Electronic Supplementary Information (ESI) for

Necroptosis-inducing iridium(III) complexes as regulators of cyclin-dependent kinases

Ruilin Guan,†[a] Lina Xie,†[a] Lili Wang,[a] Ying Zhou,[a] Yu Chen,[a]

Liangnian Ji[a] and Hui Chao*[a]

[a] *MOE Key Laboratory of Bioinorganic and Synthetic Chemistry, School of Chemistry, Sun Yat-Sen University, Guangzhou, 510275, P. R. China.*

Email: ceschh@mail.sysu.edu.cn

Supporting Experimental Procedures

Materials and Measurements

All starting materials were used as received from commercial sources unless otherwise stated. Chemical materials for synthesis is purchased from Energy Chemical (China). Cisplatin (Sigma Aldrich, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich, USA), dimethyl sulfoxide (DMSO, Sigma Aldrich, USA), propidium iodide (PI, Sigma Aldrich, USA), and 5,5′,6,6′-tetrachloro-1,1',3,3′-tetraethyl-benzimidazolyl carbocyanine iodide (JC-1, Life Technologies, USA), were used as received. Annexin V-FITC apoptosis detection kit was purchased from BD (USA). Caspase 3/7 activity kit, Caspase 8 activity kit, and LDH detection kit were purchased from Promega (USA). MitoTracker™ Green was purchased from Invitrogen. Nucleus extraction kit and cytoplasm extraction kit were purchased from Sangon Biotech (China). 2', 7'-dichlorofluorescin diacetate (DCFH-DA) kit and CFSE dye was purchased from Beyotime (China). Cell cycle analysis kit was purchased from Thermo Scientific (USA). All primary and secondary antibodies used in western blot were purchased from Abcam (USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and penicillin streptomycin were purchased from Gibco (USA). All the compounds tested were dissolved in DMSO just before the experiments, and the final concentration of DMSO was kept at 1% (v/v).

Electrospray ionization mass spectrometry (ES-MS) was recorded on a Thermo Finnigan LCQ DECA XP spectrometer (USA). The quoted m/z values represented the major peaks in the isotopic distribution. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 500 MHz spectrometer (Germany). Shifts were referenced relative to the internal solvent signals. The inductively coupled plasma mass spectrometry (ICP-MS) experiments were carried out on an Agilent's 7700x instrument. UV/Vis spectra were recorded on a Varian Cary 300 spectrophotometer (USA). Instruments for Western blot experiments are from Bio-Rad. Cell imaging experiments were carried out on a confocal microscope (LSM 880 Carl Zeiss, Germany). Wound-healing assay was conducted on an inverted fluorescence microscope (Zeiss Axio Observer D1, Germany). Caspase 3/7 activity, caspase 8 activity assay, and LDH leakage assay were conducted using a TECAN Infinite M200 PRO multifunctional reader. MTT assay was conducted on a SpectraMax Absorbance

Reader CMax Plus (Molecular devices).

Cell lines and culture conditions

Cell were obtained from Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). Cells were routinely maintained in DMEM medium (Dulbecco's modified Eagle's medium, Gibco BRL), RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium, Ham's F-12K (Kaighn's, Gibco BRL) medium and McCoy's 5A (Gibco BRL) medium containing 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin, and 100 U/mL penicillin (Gibco BRL). Cells in tissue culture flasks were incubated in a humidified incubator (Atmosphere: 5% CO_2 and 95% air; Temperature: 37 °C). Cisplatin-resistant A549R cells were cultured in DMEM with cisplatin to maintain the resistance.

MTT assay

The cytotoxicity of the complexes was determined by MTT assay. Briefly, the cells were seeded into 96-well microtiter plates at (1 × 104 cells per well), and grown for 24 h at 37 °C in a 5% CO_2 incubator conditions, and different concentrations of the complexes were added to the culture media. The plates were then incubated for 48 h in the dark. The MTT dye solution (10 μL, 5 mg/ml) was added to each well. After 4 h of incubation, the cultures were removed and 150 μL of DMSO solution was added to each well. The optical density of each well was measured on a microplate spectrophotometer at a wavelength of 595 nm. Data were reported as the means ± standard deviation (n = 3).

CFSE Staining Assay

CFSE stinging assay was conducted according to current literature.[1,2] Briefly, L02 cells were thoroughly resuspended in a 1 ml volume of phosphate buffer solution (PBS) containing 5% FBS in a fresh tube. 5mM stock solution of CFSE was added to 110 μl PBS and the mixture was subsequently added to the 1 ml PBS containing L02 cells, to give a final concentration of 5 μM. After mixing, leave cells in the dark for 5 min at room temperature. 10 ml PBS (5% FBS) was added to wash the cells by centrifugation as 300 g for 5 min and the liquid was discarded. The labelled L02 cells

were then mix with non-labelled A549R cells and seeded in appropriate containers. The cells were respectively stimulated by 2 μM **NecroIr1** and **NecroIr2** for 1 or 2 days, and then suspended and tested by flow cytometer.

