Supporting Information

Ligand field and polarity tuning of Fe(II) complexes for selective ROS-mediated anticancer activity

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Experimental Section

Materials and Instrumentation. Commercial reagents were employed as received except where purification is specifically noted. The nonheme iron(II) complexes were prepared according to the modified literature methods.[1] ^{1}H nuclear magnetic resonance (NMR) spectra were measured using a Bruker model AVANCE III 400 FT-NMR digital spectrometer. UV-vis spectra were recorded on a Hewlett Packard Agilent Cary 8454 UV-visible spectrophotometer equipped with a T2/sport temperature controlled cuvette holder. Electrospray ionization mass spectra (ESI MS) were collected on a Thermo Finnigan (San Jose, CA, USA) LTQ^{TM} XL ion trap instrument, by infusing samples directly into the source at 5.0 μL/min using a syringe pump. The spray voltage was set at 4.7 kV and the capillary temperature at 200 ^{o}C. Electrochemical measurements were performed on a CHI617B electrochemical analyzer (CH Instruments, Inc.) in CH_3CN containing 0.10 M, Bu_4NPF_6 ($TBAPF_6$) or in CH_3CN. A conventional three-electrode cell was used with a glassy carbon working electrode (surface area of 0.030 cm^2), a platinum wire as a counter electrode and an Ag/Ag^+ electrode as a reference electrode (in CH_3CN) or an Ag/AgCl, 1.0 M KCl electrode as a reference electrode (in H_2O). The glassy carbon working electrode was routinely polished with BAS polishing alumina suspension and rinsed with acetone and acetonitrile before use. The measured potentials were recorded with respect to an Ag/Ag^+ (0.010 M) reference electrode (in CH_3CN) or an Ag/AgCl, 1.0 M KCl reference electrode (in H_2O), respectively. All potentials (vs Ag/Ag^+ or Ag/AgCl) were converted to values vs Fc/Fc^+ by subtracting 0.09 V or 0.02 V. Cell viability was quantified using a microplate reader (VICTOR Nivo™, Waltham, MA, USA). Live/dead imaging was performed using Calcein-AM and PI staining, and fluorescence images were obtained with a Lionheart FX Automated Imaging Microscope (BioTek). Intracellular ROS levels were measured using a DCFDA-based assay (Abcam, #ab113851) with imaging on the Lionheart FX. Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using the JC-1 assay (Abcam, #ab113850), and fluorescence signals (red/green channels) were recorded using the same imaging platform. Western blot images were acquired using the Lumino Graph III Lite chemiluminescence system (ATTO, WSE-6370K).

Synthesis and characterization of ligand and iron(ll) complexes. The N5Me ligand was

synthesized via reductive amination of N-propylethylenediamine with 2-pyridinecarboxaldehyde under mild acidic conditions. N-Propylethylenediamine (0.500 mL, 5.00 mmol) and 2-pyridinecarboxaldehyde (1.42 mL, 15.0 mmol) were dissolved in anhydrous THF under a N_2 atmosphere. Acetic acid (0.750 mL, 13.1 mmol) was added to promote iminium formation, followed by sodium triacetoxyborohydride (3.43 g, 16.2 mmol). The reaction mixture was stirred at room temperature for 48 h under N_2. After completion, the solvent was removed under reduced pressure, and the residue was dissolved in dichloromethane (DCM) and transferred to a separatory funnel. The organic phase was washed with aqueous $NaHCO_3$ solution to neutralize residual acetic acid. The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure to afford the N5Me ligand as a powder. The [Fe(HN3O2)]2+ complex was prepared according to the modified literature methods.[1] Elemental analysis: for $C_{16}H_{21}N_3O_2$ (HN3O2) : C, 66.88; H, 7.37; N, 14.62; O, 11.14, for $C_{23}H_{29}N_5$ (N5Me) : C, 73.57; H, 7.78; N, 18.65. $[Fe(N5Me)]^{2+}$ complex was prepared by reacting N5Me ligand and equimolar $Fe(ClO_4)_2 \cdot 6H_2O$ in CH_3CN. NMR samples were prepared by dissolving the N5Me ligand in $CDCl_3$, and spectra were recorded on a 400 MHz spectrometer. The UV–vis spectra of the iron(II) complexes in CH_3CN at 20 °C exhibited characteristic absorption bands at 330 nm for **1** and at 335, 370, and 570 nm for **2**. All commercially available reagents were used as received unless otherwise noted, and solvents were dried according to standard literature procedures and distilled under Ar prior to use.

X-ray Structural Analysis. Single crystals of **2** suitable for X-ray crystallographic analysis were obtained by slow diffusion of Et_2O into a CH_3CN solution of **2** complex. These crystals were taken from the solutions by a nylon loop (Hampton Research Co.) on a handmade copper plate and mounted on a goniometer head in a N_2 cryostream. The diffraction data for **2** were collected at 173 K on a Bruker SMART AXS diffractometer equipped with a monochromator in the Mo Kα (λ = 0.71073 Å) incident beam. The CCD data were integrated and scaled using the Bruker-SAINT software package, and cell parameters were determined and refined using SHEXTL V 6.12 and Olex 2. Hydrogen atoms were located in calculated positions. The crystallographic data and selected bond distances and angles are listed in Tables S1 and S2 in the SI and Table 1 in the text. Full crystallographic details can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif (CCDC 2488729). Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, U.K.;

fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk. The X-ray measurement was performed using the Single Crystal II at the National Research Facilities and Equipment Center (NanoBio·Energy Materials Center) at Ewha Womans University.

