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Supplemental Information

Identification of a Rhodium(III) Complex as a Wee1 Inhibitor Against *TP53*-mutated Triple-negative Breast Cancer Cells

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Experimental Section

General experiment

High resolution mass spectrometry was carried out at the Mass Spectroscopy Unit at Hong Kong Baptist University, Hong Kong (China). ^{1}H and ^{13}C NMR were recorded on a 400 MHz (^{1}H) and 100 MHz (^{13}C) Bruker instrument using acetonitrile-d_3 or DMSO-d_6 as the solvent. ^{1}H and ^{13}C chemical shifts were referenced internally to solvent shift (Acetone-d_6: ^{1}H, 2.06, ^{13}C, 206.68, 29.92; DMSO-d_6: ^{1}H, 2.50, ^{13}C, 39.5). Coupling constants are typically ± 0.1 Hz for ^{1}H-^{1}H and ±0.5 Hz for ^{1}H-^{13}C couplings. The following abbreviations are used for convenience in reporting the multiplicity of NMR resonances: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. All NMR data was acquired and processed using standard Bruker software (Topspin). The elemental analysis test of complexes was performed in Atlantic Microlab, Inc. (USA).

Complex **1**: (Yield: 65%) ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.89 (d, J = 8.4 Hz, 1H), 8.82 (d, J = 4.9 Hz, 1H), 8.74 (d, J = 5.1 Hz, 1H), 8.68 (d, J = 8.3 Hz, 1H), 8.62 (d, J = 4.3 Hz, 4H), 8.40 (ddd, J = 8.0, 4.4, 1.4 Hz, 2H), 8.10 (dd, J = 8.4, 5.0 Hz, 1H), 8.02 (dd, J = 8.2, 5.0 Hz, 1H), 7.98 (d, J = 1.3 Hz, 1H), 7.89 (dd, J = 8.6, 5.4 Hz, 2H), 7.45 (d, J = 2.0 Hz, 1H), 7.37 (d, J = 2.0 Hz, 1H), 7.35 – 7.26 (m, 4H), 7.00 (t, J = 7.4 Hz, 2H), 6.70 (t, J = 7.6 Hz, 2H), 2.78 (s, 3H). ^{13}C NMR (101 MHz, Acetone) δ 149.46, 148.99, 140.75, 137.15, 131.64, 128.65, 128.16, 127.75, 125.13, 124.63, 18.81. MALDI-TOF-HRMS: Calcd. for $C_{43}H_{28}Cl_2RhN_4$ $[M–PF_6]^+$: 773.0746. Found: 773.0708. Anal.: ($C_{43}H_{28}Cl_2RhN_4PF_6 + H_2O$) C, H, N: Calcd. 55.17, 3.07, 6.09; found 55.67, 3.26, 6.53.

Complex **2**: (Yield: 67%) ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.93 (d, J = 8.6 Hz, 2H), 8.76 (d, J = 4.9 Hz, 2H), 8.62 (s, 4H), 8.40 (d, J = 7.9 Hz, 2H), 8.06 (dd, J = 8.5, 5.0 Hz, 2H), 7.89 (d, J = 8.6 Hz, 2H), 7.40 (d, J = 2.0 Hz, 2H), 7.36 – 7.25 (m, 4H), 7.00 (t, J = 7.5 Hz, 2H), 6.68 (d, J =

7.8 Hz, 2H), 2.74 (s, 6H). ^{13}C NMR (101 MHz, Acetone) δ 169.50, 168.49, 148.51, 148.06, 146.32, 145.21, 140.72, 137.03, 136.82, 136.00, 133.10, 131.95, 131.62, 128.67, 128.13, 127.54, 127.41, 125.10, 124.60, 123.84, 119.75, 15.47. MALDI-TOF-HRMS: Calcd. for $C_{44}H_{30}Cl_2RhN_4$ $[M–PF_6]^+$: 787.0903 Found: 787.0829; Anal.: ($C_{44}H_{30}Cl_2RhN_4PF_6 + H_2O$) C, H, N: Calcd. 55.54, 3.39, 5.89; found 55.41, 3.52, 6.29.

Complex **3**: (Yield: 75%) ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.83 (s, 2H), 8.82 – 8.80 (m, 2H), 8.62 (s, 4H), 8.41 (d, J = 7.9 Hz, 2H), 8.18 (s, 2H), 8.08 (dd, J = 7.9, 5.3 Hz, 2H), 7.88 (d, J = 8.7 Hz, 2H), 7.43 (d, J = 2.0 Hz, 2H), 7.33 (t, J = 7.5 Hz, 2H), 7.29 (dd, J = 8.7, 2.0 Hz, 2H), 7.01 (t, J = 7.5 Hz, 2H), 6.71 (d, J = 7.8 Hz, 2H). 13C NMR (101 MHz, Acetone) δ 169.02, 168.72, 168.46, 149.89, 148.07, 146.34, 146.03, 140.76, 140.17, 137.05, 136.06, 131.70, 131.65, 131.63, 128.88, 128.67, 128.13, 127.70, 127.56, 125.16, 124.56, 119.76, 119.74. MALDI-TOF-HRMS: Calcd. for $C_{42}H_{26}Cl_2RhN_4$ [M–PF_6]+: 759.0590 Found: 759.0598; Anal.: ($C_{42}H_{26}Cl_2F_6RhN_4P + H_2O$) C, H, N: Calcd. 54.63, 3.06, 6.07; found 54.90, 3.16, 6.27.