Cellular ROS detection

A549R cells plated into 6-well plates were respectively treated with **NecroIr1** and **NecroIr2** at the indicated concentrations for 24 h. Then cells were stained with H_2DCFDA (1 μM) for 20 min at 37 °C in the dark and washed twice with serum-free DMEM. The fluorescence intensity of DCF in A549R cells was measured by flow cytometer.

ICP-MS measurement

The cellular uptake capacity of complexes was measured by determination of intracellular iridium contents. Briefly, A549R cells were incubated in 100 mm dishes overnight. The medium was removed and replaced with medium/DMSO (v/v, 99:1) containing **NecroIr1** or **NecroIr2** (5 μM). After 30 min incubation, the cells were trypsinized and collected in PBS (3 mL). Mitochondria and mitochondria-free cytosol were isolated from treated cells using the mitochondria isolation kit (Sangon Biotech, China) according to the manufacturer's instructions. Nuclear fractions were separated using a nucleoprotein extraction kit (Sangon Biotech, China) according to the manufacturer's instructions. The samples were digested with 50% HNO_3 and 10% H_2O_2 at RT for two days. Each sample was diluted with MilliQ H_2O to obtain 2% HNO_3 sample solutions. The iridium and ruthenium content were measured using inductively coupled plasma mass spectrometry (ICP-MS Thermo Elemental Co., Ltd.). Data were reported as the means ± standard deviation (n = 3).

Colocalization assay

The cells were incubated with **NecroIr1** or **NecroIr2** (5 μM) at 37 °C for 30 min and then co-incubated with MitoTracker™ Green FM (MTG) at 37 °C for 30 min, then washed by PBS three times and visualized by laser confocal microscopy with a 63 × oil-immersion objective lens immediately. The excitation wavelengths for **NecroIr1** and **NecroIr2** were 405 nm, while the excitation wavelength of MTG is 488 nm. Emission filter: (peak wavelength of **NecroIr1** and **NecroIr2**) ± 20 nm respectively,

and 520 ± 20 nm for MTG.

Analysis of MMP

A549R cells were seeded into confocal dish and treated with **NecroIr1** and **NecroIr2** (1.5 μM and 3 μM) for 24 h. After incubation, the treated cells were stained with JC-1 for 30 min protected from light and immediately wash with the JC-buffer. The cells were collected in flow cytometry tubes and immediately analyzed by a flow cytometer.

Annexin V/PI staining assay

The assay was performed according to the manufacturer's (Thermo, USA) protocol. Cells treated with **NecroIr1** and **NecroIr2** (1.5 μM and 3 μM) for 24 h and harvested in tube stained with annexin V reagents at room temperature for 15 min in the dark. The samples were immediately analyzed by flow cytometer.

Caspase 3/7 activity assay and caspase 8 activity assay

Caspase 3/7 activity and caspase 8 activity was measured using the corresponding Caspase-Glo® Assay kit (Promega, USA) according to the manufacturer's instructions. Cells cultured in 96 well plates were treated with **NecroIr1** and **NecroIr2** (1.5 μM and 3 μM) for 24 h. After incubation, 100 μL of corresponding Caspase Glo® reagents were added to each well containing 100 μL culture medium. The mixture was incubated at room temperature for 1 h and then luminescence was measured using a micro-plate reader (TECAN Infinite M200 PRO).

Visualization of the intracellular calcium level

Visualization of the intracellular calcium level experiment was conducted according to the literature.[3] Cells were plated onto 35 mm glass bottom dishes (Cellvis) and allowed to adhere for 24 h. The culture media was changed with fresh medium or Ca^{2+}-free medium. The cells were incubated with **NecroIr1** (3 μM) and **NecroIr2** (1.5 μM) for 24 h in the dark at 37 °C. 2.0 μM Fluo-4 AM was added and continued to incubate at 37°C FOR an additional 50 min. Cells were washed with PBS and the images were captured by confocal laser scanning microscope.

Western blot

The A549R cells were seeded into 100 mm tissue culture plates (Costar), incubated for 24 h, and then treated with **NecroIr1** and **NecroIr2** (1.5 μM and 3 μM) for 24 h. The cells were washed with ice-cold PBS and lysed by incubation in radio immune precipitation assay buffer (RIPA) with a protease inhibitor cocktail (Sigma) for 30 min on ice. The lysates were centrifuged at 15000 rpm for 15 min at 4°C, and the protein concentrations were quantified by a BCA protein assay reagent kit (Novagen Inc, USA). The proteins were fractionated by electrophoresis in a 10% acrylamide gel, transferred to PVDF membranes, and subjected to Western blot analysis. Primary antibodies were diluted in TBS containing 5% skim milk and 0.1% Tween-20 and then incubated with the membrane overnight at 4°C. Horseradish peroxidase conjugated secondary antibodies (Abcam) were used. The bound immune complexes were detected using an ECL prime Western blot detection reagent (Thermo, USA) and visualized by Omega Lum C Imaging System (Aplegen, USA).

