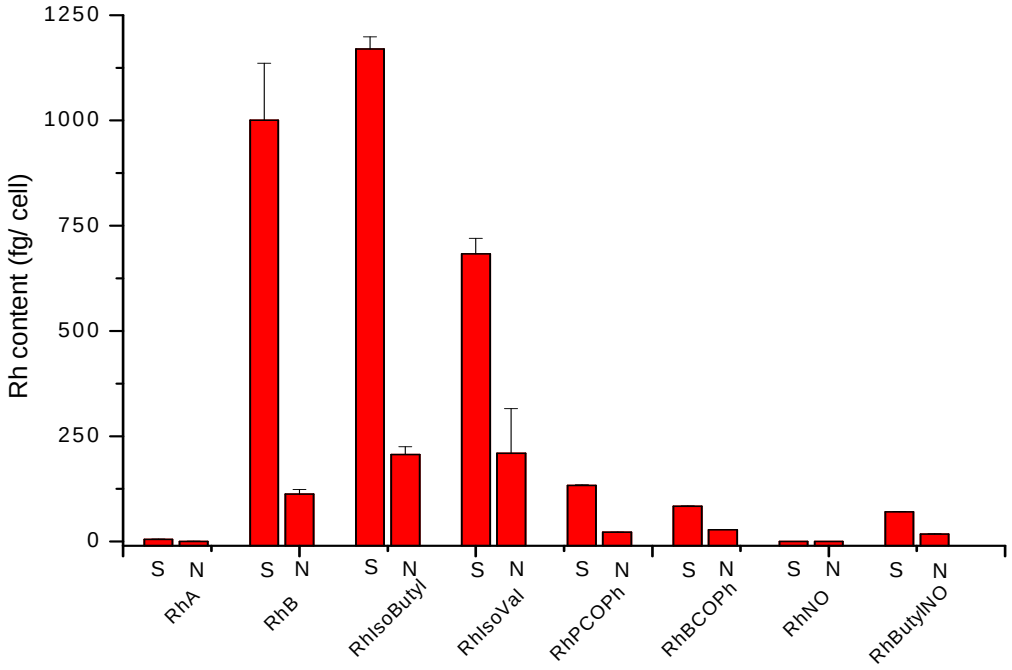


Fig. S6 Rh content of cells treated with various dirhodium(II,II) complexes. HeLa cells (5 x 10^6 cells/ 10 cm dish) were treated with 10 μM of dirhodium(II,II) carboxylates and dirhodium(II,II) carboxamidates for 2 h. Cells were lysed, and the nuclear (N) and postnuclear fraction (S, as cytoplasmic fraction) were isolated and acid-digested. The Rh contents were determined by ICP-MS.



	Postnuclear supernatant (fg Rh/cell)	Nuclear pellet (fg Rh /cell)	% in Nuclear fraction
RhA	5.7	0.61	10
RhB	1001	113	15
RhIsoButyl	1170	207	15
RhIsoVal	683	210	24
RhPCOPh	133	22	14
RhBCOPh	84	28	25
RhNO	0.37	0.073	16
RhButyNO	71	18	20

Table S1. Crystal data and structural refinement for RhNO and RhButylNO

	RhNO	**RhButylNO**
Formula	$C_{20}H_{30}N_6O_4Rh_2 \cdot 2(H_2O)$	$C_{32}H_{60}N_4O_6Rh_2$
Weight	660.35	802.66
Crystal system, space group	Monoclinic, $P2_1/c$	Monoclinic, $P2_1/c$
***a*, Å**	16.3720 (4)	6.9865 (4)
***b*, Å**	8.7441 (2)	19.2728 (11)
***c*, Å**	17.2355 (5)	14.2390 (8)
α , β , γ, °	90, 104.039(1), 90	90, 103.327(2), 90
***V* , Å^3**	2393.71(11)	1865.64(18)
Radiation	Cu *K*α radiation, λ = 1.54178 Å	Cu *K*α radiation, λ = 1.54178 Å
Z, ρ_{calc}, g cm^{-3}	4, 1.832	2, 1.429
T, K	100	173
F(000)	1336	836
R_{int}	0.044	0.065
Data/parameters/restraints	4091/321/12	3193/230/71
Goodness-of-fit	1.09	1.07
R1, wR2	0.030, 0.090	0.061, 0.165
Largest peak/hole, Å^{-3}	0.92, -1.03	1.24, -1.70