Detection of hydroxyl radical Methylene blue stock solution of 0.010 mM was prepared by dissolving 0.0018 g of methylene blue in 2.0 mL of distilled water. 10 μL of methylene blue stock solution was added to aqueous solution of **1** or **2**. Then, hydrogen peroxide (2.0 μL, 0.019 mM) was added and reaction mixture was stirred at RT for 4 h. Terephthalic acid (3.4 mg, 10 mM) was added to aqueous solution of **1** or **2**. The reaction mixture was stirred at RT for 24 h after the addition of hydrogen peroxide. (2.0 μL, 0.019 mM).

Cell culture Human renal cancer cell lines (786-O, A498, and Caki-1) and human fibroblast cell (CCD-986Sk) were cultured as monolayers. 786-O cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Welgene), A498 and Caki-1 cells in Dulbecco's Modified Eagle Medium (DMEM, Welgene), and CCD-986Sk cells in Iscove's Modified Dulbecco's Medium (IMDM, Welgene). All media were supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin–streptomycin (Welgene). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability Cells were seeded in 96-well plates and incubated for 24 h for adherence. After treatment with **1** and **2** complexes or DW as a control for 24 h at 37 °C under 5% CO_2. All iron complexes used in biological assays were obtained as crystalline solids, and stock solutions were freshly prepared prior to use. Cell viability was evaluated using the WST-8 assay (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt; Dojindo). Absorbance was measured at 450 nm using a microplate reader (VICTOR Nivo™, Waltham, MA, USA). WST-8 assay was performed with three independent biological replicates (n = 3). Data are presented as mean ± standard deviation (SD).

PI and calcein AM staining Live/dead cell staining was performed using calcein acetoxymethyl ester (Calcein-AM) to identify viable cells and propidium iodide (PI) to detect

membrane-compromised dead cells. Briefly, 786-O cells were seeded at a density of 5,000 cells/well in a black 96-well plate and incubated overnight to allow for cell attachment. Cells were then treated with DW, and **2** (5 μM) for a total of 24 h. Following the incubation, cells were stained with Calcein-AM (Thermo Fisher, USA) and PI (Thermo Fisher, USA) at 37 °C for 30 min under light-protected conditions. Fluorescence images were acquired using the Lionheart FX Automated Imaging Microscope (BioTek).

Intracellular reactive oxygen species assay To investigate intracellular ROS levels, a DCFDA-based assay was performed using the Cellular ROS Detection Assay Kit (Abcam, #ab113851) in accordance with the manufacturer's instructions. 786-O cells were seeded in black 96-well plates at a density of 5,000 cells/well and incubated overnight to allow for adherence. Cells were then treated with **1** and **2** (5, 10, and 25 μM) and incubated for 24 h. After incubation, DCFDA was added to each well to a final concentration of 25 μM and incubated for 20 min. The DCFDA-containing medium was then removed, and the cells were washed once with PBS. Fluorescence images were then acquired using the Lionheart FX Automated Imaging Microscope (BioTek). DCFDA assay was performed with three independent biological replicates (n = 3). Data are presented as mean ± standard deviation (SD).

Mitochondrial membrane potential assay The mitochondrial membrane potential (ΔΨm) was assessed using the JC-1 staining assay (Abcam #ab113850). Briefly, 786-O cells were seeded into 96-well plates and incubated for 24 h to allow for attachment. The cells were treated with **1** and **2** (5, 10, and 25 μM) and incubated for 24 h. To evaluate mitochondrial depolarization, 100 μL of JC-1 solution was added to each well containing the nanoparticle-treated medium, resulting in a final JC-1 concentration of 5 μg/mL. The plate was then incubated at 37 °C for 15 min under standard culture conditions. Prior to fluorescence measurement, the JC-1-containing medium was removed, and the cells were washed with PBS. JC-1 aggregates were detected at 586/647 nm (red fluorescence), and JC-1 monomers were measured at 469/525 nm (green fluorescence). Fluorescence images were then acquired using the Lionheart FX Automated Imaging Microscope (BioTek).

Flow Cytometry Analysis. Cells were trypsinized and washed with cold phosphate-buffered saline (PBS). The cells were fixed by adding 70% cold ethanol drop by drop while vortexing, and the fixed cells were incubated at 4 °C overnight. After washing with cold PBS, the cells were stained with PI/RNase staining buffer (550825, BD) at room temperature for 15 min. The analysis was conducted using a BD FACS LSRFortessa high-parameter flow cytometer (Becton Dickinson Biosciences) at the Fluorescence Core Imaging Center, Ewha Womans University.