LDH leakage assay

A549R cells incubated with **NecroIr1** and **NecroIr2** (1.5 μM and 3 μM) for 24 h in black-walled glass-bottomed 96-well plates. Subsequently, the cells were treated with CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin (Promega) according to the manufacturer's protocol and the fluorescence in RLUs was quantified by an Infinite M200 PRO (TECAN, Swiss).

Cell cycle analysis

Briefly, A549R cells after treatment with **NecroIr1** (1.5 μM and 3 μM) and **NecroIr2** (0.75 μM and 1.5 μM) for 24 h were washed in cold PBS and fixed in 70% ethanol at -20 °C overnight. The fixed cells were washed twice with PBS, re-suspended in 1 ml cell cycle detection solution containing PI (Thermo Scientific). This solution was incubated at room temperature for 30 min, and the cells were analyzed via flow cytometry on a FACS Canto II flow cytometer (BD Biosciences, USA).

Cell Treatment with EdU

To measure anti-proliferation activity of **NecroIr1** and **NecroIr2**, Cell-Light EdU Apollo488 In Vitro Kit (RiboBio, China) was used accoding to the manufacturer's instructions. Briefly, after incubation with **NecroIr1** (0.75 μM, 1.5 μM and 3 μM),

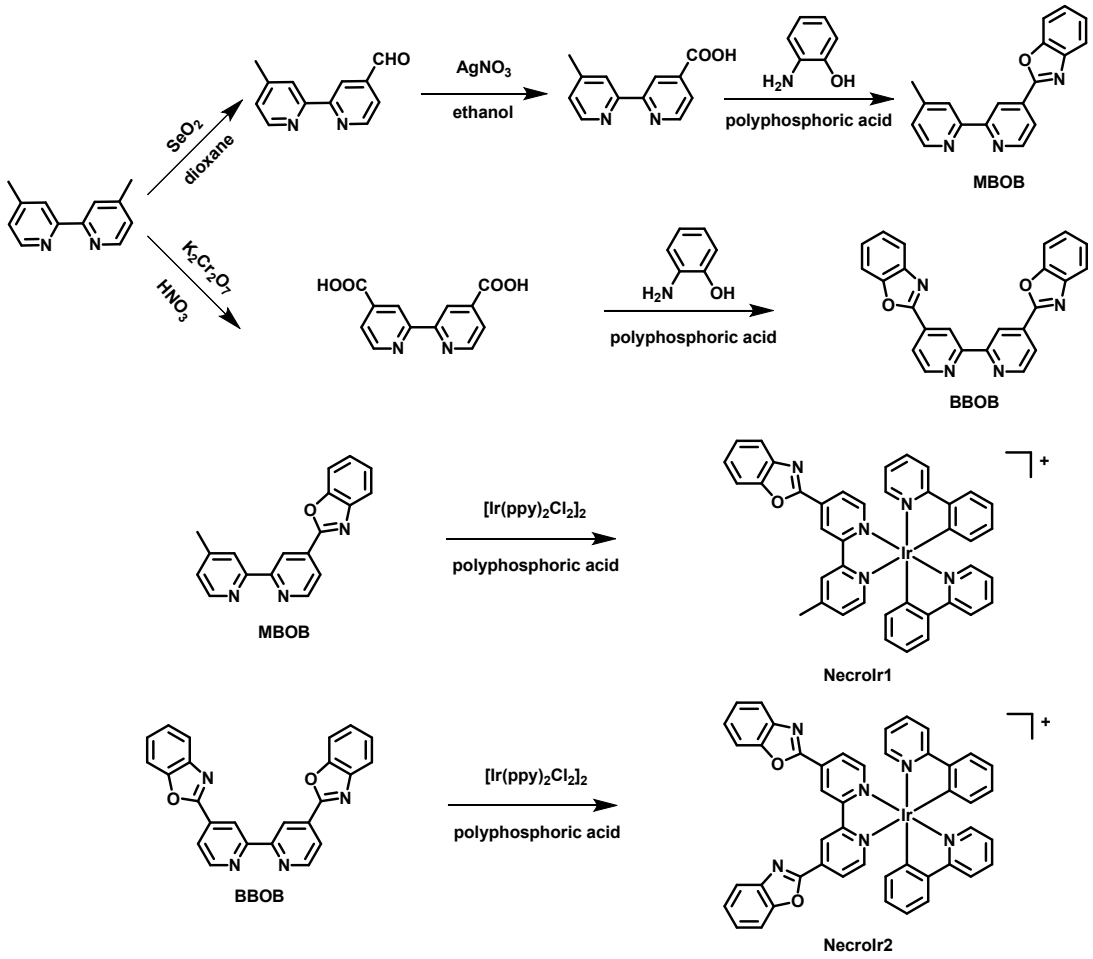
NecroIr2 (0.375 μM, 0.75 μM and 1.5 μM) or CDDP (22.5 μM, 45 μM, and 90 μM) for 24 h, A549R cells in a 96-well plate were washed with 1× PBS, and 200 μL of medium containing EdU was then added to each well. The cells was incubated for 24 h and then washed with 1× PBS followed by cell fixation with 4% polyphosphoric acid. After 15 min of incubation, the cells were washed with 3% BSA twice, followed by cell permeabilization using 0.5% Triton X-100 in 1× PBS and incubation for 20 min. The cells were then washed with 3% BSA twice, and 200 μL of the reaction mixture was added, followed by 30 min of incubation and washing once with 3% BSA. For nucleus staining, 200 μL of Hoechst 33342 solution was added to each well, and cells were incubated for another 30 min and washed twice with 200 μL of 1× PBS. The cells were finally imaged under a confocal microscope (LSM 880 Carl Zeiss, Germany).