Complex **4**: (Yield: 51%) ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.65 – 8.56 (m, 4H), 8.44 (d, J = 8.8 Hz, 2H), 8.17 (d, J = 7.9 Hz, 2H), 7.96 (d, J = 8.7 Hz, 2H), 7.90 (s, 2H), 7.84 (d, J = 8.3 Hz, 2H), 7.73 (d, J = 2.0 Hz, 2H), 7.41 (dd, J = 8.7, 2.0 Hz, 2H), 7.19 (t, J = 8.0 Hz, 2H), 6.93 (t, J = 7.9 Hz, 2H), 6.54 (d, J = 7.9 Hz, 2H), 2.19 (s, 6H). ^{13}C NMR (101 MHz, Acetone) δ 168.18, 166.80, 166.48, 163.72, 147.17, 145.68, 145.54, 139.92, 139.18, 135.91, 134.14, 130.61, 130.52, 128.56, 127.66, 127.42, 127.17, 126.47, 126.31, 123.84, 123.46, 118.13, 23.83. MALDI-TOF-HRMS: Calcd. for C44H30Cl2RhN4 [M–PF_6]$^+$: 787.0903. Found: 787.0933. Anal.: ($C_{44}H_{30}Cl_2F_6RhN_4P + H_2O$) C, H, N: Calcd. 55.60, 3.29, 5.89; found 55.82, 3.61, 6.05.

Complex **5**: (Yield: 63%) ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.63 (d, J = 5.1 Hz, 2H), 8.60 (s, 4H), 8.38 (d, J = 7.9 Hz, 2H), 8.30 (s, 2H), 7.92 – 7.86 (m, 4H), 7.42 (d, J = 2.1 Hz, 2H), 7.33 – 7.25 (m, 4H), 6.98 (t, J = 7.5 Hz, 2H), 6.67 (d, J = 7.9 Hz, 2H), 2.84 (s, 6H). ^{13}C NMR (101 MHz, Ace-tone) δ 168.44, 150.35, 149.25, 148.06, 146.25, 145.89, 140.64, 137.00, 135.92, 131.57, 131.53, 130.88, 128.60, 128.20, 128.06, 127.52, 124.98, 124.56, 119.69, 19.03. MALDI-TOF-HRMS: Calcd. for $C_{44}H_{30}Cl_2RhN_4$ [M–PF_6]$^+$: 787.0903. Found: 787.0839. Anal.: ($C_{44}H_{30}Cl_2F_6RhN_4P + 0.5H_2O$) C, H, N: Calcd. 56.13, 3.21, 5.95; found 55.87, 3.43, 6.33.

Stability analysis

Complex **1** was stored in [d_6] DMSO/D_2O (v/v = 9:1) at 298 K for seven days. ^{1}H NMR experiments were carried out on a 400 MHz (^{1}H) Bruker instrument. Additionally, complex **1** was also stored in acetonitrile/Tris-HCl buffer (v/v = 8:2, 10 μM) or Tris-HAc buffer (pH=7.4) at 298K for seven days and 48 h, respectively. Absorption spectra were recorded on a Cary UV-100 Spectrophotometer.

Solubility analysis

The solubility of complex **1** was measured according to the reported method[1]. Stock solution of complex **1** was prepared in DMSO (10 mM). Different diluted solutions (0.5 to 50 μM) were prepared in10 mM HEPES, pH=7.0 solution. The saturated solutions were prepared by the addition of excess complex **1** in 10 mM HEPES, followed by filtration using 0.3 μm filter disc. The UV/Vis spectra were acquired from 200 to 800 nm.

Cells and reagents

All the cell lines used in our experiments were purchased from American Type Culture Collection (Manassas, VA, USA). LO2 cells and breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-10A, and MCF-7 cells) used here were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. The ligands **7-11** (purity >90%) were purchased from J&K Scientific Ltd., Hong Kong. Compound **6** (MK-1775) was purchased from Selleckchem (Shanghai, China). The Wee1 Kinase Assay/Inhibitor screening Kit (CycLex: Catalog # KA0072 V.01) was purchased from MBL International (San Diego, CA). All the complexes were immersed in dimethyl sulfoxide (DMSO), MTT kit from Sigma-Aldrich (Santa Clara, CA). Antibodies for PARP (tPARP), activated caspase-3, activated caspase-7, were got from Santa Cruz Biotechnologies, phospho-Histone H3(Ser10), caspase-8 and Phospho-Chk1(Ser345) were purchased from Beyondtime Biotechnologies, Wee1, and Bcl-2 from Abcam, rabbit γH2AX, total cdc2, and phospho-cdc2(Y15) antibodies were purchased from Cell Signaling Technology.