Western blot analysis 786-O cells treated with **2** and DW as control were harvested for protein analysis. Total proteins were extracted using an RNA/Protein extraction kit (MACHEREY-NAGEL) according to the manufacturer's instructions. Protein concentrations were determined using the BCA assay for normalization. Equal amounts of protein lysates were separated on 12% SDS-PAGE gels (Bio-Rad, #BR4561043) and transferred onto polyvinylidene difluoride (PVDF) membranes (GenDEPOT, #W7031-540). Membranes were blocked and primary antibodies were diluted in PBS containing 1% skim milk and 1% Tween-20 (Sigma), followed by overnight incubation at 4 °C. The following primary antibodies were used: β-actin (Abcam, #ab8227), Bcl-2 (Abcam, #ab182858), Cleaved Caspase-9 (Cell Signaling Technology, #7237), Cleaved Caspase-3 (Cell Signaling Technology, #9661). After washing, the membranes were incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in PBS containing 2% skim milk and 1% tween-20. Protein bands were visualized using a chemiluminescent detection reagent (ATTO) and imaged using the Lumino Graph III Lite imaging system (WSE-6370K, ATTO, Japan). Band intensities were quantified using ATTO imaging software, background-corrected, normalized to β-actin, and expressed relative to the control.

Statistical analysis Statistical analysis was performed using a two-tailed paired Student's *t*-test in GraphPad Prism 11 software (San Diego, CA, USA).

Table S1 Partition Coefficients (log P_{calc}) for **1** and **2**.

complex	**Partition coefficient** (log P_{calc})
1	-0.170
2	2.27

Table S2. Crystallographic data and refinements for **2**.

	2
Empirical formula	$C_{23}H_{29}C_{l2}FeN_5O_8$
Formula weight	671.316
Temperature (K)	273.15
Wavelength (Å)	0.71073
Crystal system/space group	monoclinic, P21/c
Unit cell dimensions	
a (Å)	17.8269(11)
b (Å)	9.5462(6)
c (Å)	17.5337(11)
α (°)	90
β (°)	93.321(2)
γ (°)	90
Volume (Å^3)	2978.9(3)
Z	4
Calculated density (g/cm^{-3})	1.496
Absorption coefficient (mm^{-1})	0.736
Reflections collected	49108
Independent reflections	5289
Goodness-of-fit on F^2	1.037
R [F^2 > 2sigma(F^2)]	0.0506
wR^2	0.0618

Table S3. Selected bond distances (Å) and angles (°) for **2**.

	2
Bond Distances (Å)	
Fe1-N1	1.977(3)
Fe1-N2	1.999(3)
Fe1-N3	1.954(3)
Fe1-N4	1.987(3)
Fe1-N5	1.937(3)
Fe1-N6	2.065(3)
Bond Angles (°)	
N2-Fe1-N1	81.94(11)
N3-Fe1-N1	88.52(11)
N3-Fe1-N2	85.29(11)
N4- Fe1-N1	95.33(11)
N4-Fe1-N2	93.92(11)
N4-Fe1-N3	175.92(11)
N5-Fe1-N1	95.55(11)
N5-Fe1-N2	177.49(11)
N5-Fe1-N3	94.58(11)
N5-Fe1-N4	112.29(14)
N6-Fe1-N1	168.03(11)
N6-Fe1-N2	86.27(11)
N6-Fe1-N3	92.55(11)
N6-Fe1-N4	83.40(10)
N6-Fe1-N5	96.24(11)

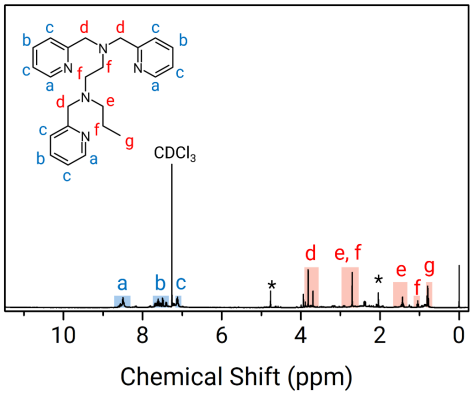

Fig. S1 ^{1}H NMR spectrum of N5Me in $CDCl_3$. * the signal is the remnant of the suppressed DW signal.

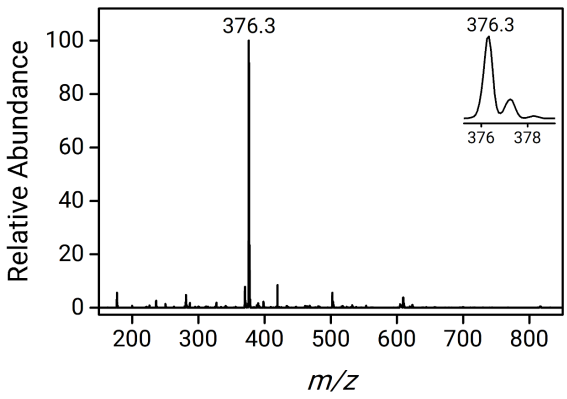

Fig. S2 ESI MS spectra of N5Me ligand recorded in CH_3CN. The prominent ion peak at 376.3 corresponds to $[N5Me]^+$ (calculated *m/z* of 376.2). The insets show the observed isotopic distribution patterns of N5Me.