Wound-healing assay

A549R cells were seeded in a 6-well plate and supplemented with culture media with 10% FBS. After incubated for 24 h, the culture media was removed and maintained in 1% FBS culture media. The cells were wounded by a 200 μL pipette tip to made a cross line and the scratched cells were removed by washing the cells with PBS twice. Then the cells were maintained in 1% FBS culture media containing **NecroIr1** and **NecroIr2** (0.5 μM and 1 μM) and incubated for 24 h. Cell migration was recorded and photographed through an inverted microscope (Carl Zeiss, Gottingen, Germany).

Synthesis and characterization

$[Ir(ppy)_2Cl]_2$,[4] 4-methyl-4'-aldehyde-2,2'-bipyridine,[5] 4-methyl-4'-carboxyl-2,2'-bipyridine,[6] 4,4'-carboxyl-2,2'-bipyridine,[7] 4-methy-4'-benzoxazolyl-2,2'-bipyridine (MBOB),[7] and 4-methy-4'-benzoxazolyl-2,2'-bipyridine (BBOB)[7] were synthesized according to the published methods. The synthetic route used to access **NecroIr1** and **NecroIr2** is illustrated in Scheme S1.



Scheme S1. Synthesis of complex **NecroIr1** and **NecroIr2**.

Synthesis of Ir(III) complexes

The synthesis of the Ir(III) complexes was achieved from the reaction of cyclometalated chloride-bridged dimer $[Ir(ppy)_2Cl_2]_2$ and the corresponding ancillary ligands (MBOB and BBOB). In a general procedure, 0.12 mmol $[Ir(ppy)_2Cl_2]_2$ and 0.1 mmol ligand were placed in a 50 mL three-necked flask with 20 mL of methanol and chloroform (1:1, v/v) and refluxed for 6 h at 65°C under argon. Then the solvent was removed under reduced pressure, the crude product was purified by

column chromatography on aluminum oxide with CH_2Cl_2/CH_3CH_2OH (10:1, v/v) as the eluent. The obtained complex was then recrystallized with a mixture of CH_2Cl_2/toluene to obtain the Ir(III) complexes.

[Ir(ppy)$_2$(MBOB)]Cl (NecroIr1): yield 51%. Anal. Calc. for $C_{40}H_{29}ClIrN_5O$ (%) C, 58.35; H, 3.55; N, 8.51; Found (%):C, 58.19; H, 3.74; N, 8.83. ES-MS (CH_3OH) m/z: 788.5 $[M\text{-}Cl]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ 9.45 (s, 1H), 9.17 (s, 1H), 8.38 (d, *J* = 5.3 Hz, 1H), 8.29 (t, *J* = 8.8 Hz, 2H), 8.10 (d, *J* = 5.8 Hz, 1H), 7.95 (dt, *J* = 14.9, 7.4 Hz, 6H), 7.77 (d, *J* = 5.8 Hz, 1H), 7.73 (d, *J* = 5.5 Hz, 1H), 7.67 (d, *J* = 5.7 Hz, 1H), 7.60 (t, *J* = 7.2 Hz, 2H), 7.54 (t, *J* = 7.7 Hz, 1H), 7.20 – 7.12 (m, 2H), 7.04 (q, *J* = 7.5 Hz, 2H), 6.93 (q, *J* = 6.8 Hz, 2H), 6.20 (dd, *J* = 18.0, 7.4 Hz, 2H), 2.60 (s, 3H).

[Ir(ppy)$_2$(BBOB)]Cl (NecroIr2): yield 69%. Anal. Calc. for $C_{46}H_{30}ClIrN_6O_2$ (%):C, 59.64; H, 3.26; N, 9.07, Found (%): C, 59.33; H, 3.42 N, 9.18. ES-MS (CH_3OH): m/z 891.6 $[M\text{-}Cl^-]^+$. ^1H NMR (500 MHz, DMSO-d_6) δ 9.74 (s, 2H), 8.43 (dd, *J* = 5.7, 1.6 Hz, 2H), 8.29 (d, *J* = 8.2 Hz, 2H), 8.13 (d, *J* = 5.8 Hz, 2H), 8.00 (d, *J* = 7.8 Hz, 2H), 7.98 – 7.92 (m, 6H), 7.78 (d, *J* = 5.5 Hz, 2H), 7.60 (t, *J* = 7.8 Hz, 2H), 7.54 (t, *J* = 7.6 Hz, 2H), 7.15 (t, *J* = 7.2 Hz, 2H), 7.05 (t, *J* = 7.6 Hz, 2H), 6.94 (t, *J* = 7.5 Hz, 2H), 6.19 (d, *J* = 7.4 Hz, 2H).