Wee1 inhibitor screening assay

Wee1 inhibitor screening assay was conducted using a kit following the manufacturer's instructions. Briefly, wells pre-coated with a recombinant cdc2 substrate were added kinase reaction buffer, compounds **1-11** (3.0 μM), and Wee1 kinase. The reaction was incubated at 30 °C for 60 min. After washing with TBST buffer four times, anti-p-cdc2 (Y15) antibody were added into each well followed by incubation at room temperature for 60 min. The wells were washed with PBS buffer four times, horseradish peroxidase-labeled secondary antibody was added, and the reaction was incubated at room temperature for 60 min. After washing with PBST buffer five times, the colorless substrate tetramethylbenzidine (TMB) was added. Wee1 kinase activity was evaluated by measuring the intensity of colored solution by spectrophotometry. All of assays were performed in triplicate and the activity measured for the DMSO control was taken to be 100%.

Cellular thermal shift assay

Cellular thermal shift assay was performed to monitor the target engagement of complexes in MDA-MB-231 cell lysates. Briefly, cell lysates from 2×10^6 MDA-MB-231 cells were collected, diluted in PBS and separated into aliquots. Each aliquot was treated with DMSO, complex **1** or compound **6** (3 μM). After 30 min incubation at room temperature, the treated lysates were further divided into 50 μL aliquots and heated individually in PCR tubes at different temperatures. The heated lysates were centrifuged, and the supernatants were analyzed SDS-PAGE followed by immunoblotting analysis by probing with Wee1 antibody (1: 1,000, Abcam, Cambridge, MA).

Wee1 knockdown assay

MDA-MB-231 cells were seeded in 6 well plates at 80% confluence in RPMI-1640 medium for 24h. Lipo3000 reagent, Wee1 siRNA (sense strand: 5'-CGACAGACUCCUCAAGUGA-3'),[2] and control siRNA (Santa Cruz SC-35847) were gently mixed and incubated for 20 min at room temperature. Remove growth medium from cells and replace with 0.5 ml of fresh medium.

Then the mixture 500 μL were added to each well. Cells were incubated at 37°C in a CO_2 incubator for 48 h post-transfection before the further research.

MTT assay

Cells were seeded in a 96-well plate, allowed to adhere overnight, and treated with the indicated concentrations of complex **1** for 48 h. Cell viability was determined using a Cell Counting Kit (Dojindo Molecular Technologies) according to the manufacturer's instructions. The subsequent absorbance was measured on a microplate reader at a wavelength of 450 nm.

Western blotting assay

Total cellular proteins were extracted with RIPA lysis buffer, and 20 mg were used. The membranes were probed with antibodies against PARP (tPARP), cleaved caspase-3, cleaved caspase-7, phospho-Histone H3(Ser10), caspase-8, Phospho-Chk1(S345), Wee1, and Bcl-2, rabbit γ-H2AX, total cdc2, and phospho-cdc2(Y15) antibodies, followed by anti-mouse or rabbit horseradish peroxidase-conjugated immunoglobulin (Ig) G and developed with the enhanced chemiluminescent method. GAPDH signal served as a loading control.

Apoptosis analysis

MDA-MB-231 cells were treated with different concentrations of **1** for 24 h. Compound **6** was used as a positive control. The cells were stained with anti-Annexin V antibody and propidium iodide (PI). Finally, the cells were analyzed by flow cytometry. Cells were washed twice in cold 1 × PBS. Then, 1×10^6 cells per mL were resuspended in 1 × binding buffer, and 100 μL of the cell suspension was mixed with 5 μL FITC and 5 μL PI using the FITC Annexin V apoptosis detection kit (BD 556547, San Jose, CA, USA), according to the manufacturer's instructions. Stained cells were analyzed using a FACSCalibur (BD Bioscience) flow cytometer.

Cell cycle analysis

Propidium iodide staining was conducted for detection of DNA content. Following 1 (1.0 and 3.0 μM) and compound 6 (3.0 μM) treatment for 24 h, harvested MDA-MB-231 cells were washed in ice-cold PBS, fixed in 70% ethanol, permeabilized with 0.25% Triton X-100 in PBS, and incubated with phospho-Histone H3(Ser10) antibody conjugated to Alexa Flour 488 (#9708; at concentration 1:100, from Cell Signaling Technology) for 2 h at 4℃. DNA was stained with 20 mg/mL propidium iodide (PI, Sigma-Aldrich) in the presence of 100 mg/mL RNase A (Sigma-Aldrich). Cellular DNA content was detected on a FACSCalibur (BD Bioscience) flow cytometer and analyzed with Flo-Jo software (FloJo).