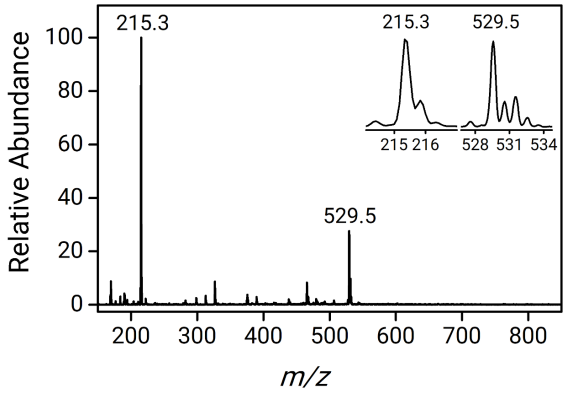

Fig. S3 ESI MS spectra of **2** recorded in CH_3CN. The prominent ion peak at 215.3 corresponds to $[Fe(N5Me)]^{2+}$ (calculated *m/z* of 215.5), and the peak at 529.5 corresponds to $[Fe(N5Me)(ClO_4)_2]^{+}$ (calculated *m/z* of 530.1). The insets show the observed isotopic distribution patterns of **2**.

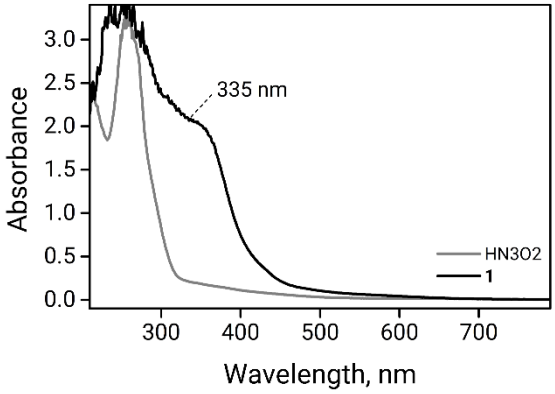

Fig. S4 UV-vis spectra of HN3O2 ligand (0.5 mM, gray) and **1** (0.5 mM, black) in CH_3CN at 20 °C.

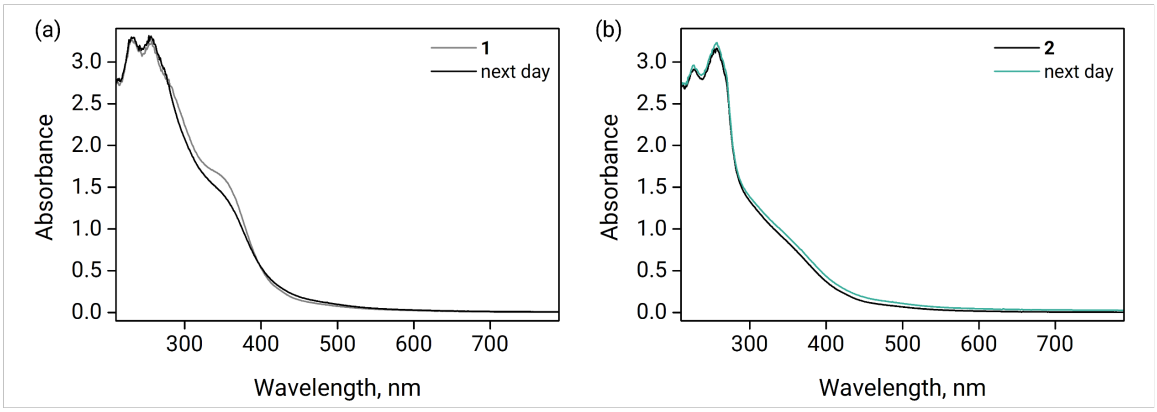

Fig. S5 UV-vis spectra of **1** (0.5 mM, gray) and **2** (0.5 mM, teal) in DW at 20 ºC. black lines represent spectra after 24 h.

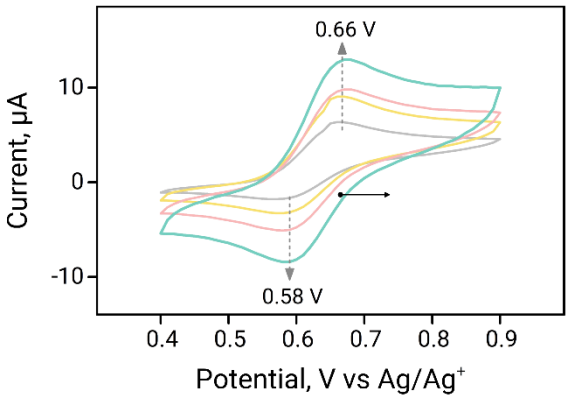

Fig. S6 Cyclic voltammogram of **2** (2.0 mM) in CH_3CN with a glassy carbon working electrode at 20 °C. Scan rates were 0.10 V/s (gray), 0.20 V/s (yellow), 0.50 V/s (pink), 1.0 V/s (teal).