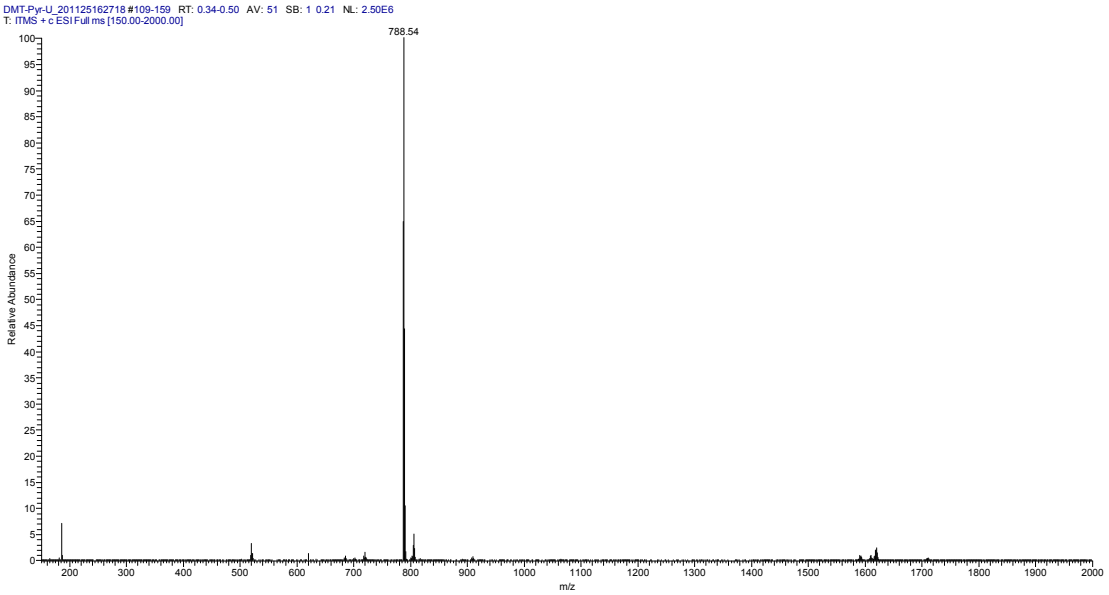


Figure S1. ES-MS characterization of **NecroIr1**.