Immunofluorescence assay

The cell damage and prolonged mitosis were analyzed according to previous methods with minor modifications.[3] For phosphorylated histone 3 (pH3) and γ-H2AX activation, MDA-MB-231 cells were seeded in 35 ml plates at a density of (6×10^4) cells per ml overnight and treated with DMSO, complex **1** (1.0 and 3.0 μM), and **6** (MK-1775, 3.0 μM). After fixation with 4% formaldehyde, cells were permeabilized with 0.5% Triton X-100 and blocked with 5% BSA.

Then, the cells were incubated with phospho-Histone H3 (Ser10) (1:400) antibody (Beyondtime Biotechnology) and conjugated with secondary antibodies labeled with AlexaFluor 488, green (Cell Signaling Technology). As for γ-H2AX, after fixation in 4% paraformaldehyde and permeabilization in 0.2% Triton X-100 in PBS for 30 minutes. Cells were incubated with Fluorescein isothiocyanate (FITC)-conjugated mouse anti-γH2AX(Ser139) monoclonal antibody (1:500) (BioLegend) overnight. Cell nuclei were counterstained with 4, 6-diaidino-2-phenylindole (DAPI), and then stained with DAPI for 1 h. The photos and density values of fluorescence were acquired on a Leica confocal microscope.

Statistical analysis

All statistical tests were conducted with GraphPad Prism version 5.0. Statistical significance was determined using the Student's T-test for experiments comparing two groups. Comparisons among groups were analyzed using analysis of variance (ANOVA). Comparisons between different groups were analyzed using 2- way ANOVA. Unless stated otherwise, *P* values were 2-tailed and considered significant if $P < 0.05$. Error bars represent SEM of 3 experiments unless stated otherwise.

References

1. B. Hoelke, S. Gieringer, M. Arlt, C. Saal, *Anal. Chem.*, 2009, **81**, 3165–3172.
2. W. N. Pappano, Q. Zhang, L. A. Tucker, C. Tse and J. Wang, *BMC cancer,* 2014, **14**, 430.
3. J. M. Kreahling, J. Y. Gemmer, D. Reed, D. Letson, M. Bui and S. Altiok, *Mol. Cancer Ther*., 2012, **11**, 174-182.

Table S1. *TP53* status, tumor type and IC_{50} values for the cell lines under investigation. IC_{50} values for complex **1** were determined from the MTT assay. Data shown as mean ± SD, n =3.

Cell lines	*TP53* status	Tumor type	IC_{50} (μM)
MCF-7	WT	Luminal A	2.44 ± 0.22
MCF- 10A	WT	TNBC	3.86 ± 0.42
MDA-MB-468	Mut	TNBC	0.55 ± 0.01
MDA-MB-231	Mut	TNBC	0.35 ± 0.03
LO2	WT	-	5.08 ± 0. 75

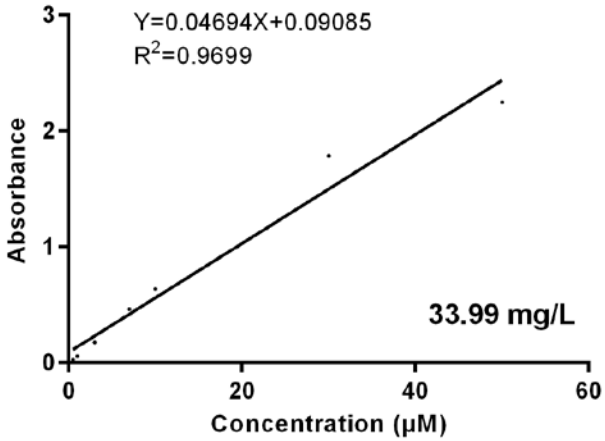

Fig. S1. Calibration of UV-spectroscopic measurement for solubility of complex **1** at λ = 277 nm in 10 mM HEPES, pH=7.0 solution.