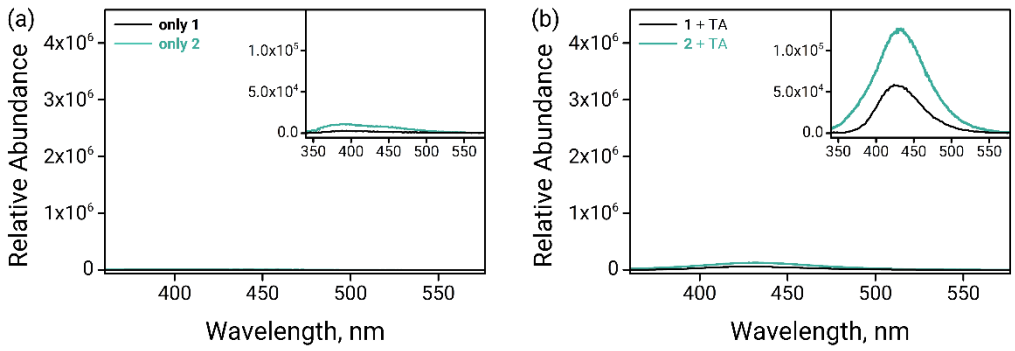

Fig. S7 Fluorescence spectra of **1** (25 μM, black) and **2** (teal, 25 μM) at 20 °C under three conditions. (a) only Fe complex (b) with terephthalic acid (TA, 10.0 mM).

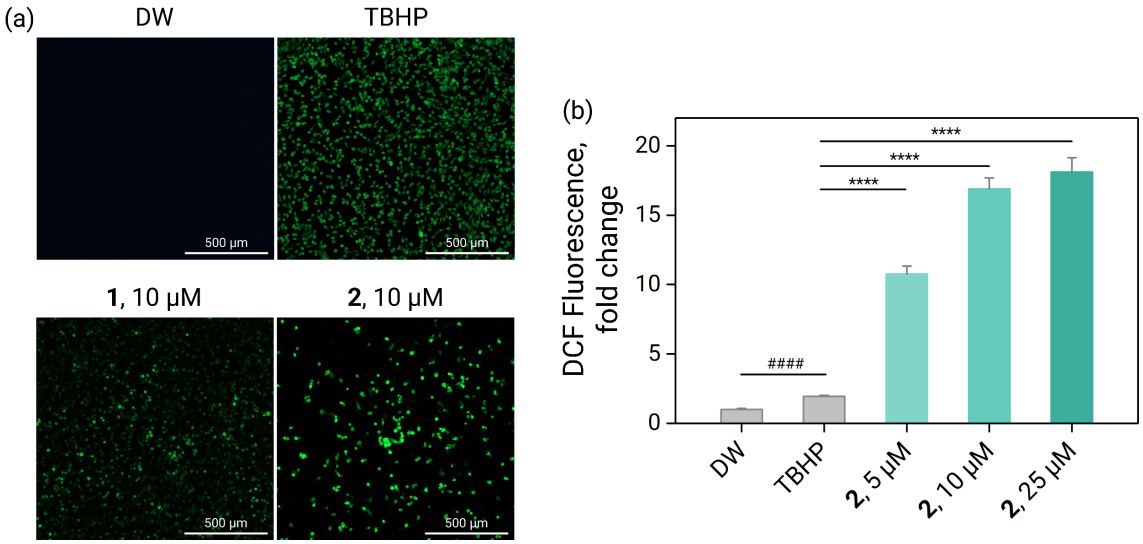

Fig. S8 (a) Fluorescence images of 786-O treated with DW, TBHP, **1**, and **2** (10 μM). (b) Fluorescence intensity of 786-O treated with TBHP and **2** at different concentration (5.0, 10, 25 μM). (n = 4, $^{\#\#\#\#}p < 0.001$ vs DW, ****$p < 0.0001$ vs TBHP, student's *t*-test).

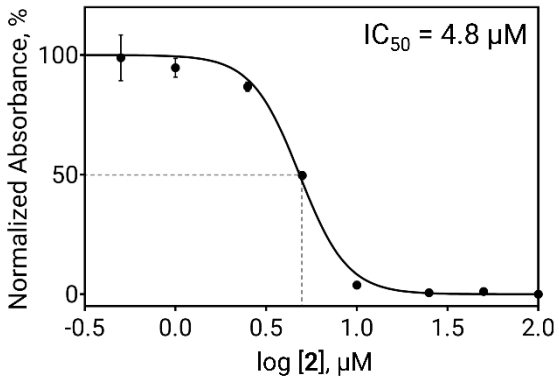

Fig. S9 Plot of normalized absorbance of 786-O cells with respect to the log concentration of **2** in order to determine the IC_{50} value.