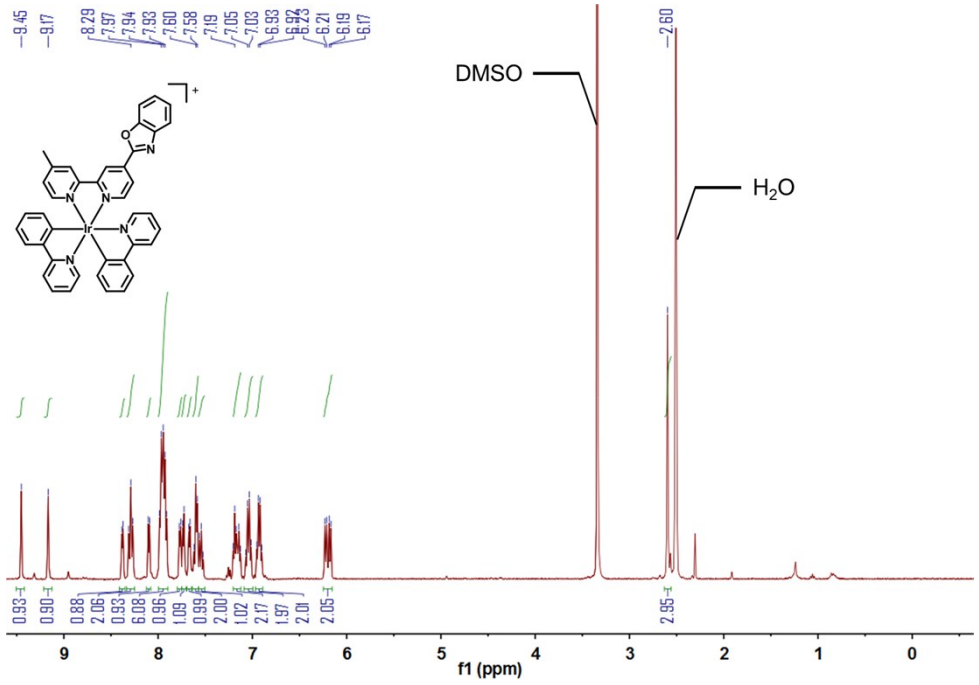


Figure S2. ^{1}H NMR spectrum of **NecroIr1** in DMSO-d_6**.**

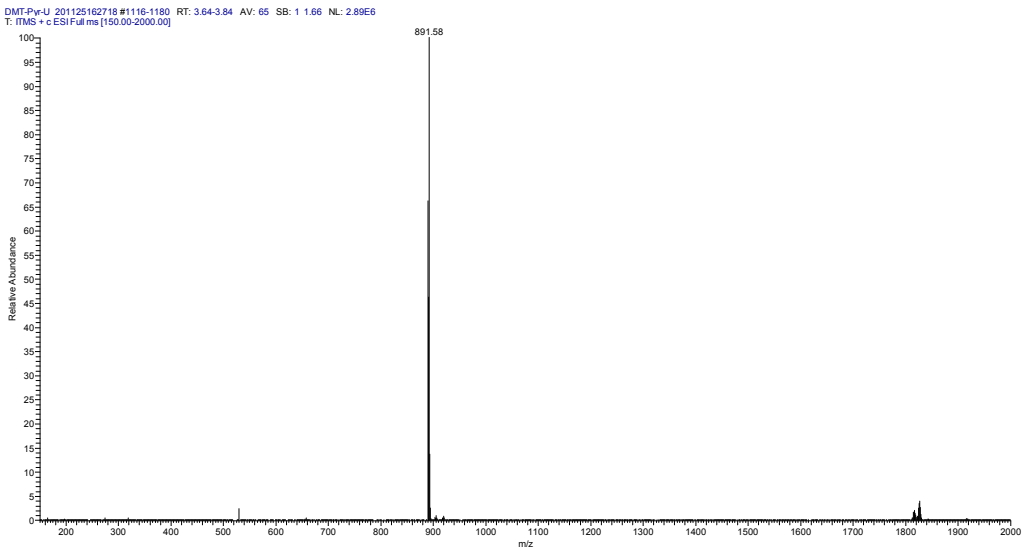


Figure S3. ES-MS characterization of **NecroIr2**.

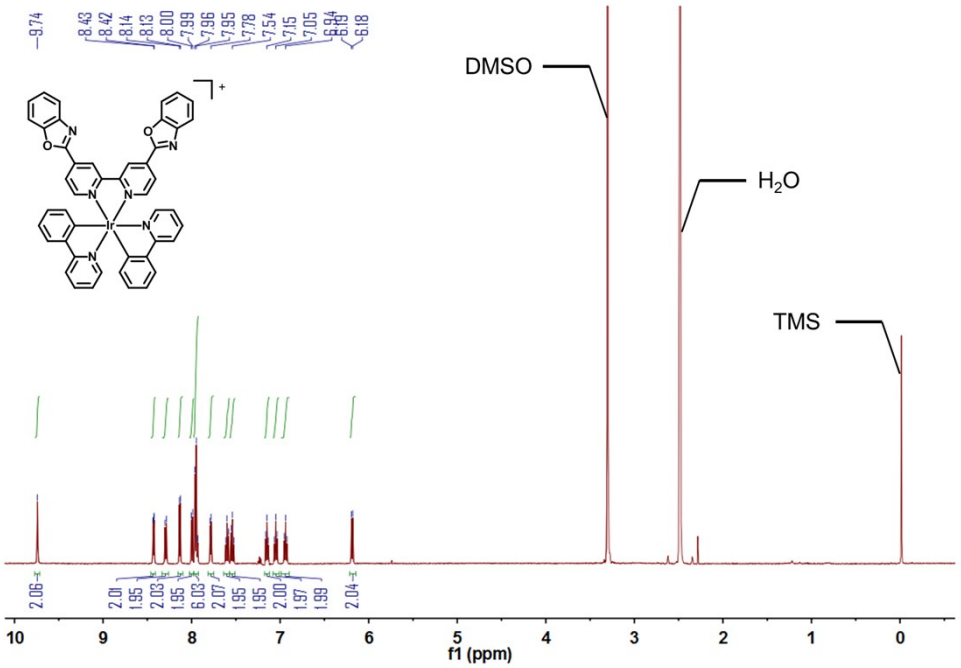


Figure S4. ^{1}H NMR spectrum of **NecroIr2** in DMSO-d_6.

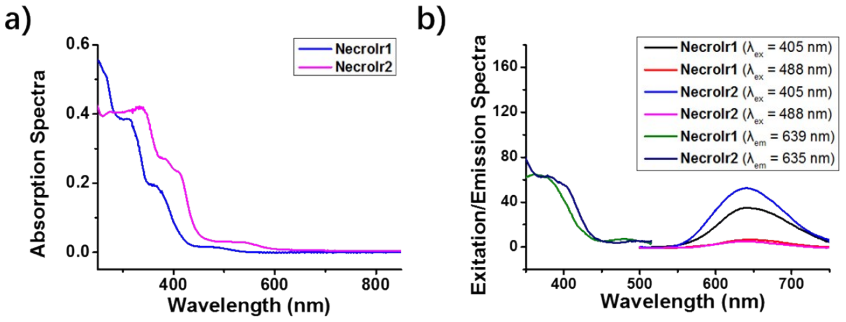


Figure S5. (a) Absorption and (b) excitation/emission spectra of **NecroIr1** and **NecroIr2** (10 μM) measured in CH_3OH at 298 K.