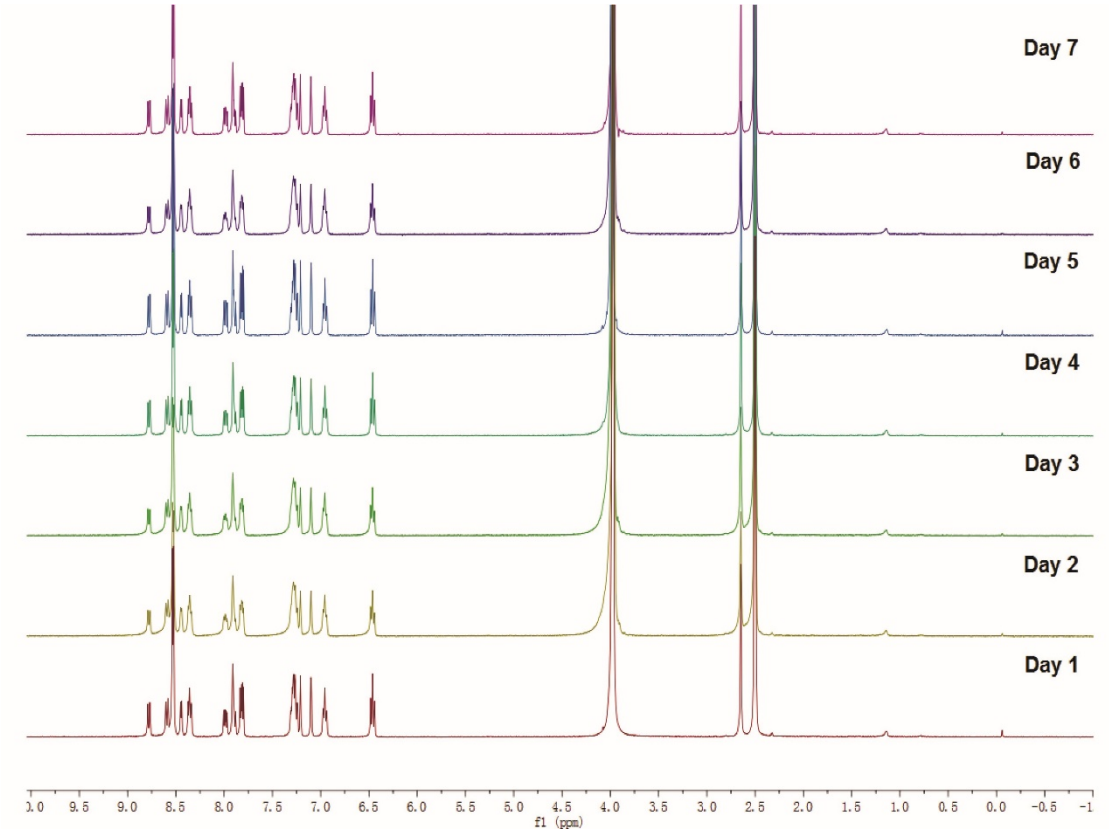

Fig. S2. ^{1}H NMR spectra of the complex **1** at a concentration of 5 mM in 90% DMSO-d_6/10% D2O at 298 K over 7 days.

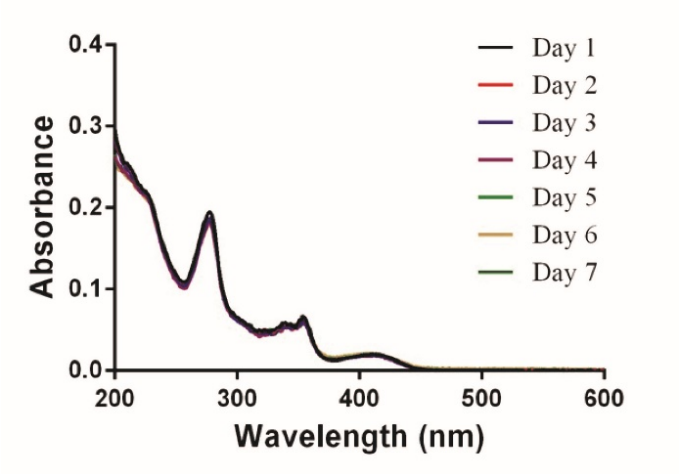

Fig. S3. UV-Vis absorption spectra of complex **1** (10 μM) in 80% acetonitrile/20% 20 mM Tris-HCl buffer (20 mM NaCl, pH 7.4) at 298 K over 7 days.

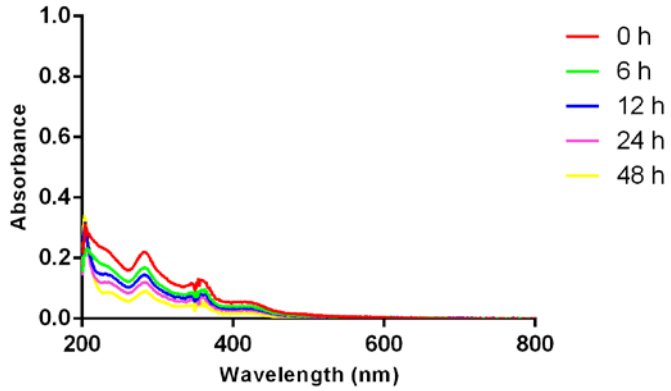

Fig. S4. UV/Vis absorption spectra of complex **1** (5 μM) in Tris-HAc buffer (10 mM, pH = 7.4) at 298 K over 48 h.