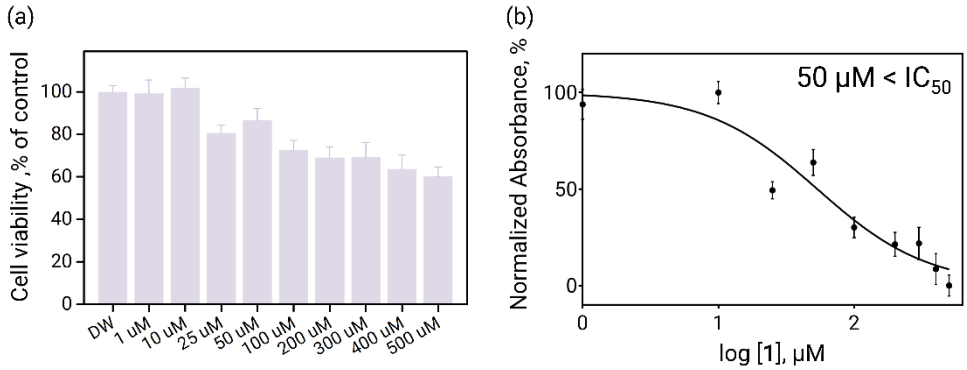

Fig. S10 Effects of **1** on cell viability. (a) 786-O cells were treated with different concentrations (1, 10, 25, 50, 100, 200, 300, 400, and 500 μM) of **1** for 24 h. (b) Plot of normalized absorbance of 786-O cells with respect to the log concentration of **1** in order to determine the IC_{50} value.

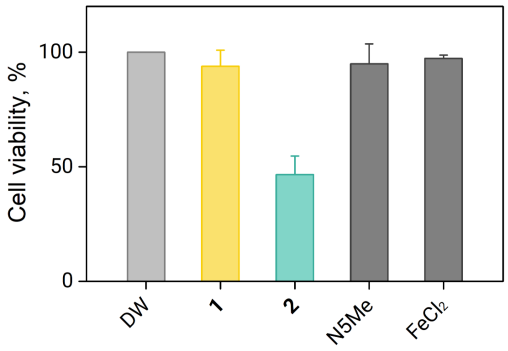

Fig. S11 Effects of Fe complexes (**1** and **2**), N5Me, and $FeCl_2$ on cell viability assessed using a WST-8 assay. 786-O cells were treated with each compound (5.0 μM) for 24 h.

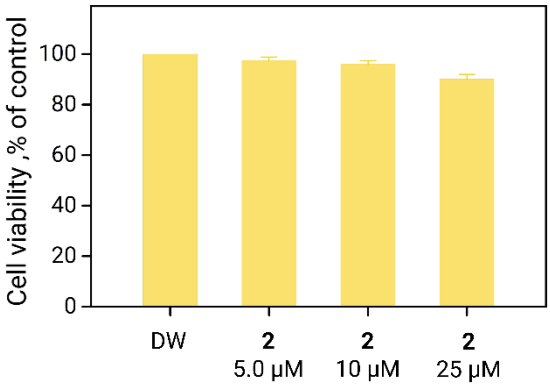

Fig. S12 Fibroblast cell line (CCD-986Sk) was treated with 5.0, 10, 25 µM of **2** for 24 h.

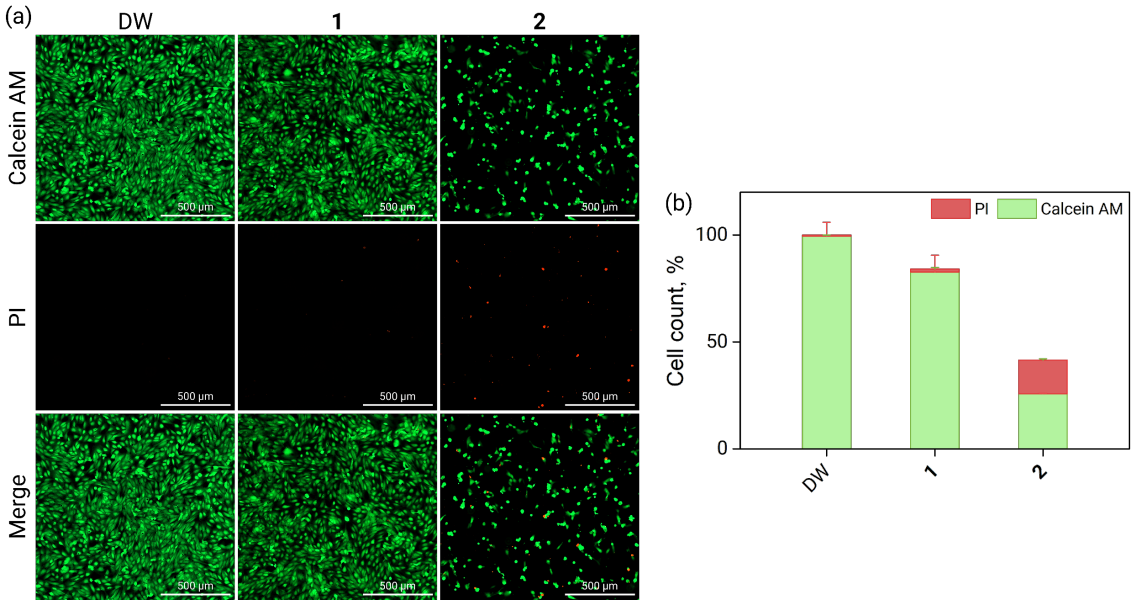

Fig. S13 (a) Fluorescence images of 786-O cells treated with **1** and **2** (5.0 μM) and stained with Calcein AM/PI. (b) Quantification of live (Calcein AM) and dead (PI) cells based on cell counting and fluorescence intensity analysis of the images in (a).