Table S2. Selected bond distances (Å) and bond angles (°) for RhNO and RhButylNO

RhNO	RhButylNO
Bond distances (Å)	
Rh1—N1 1.998 (3) N6—C17 1.136 (5) Rh1—N2^{i} 2.006 (3) O1—C1 1.283 (4) Rh1—O2 2.082 (2) O1—Rh1^{i} 2.083 (2) Rh1—O1^{i} 2.083 (2) O2—C5 1.277 (4) Rh1—N5 2.234 (3) O3—C9 1.281 (4) Rh1—Rh1^{i} 2.4509 (5) O3—Rh2^{ii} 2.075 (3) Rh2—N3 2.006 (3) O4—C13 1.279 (4) Rh2—N4 2.012 (3) O4—Rh2^{ii} 2.074 (3) Rh2—O4^{ii} 2.074 (3) C1—C2 1.509 (4) Rh2—O3^{ii} 2.075 (3) C2—C3 1.529 (5) Rh2—N6 2.231 (3) C3—C4 1.537 (5) Rh2—Rh2^{ii} 2.4558 (5) C5—C6 1.516 (5) N1—C1 1.297 (4) C6—C7 1.521 (5) N1—C4 1.464 (4) C7—C8 1.540 (5) N2—C5 1.304 (4) C9—C10 1.504 (5) N2—C8 1.458 (4) C10—C11 1.547 (5) N2—Rh1^{i} 2.006 (3) C11—C12 1.540 (5) N3—C9 1.304 (4) C13—C14 1.503 (5) N3—C12 1.462 (4) C14—C15 1.519 (5) N4—C13 1.300 (4) C15—C16 1.541 (5) N4—C16 1.445 (4) C17—C18 1.463 (6) N5—C19 1.115 (6) C19—C20 1.444 (7)	Rh1—N1 2.000 (5) C3—C4 1.524 (12) Rh1—N2^{i} 2.014 (5) C2—C5A 1.28 (2) Rh1—O2 2.073 (4) C2—C5B 1.478 (18) Rh1—O1 2.076 (4) C5A—C6A 1.535 (18) Rh1—O3 2.305 (4) C6A—C7A 1.546 (19) Rh1—Rh1^{i} 2.4432 (7) C7A—C8A 1.521 (19) N1—C1 1.297 (8) C5B—C6B 1.548 (17) N1—C4 1.470 (8) C6B—C7B 1.501 (17) N2—C9 1.302 (8) C7B—C8B 1.546 (16) N2—C12 1.452 (8) C9—C10 1.503 (9) N2—Rh1^{i} 2.014 (5) C10—C13 1.424 (12) O1—C1^{i} 1.287 (8) C10—C11 1.483 (12) O2—C9 1.285 (8) C11—C12 1.532 (10) C1—O1^{i} 1.287 (8) C13—C14 1.506 (13) C1—C2 1.523 (11) C14—C15 1.397 (17) C3—C2 1.511 (12) C15—C16 1.467 (16)
Bond angles (°)	
N1—Rh1—N2^{i} 90.87 (11) C9—N3—Rh2 123.7 (2) N1—Rh1—O2 89.02 (10) C12—N3—Rh2 124.6 (2) N2^{i}—Rh1—O2 175.56 (9) C13—N4—C16 111.7 (3) N1—Rh1—O1^{i} 175.42 (9) C13—N4—Rh2 123.1 (2) N2^{i}—Rh1—O1^{i} 88.61 (10) C16—N4—Rh2 124.9 (2) O2—Rh1—O1^{i} 91.14 (9) C19—N5—Rh1 152.0 (3) N1—Rh1—N5 98.43 (11) C17—N6—Rh2 169.3 (3) N2^{i}—Rh1—N5 98.54 (11) C1—O1—Rh1^{i} 115.26 (19) O2—Rh1—N5 85.86 (10) C5—O2—Rh1 115.1 (2) O1^{i}—Rh1—N5 86.15 (10) C9—O3—Rh2^{ii} 115.7 (2) N1—Rh1—Rh1^{i} 85.56 (7) C13—O4—Rh2^{ii} 115.8 (2) N2^{i}—Rh1—Rh1^{i} 85.32 (7) O1—C1—N1 125.4 (3) O2—Rh1—Rh1^{i} 90.25 (6) O1—C1—C2 121.5 (3) O1^{i}—Rh1—Rh1^{i} 89.86 (6) N1—C1—C2 113.0 (3) N5—Rh1—Rh1^{i} 174.36 (9) C1—C2—C3 103.5 (3) N3—Rh2—N4 90.09 (11) C2—C3—C4 104.7 (3) N3—Rh2—O4^{ii} 90.31 (12) N1—C4—C3 106.1 (3) N4—Rh2—O4^{ii} 175.31 (10) O2—C5—N2 125.8 (3) N3—Rh2—O3^{ii} 175.23 (10) O2—C5—C6 121.9 (3) N4—Rh2—O3^{ii} 90.58 (11) N2—C5—C6 112.3 (3) O4^{ii}—Rh2—O3^{ii} 88.64 (12) C5—C6—C7 103.1 (3) N3—Rh2—N6 95.96 (12) C6—C7—C8 104.7 (3) N4—Rh2—N6 97.11 (11) N2—C8—C7 105.1 (3) O4^{ii}—Rh2—N6 87.50 (10) O3—C9—N3 125.1 (3) O3^{ii}—Rh2—N6 88.64 (11) O3—C9—C10 121.8 (3) N3—Rh2—Rh2^{ii} 85.37 (8) N3—C9—C10 113.1 (3) N4—Rh2—Rh2^{ii} 85.48 (8) C9—C10—C11 103.5 (3) O4^{ii}—Rh2—Rh2^{ii} 89.89 (7) C12—C11—C10 104.0 (3) O3^{ii}—Rh2—Rh2^{ii} 89.98 (6) N3—C12—C11 106.3 (3) N6—Rh2—Rh2^{ii} 177.08 (9) O4—C13—N4 125.7 (3) C1—N1—C4 111.7 (3) O4—C13—C14 121.1 (3) C1—N1—Rh1 123.8 (2) N4—C13—C14 113.2 (3) C4—N1—Rh1 124.4 (2) C13—C14—C15 103.1 (3) C5—N2—C8 111.9 (3) C14—C15—C16 105.1 (3) C5—N2—Rh1^{i} 123.5 (2) N4—C16—C15 105.7 (3) C8—N2—Rh1^{i} 124.3 (2) N6—C17—C18 179.7 (5) C9—N3—C12 111.7 (3) N5—C19—C20 179.0 (6)	N1—Rh1—N2^{i} 93.5 (2) C2—C3—C4 107.7 (6) N1—Rh1—O2 87.94 (19) N1—C4—C3 104.7 (6) N2^{i}—Rh1—O2 175.65 (17) C5A—C2—C5B 49.6 (12) N1—Rh1—O1 175.60 (17) C5A—C2—C3 125.6 (12) N2^{i}—Rh1—O1 87.98 (19) C5B—C2—C3 125.5 (10) O2—Rh1—O1 90.33 (18) C5A—C2—C1 131.4 (12) N1—Rh1—O3 96.02 (17) C5B—C2—C1 115.0 (8) N2^{i}—Rh1—O3 96.31 (17) C3—C2—C1 101.2 (7) O2—Rh1—O3 87.63 (15) C2—C5A—C6A 127 (2) O1—Rh1—O3 87.95 (16) C5A—C6A—C7A 139 (3) N1—Rh1—Rh1^{i} 85.16 (13) C8A—C7A—C6A 112 (2) N2^{i}—Rh1—Rh1^{i} 85.33 (14) C2—C5B—C6B 113.8 (14) O2—Rh1—Rh1^{i} 90.69 (11) C7B—C6B—C5B 115.7 (15) O1—Rh1—Rh1^{i} 90.81 (11) C6B—C7B—C8B 109.3 (16) O3—Rh1—Rh1^{i} 177.91 (10) O2—C9—N2 126.1 (5) C1—N1—C4 111.2 (5) O2—C9—C10 120.9 (6) C1—N1—Rh1 123.6 (4) N2—C9—C10 113.1 (6) C4—N1—Rh1 125.1 (4) C13—C10—C11 130.0 (9) C9—N2—C12 111.3 (5) C13—C10—C9 117.2 (8) C9—N2—Rh1^{i} 122.9 (4) C11—C10—C9 102.9 (6) C12—N2—Rh1^{i} 125.5 (4) C10—C11—C12 106.6 (6) C1^{i}—O1—Rh1 114.0 (4) N2—C12—C11 104.8 (6) C9—O2—Rh1 114.7 (3) C10—C13—C14 122.2 (10) O1^{i}—C1—N1 126.3 (6) C15—C14—C13 120.3 (11) O1^{i}—C1—C2 119.6 (6) C14—C15—C16 118.8 (15) N1—C1—C2 114.1 (6)
(^{i}) −*x*+2, −*y*+2, −*z*; (^{ii}) −*x*+1, −*y*, −*z*.	Symmetry code: (^{i}) −*x*, −*y*+1, −*z*+2.