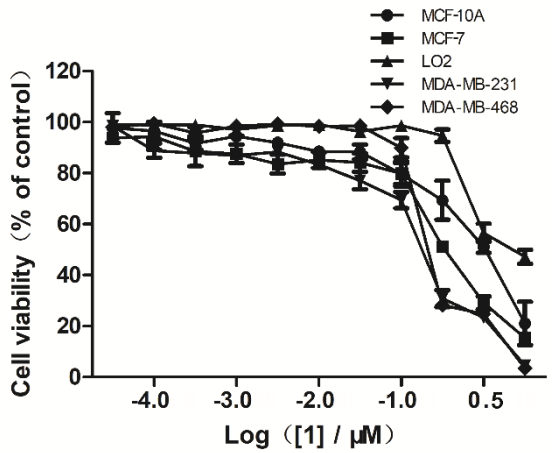

Fig. S5. The cytotoxicity effect of **1** on MCF-10A cells, MCF-7 cells, MDA-MB-231 cells, MDA-MB-468 cells, and LO2 cells. Breast cancer and normal cell lines were treated with the

indicated concentrations of **1** for 48 h. Cell viability was evaluated by the MTT assay. Data are presented as mean ± SE (n=3).

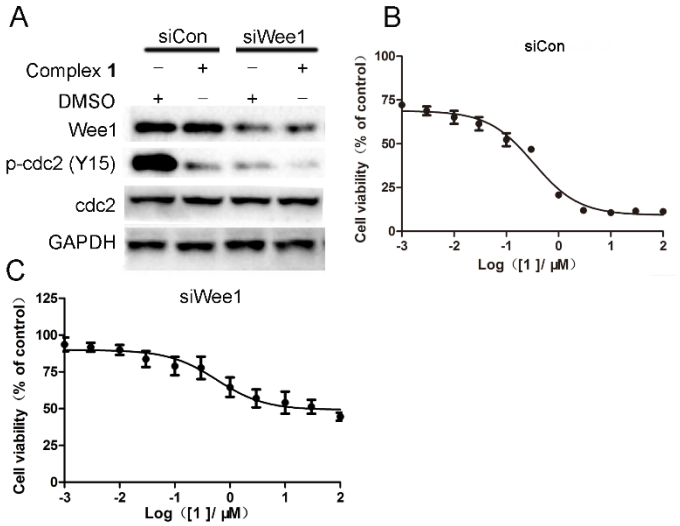

Fig. S6. Wee1 knockdown decreases the cytotoxicity effect of complex **1** on MDA-MB-231 cells. (A) Wee1 siRNA treatment produces efficient target knockdown in MDA-MB-231 cells. Total cdc2 and GAPDH were blotted to control for total protein levels. (B-C) The cytotoxicity effect of complex **1** on siCon- or siWee1-treatement MDA-MB-231 cells. Complex **1** inhibited the growth of on siCon- or siWee1-treatement MDA-MB-231 cells with an IC_{50} value of 0.33 ± 0.04 μM (B), and 41.69 ± 5.04 μM (C), respectively. Data are presented as mean ± SEM (n=3). Wee1 siRNA: SiWee1; Control siRNA: siCon.

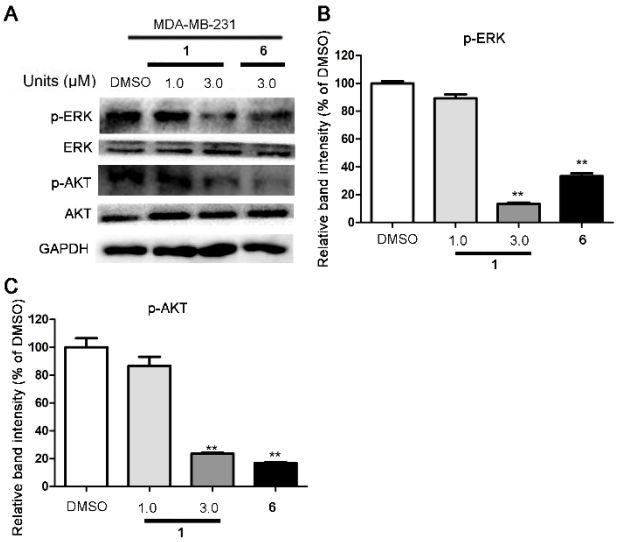

Fig. S7. Complex **1**-induced cytotoxic effect is mediated by ERK and AKT signaling in MDA-MB-231 cells. After treatment with complexes **1** and **6**, the protein levels of AKT/p-AKT and ERK/p-ERK, were detected by western blotting. (A) Phosphorylation of ERK and AKT. (B-C) Densitometry analysis of p-ERK, and p-AKT. Error bars represent the standard deviations of the results from three independent experiments. $^{**}p < 0.01$ compared to DMSO treatment.