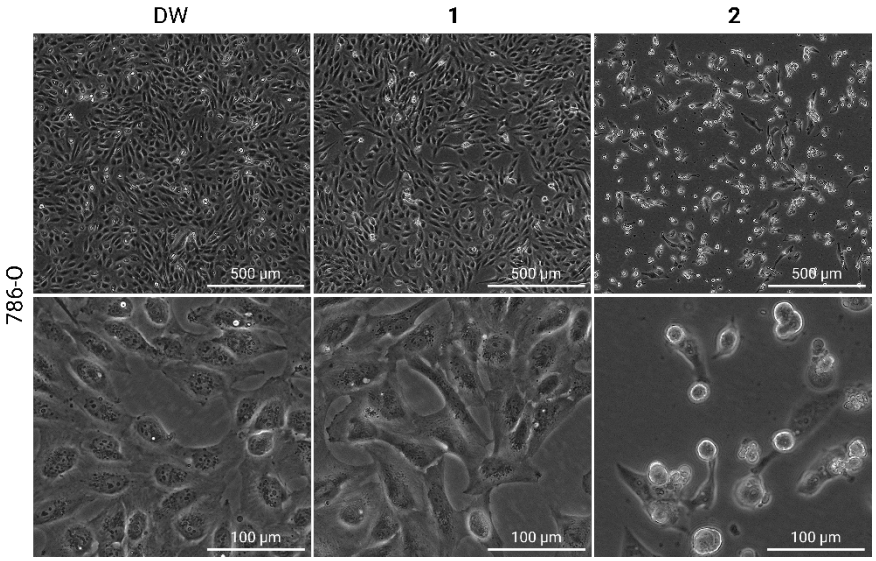

Fig. S14 Microscopic images of 786-O cells upon incubation with **1** and **2** (5.0 µM) for 24 h. Scale bar: 500 and 100 µm.

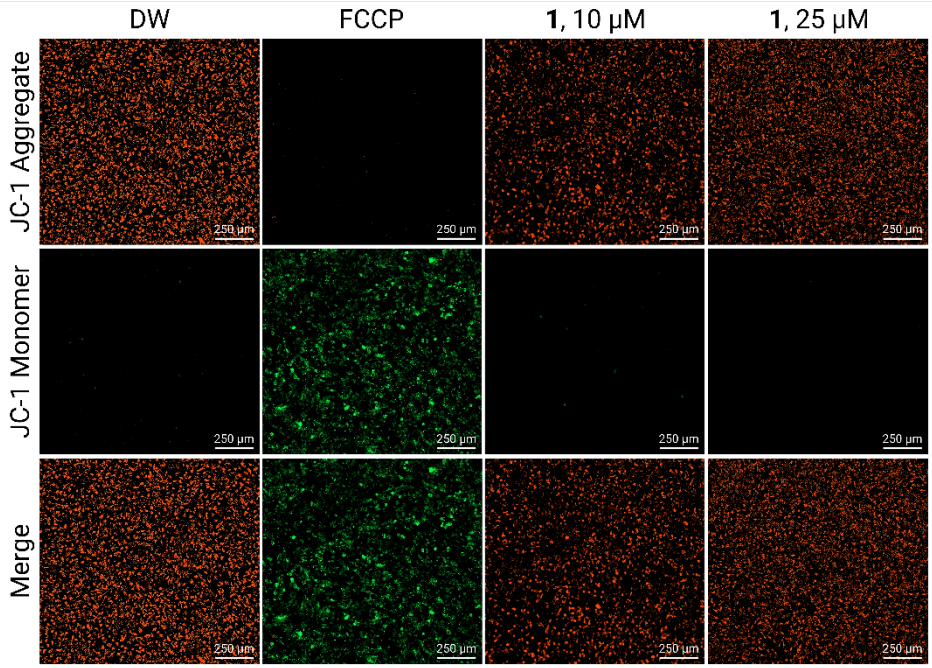

Fig. S15 JC-1 staining of 786-O cells treated with 5.0 μM and 25 μM of **1** for 24 h. Scale bar: 250 μm