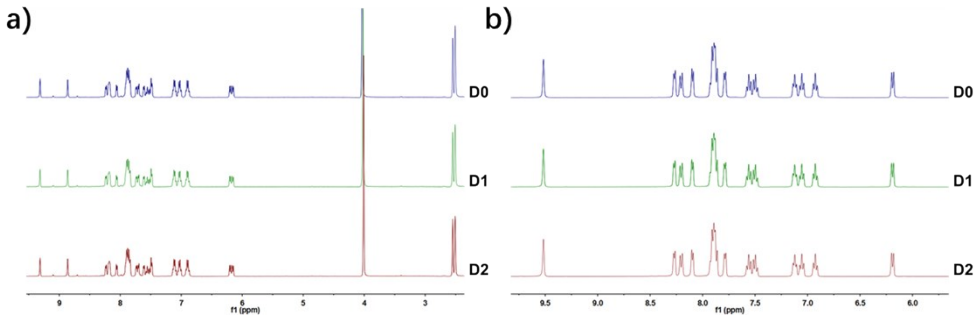


Figure S6. Stability analysis of a) **NecroIr1** and b) **NecroIr2** at room temperature. ^1H NMR spectrum in DMSO/D_2O = 5:1 (V/V) over 2 days.

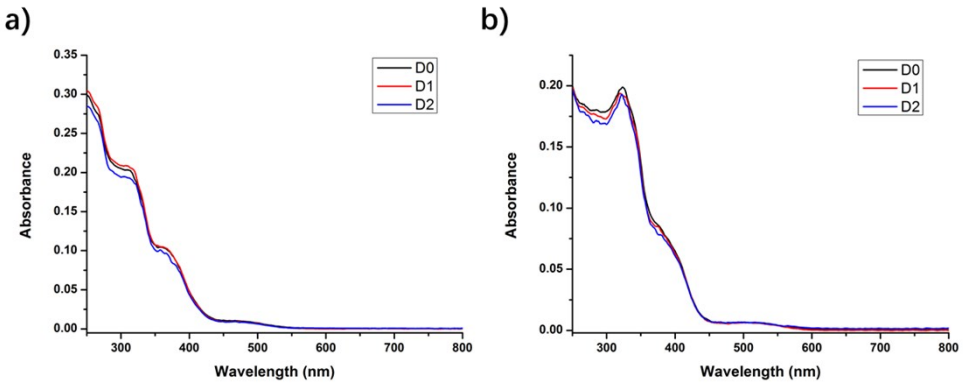


Figure S7. Stability analysis of a) **NecroIr1** and b) **NecroIr2** (10 μM) at room temperature measured in H_2O over 2 days.

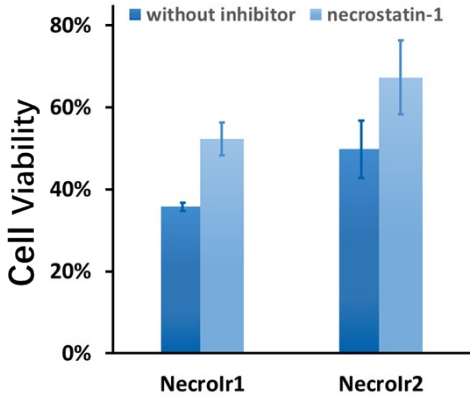


Figure S8. Cell viability with necrostatin-1 coincubation. A549R cell were respectively incubated with **NecroIr1** (5 μM) and **NecroIr2** (1.5 μM) for 24 h, with or without coincubation of necrostatin-1 (60 μM).