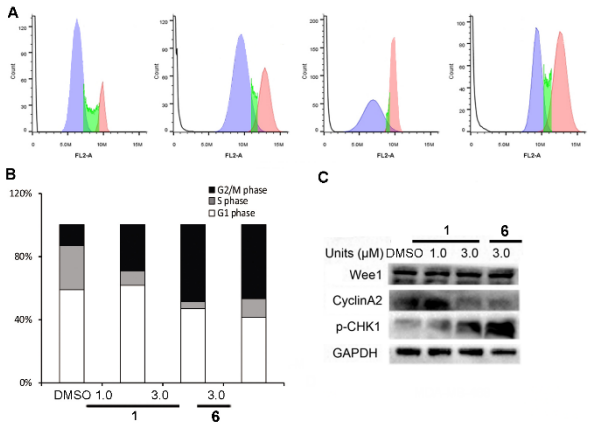

Fig. S8. Complex **1** promotes premature mitosis of *TP53*^{MUT}-MDA-MB-231 cells. MDA-MB-231 cells were treated with DMSO, **1** (1.0 and 3.0 μM) or **6** (3.0 μM). After 24 h, cell cycle analysis was performed using PI staining followed by flow cytometry. (A) Histogram representing the distribution of cell cycle. (B) The percentage distribution of cells in the G0/G1, S, and G2/M phases are shown. * $p < 0.05$. (C) The effects of complex **1** on the expression of cell cycle related proteins.

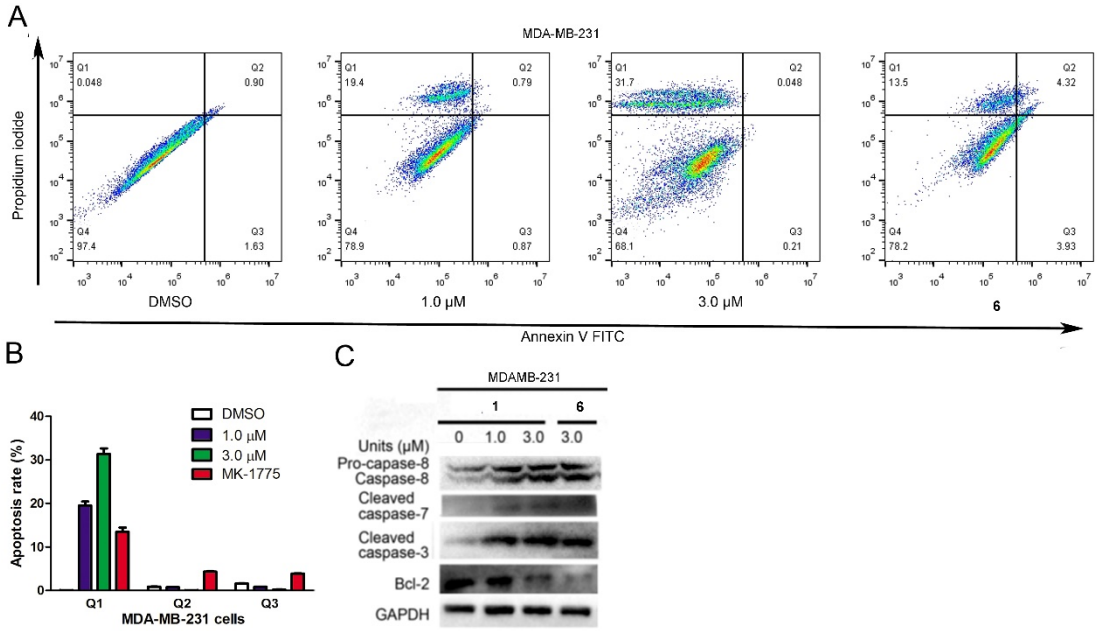

Fig. S9. The effects of complex 1 on apoptosis of MDA-MA-231 cells. MDA-MB-231 cells were treated with DMSO, **1** (1.0 and 3.0 μM) or **6** (3.0 μM). After 24 h, cells apoptosis was detected by flow cytometry and western blot. (A) Histogram representing the distribution of cell apoptosis. Q1: Dead cells; Q2: Early apoptosis; Q3: Late apoptosis, and Q4: Live cells. (B) The percentage distribution of cells in the different steps of apoptosis was shown. * $p < 0.05$. (C) The effects of complex **1** on the expression of cell cycle related proteins.