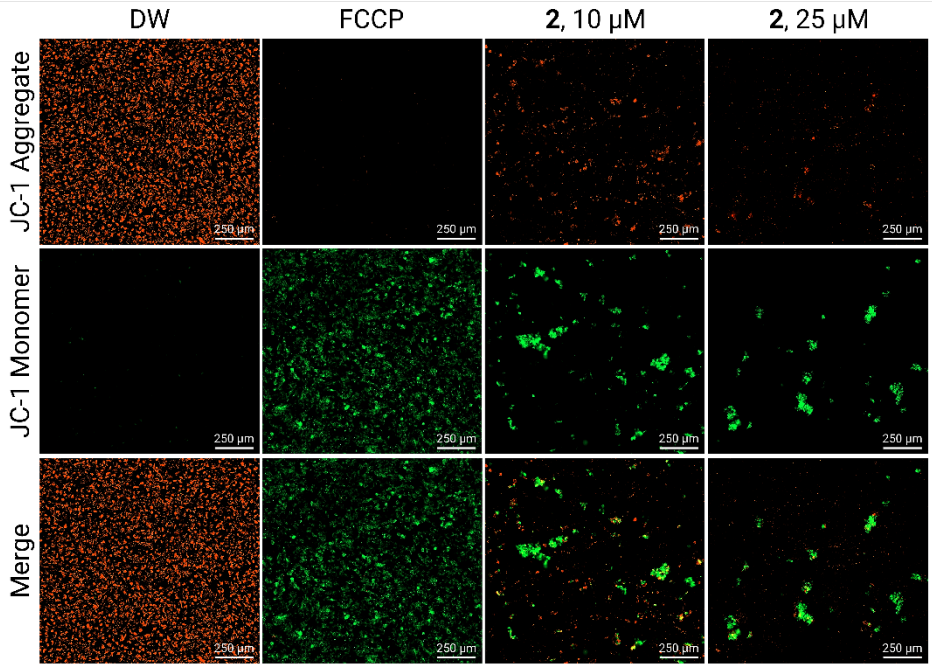

Fig. S16 JC-1 staining of 786-O cells treated with 5.0 μM and 25 μM of **2** for 24 h. Scale bar: 250 μm

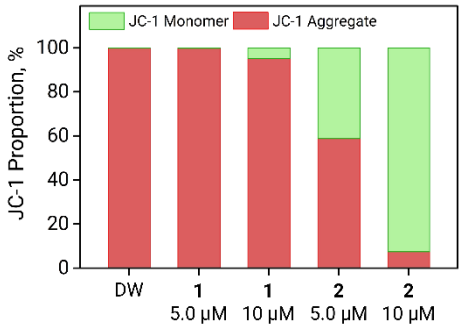

Fig. S17 JC-1 staining analysis of mitochondrial membrane potential ($\Delta\Psi_m$) in 786-O cells treated with **1** or **2** (5.0 or 10 μM). The proportions of JC-1 aggregates (red) and JC-1 monomers (green) are shown for each condition.

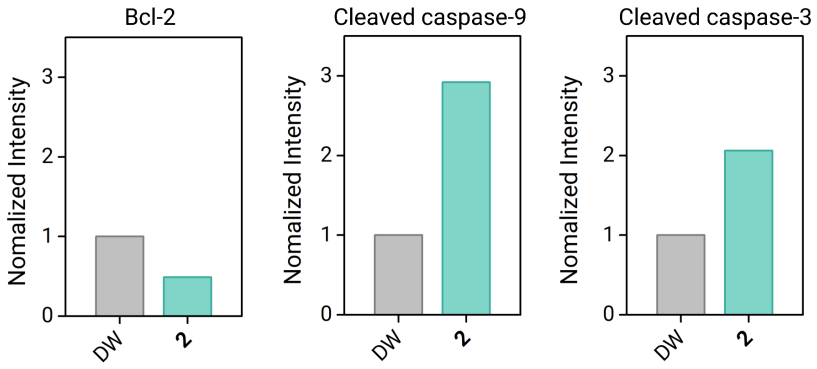

Fig. S18 Quantification of protein expression in 786-O cells treated with 2 (5.0 μM) by Western blot. Protein levels of Bcl-2, cleaved caspase-9, and cleaved caspase-3 were normalized to β-actin.

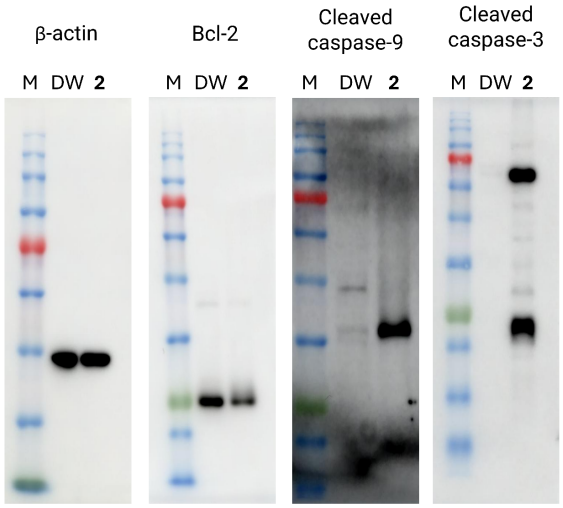

Fig. S19 Western blot raw data.

References

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