Table S3. Cytotoxicities of dirhodium(II,II) carboxylates and dirhodium(II,II) carboxamidates towards human cervical epithelioid carcinoma cells (HeLa), human hepatocellular carcinoma cells (HepG2), human lung epithelioid carcinoma cells (NCI-H460), human nasopharyngeal carcinoma cells (SUNE1) and human normal lung fibroblast cells (CCD-19Lu) respectively. Cells were treated with various concentrations of the dirhodium(II,II) complexes for 48 h, and the cell viabilities were measured by MTT assays to determine the IC_{50} values.

IC_{50} (μM)	***HeLa***	***HepG2***	***NCI-H460***	***SUNE1***	***CCD-19Lu***
RhA	76	17.15	85.82	82.52	42.48
RhB	0.45	0.53	0.25	0.30	0.54
RhIsoVal	0.55	0.42	0.23	0.36	0.51
RhIsoButyl	0.44	0.54	0.24	0.37	0.52
RhPCOPh	3.18	3.08	2.44	3.90	2.66
RhBCOPh	2.12	3.14	3.25	2.00	1.12
RhPentCOPh	2.48	4.03	2.96	3.60	0.55
RhNO	>100	>100	>100	>100	>100
RhButylNO	0.68	1.26	1.16	1.47	1.58

Table S4: The up-regulated and down-regulated genes lists prepared according to the Minimum Information About the Microarray Gene Experiment (MIAME) guidelines of the Microarray Gene Expression Data Society (MGED). The excel file is submitted for peer-review purpose. Upon publication, complete microarray data for each sample will be made available through National Cancer for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/).

Table S5: The sequences of primers used in semiquantitative RT-PCR experiments

Gene symbol	Primer sequence	Product size (bp)
ho-1	Left: GAGGTGCACACCCAGGCAGAGAAT Right: TAGCGCTGCATGGCTGGTGTGTAG	275
atf3	Left: CAGGATGCCCACCGTTAGGATTCA Right: TTTGCAACAGAGGACCTGCCATCA	430
chop	Left: TCTGGCTTGGCTGACTGAGGAGGA Right: TGGTGCAGATTCACCATTCGGTCA	327
stc2	Left: GCAGAATACAGCGGAGATCCAGCA Right: CACAGGTCAGCAGCAAGTTCACGA	404
erg-1	Left: TCGCCTGCGACATCTGTGGAAGAA Right: GTGGAGGCGCTGAAGGAGTTGGTG	391
asns	Left: TTGGAATGCAGCCAATTCGAGTGA Right: AAGGGAGTCGCGGAGTGCTTCAAT	352
xbp1	Left: CTGGAACAGCAAGTGGTAGA Right: CTGGGTCCTTCTGGGTAGAC	Spliced 389 Unspliced 424
dkk1	Left: CTGGGAGTGAGCGCCACCTTGAAC Right: CGCTTCCTGCAGGCGAGACAGATT	272
gapdh	Left: ACCACAGTCCATGCCTACAC Right: TTCACCACCCTGTTGCTGTA	555

Table S6: Rh content in the nuclear fraction and DNA isolated from cells treated with **RhB**. HeLa cells (5 x 10^6 cells/ 10 cm dish) were treated with 10 μM of **RhB** for 2 h. The nuclear fraction and the DNA were isolated, and then acid-digested. The Rh contents were determined by ICP-MS. The data shown are mean ±S.D for three independent determinations.

	Rh content (fg/ cell)
nuclear fraction (A)	110 ± 12
isolated DNA (B)	0.41 ± 0.01 (correspond to 46 ± 2 pg/μg DNA)
% of Rh in DNA over nuclear uptake (100 x B/A)	0.37

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