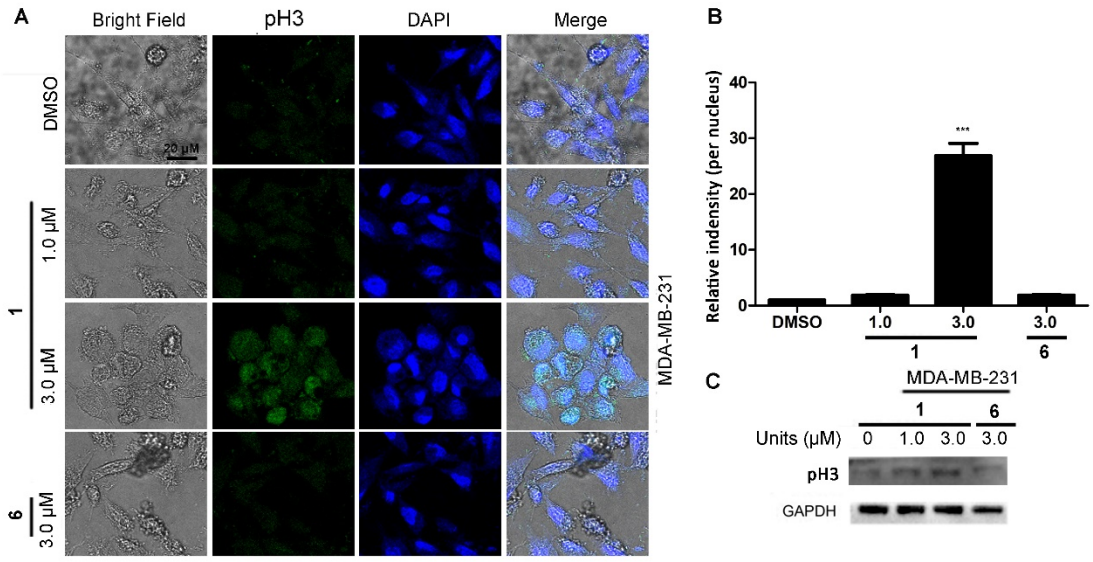

Fig. S10. Complex **1** treatment results in prolonged mitotic arrest in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with DMSO, **1** (1.0 and 3.0 μM), or **6** (3.0 μM) for 24 h and stained for pH3 (AlexaFluor 488, green) and counterstained nuclei with DAPI and visualized using a confocal laser scanning microscope (Leica TCS SP8). (B) The results in Figure S10A was quantified by Image J software. Error bars: SEM, $^{*}p < 0.05$ and $^{***}p < 0.001$. (C) Expression of pH3 (S10) in MDA-MB-231 cells was analyzed by Western blot.

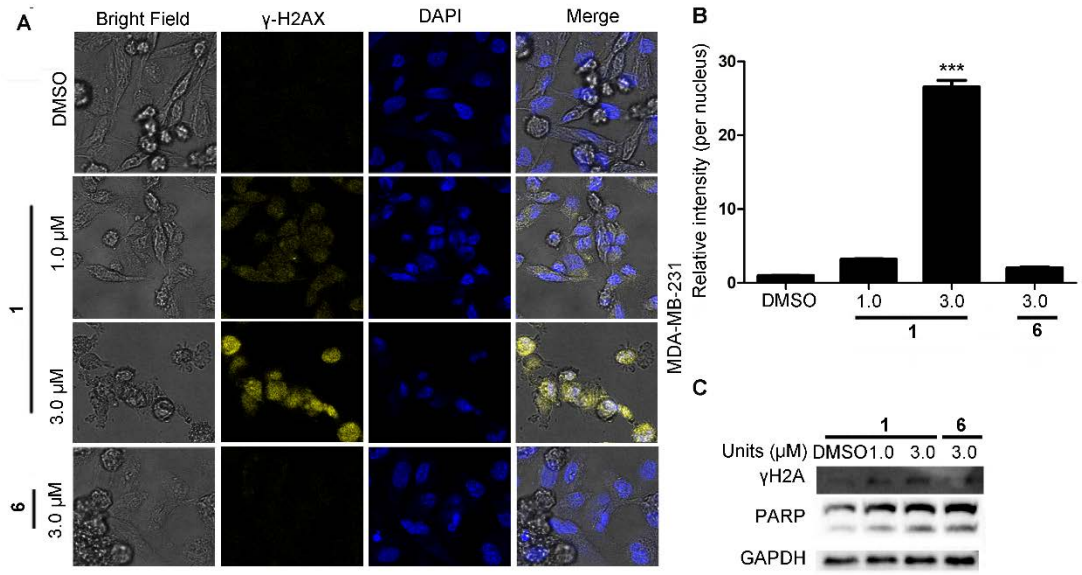

Fig. S11. Complex **1** treatment causes DNA damage in MDA-MB-231cells. (A) MDA-MB-231 cells were treated with DMSO, **1** (1.0 and 3.0 μM) or **6** (3.0 μM) for 24 h and visualized using a confocal laser scanning microscope. Cells were stained for γH2AX (FITC, orange), and nuclei were counterstained with DAPI. (B) The results in Figure S11A was quantified by Image J software. Error bars: SEM, ***$p < 0.001$. (C) Expression levels of γ-H2AX and PAPR in two TNBC cells were analyzed by Western blotting.