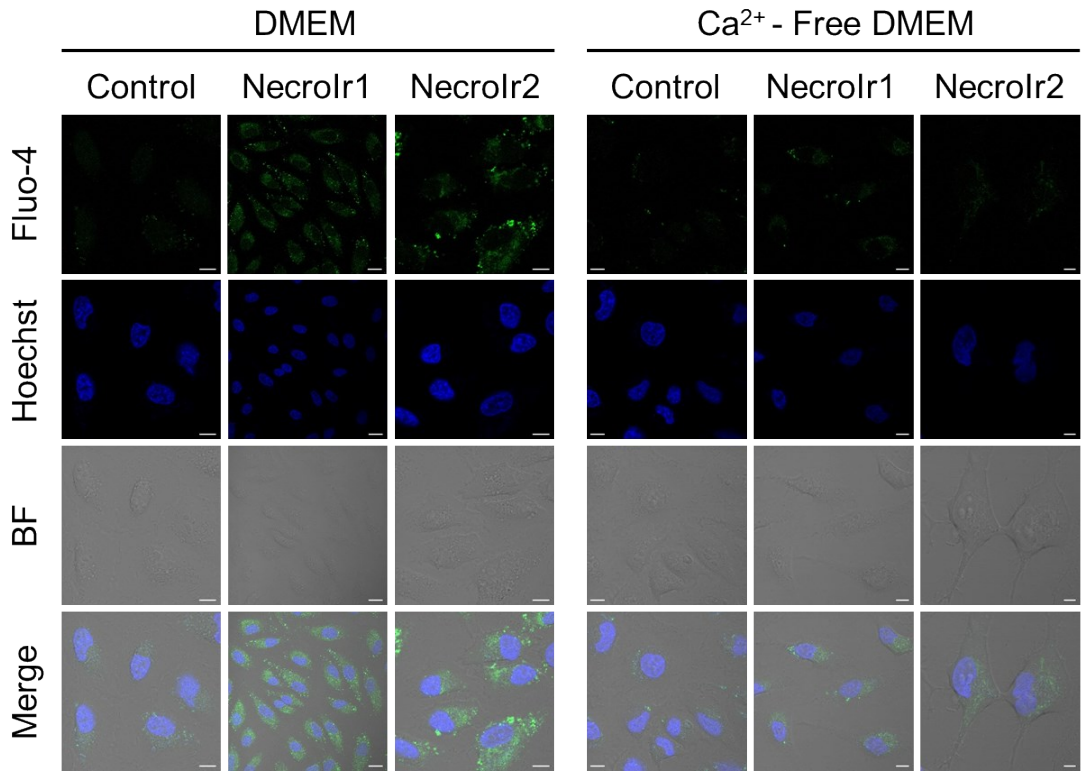


Figure S9. Confocal images of calcium influx. A549R cells were incubated in normal culture media of calcium free media with the co-treatment with 1.5 μM **NecroIr1** and **NecroIr2** for 24 h, respectively. Scale bar: 10 μm.

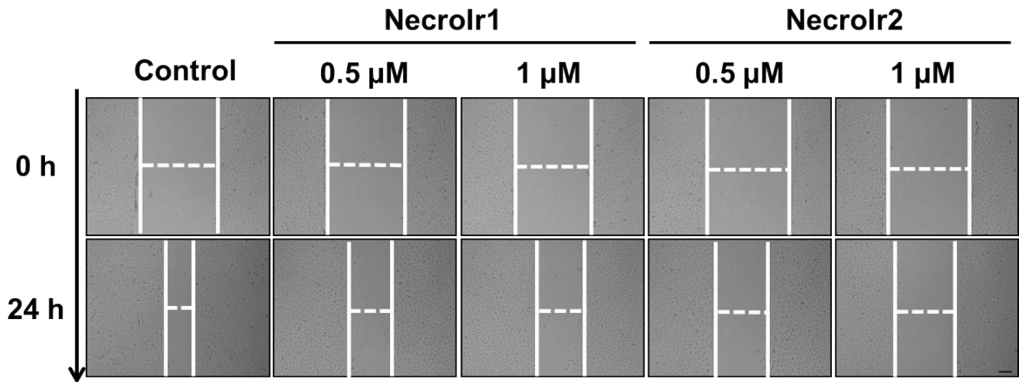


Figure S10. Cell migration tested by wound-healing assay. A549R cells were treated with **NecroIr1** and **NecroIr2** (0.5 μM and 1 μM) for 24 h, respectively. Scale bar: 100 μm.

Table S1. IC_{50} values of Ir(III) complexes (48 h, μM) [a]

	A549	A549R	L02	SGC7901	MDA-MB-231	MCF-7	SW620	HeLa
NecroIr1	1.33 ±0.12	3.21 ±0.06	8.20 ±0.27	1.45 ± 0.13	0.87 ± 0.10	2.73 ± 0.31	2.35 ± 0.11	1.76 ± 0.30
NecroIr2	0.48 ±0.09	1.20 ±0.03	7.62 ±0.15	3.09 ± 0.33	0.85 ± 0.09	7.45 ± 3.73	4.54 ± 0.38	1.16 ± 0.04
CDDP	15.05±0.17	97.79±0.43	9.49±0.13	8.11 ± 1.60	12.74 ± 0.62	18.93 ± 1.09	24.31 ± 3.00	20.20 ± 3.6

[a] IC_{50} values are drug concentrations necessary for 50% inhibition of cell viability. Data are presented as mean ± standard deviations (SD) and cell viability is assessed after 48 h of incubation.

Supporting References

1 E. D. Hawkins, M. Hommel, M. L. Turner, F. L. Battye, J. F. Markham and P. D. Hodgkin, *Nat. Protoc.*, 2007, **2**, 2057-2067.

2 B. J. Quah, H. S. Warren and C. R. Parish, *Nat. Protoc.*, 2007, **2**, 2049-2056.

3 Z. Cai, S. Jitkaew, J. Zhao, H. Chiang, S. Choksi, J. Liu, Y. Ward, L. Wu and Z. Liu, *Nat. Cell Biol.*, 2013, **16**, 55-65.

4 C. Z. Jin, J. P. Liu, Y. Chen, L. L, Zeng, R. L. Guan, C. Ouyang, L. N. and H. Chao, *Chem. Eur. J.*, 2015, **21**: 12000-120010.

5 W. S. Aldridge, B. J. Hornstein, S. Serron, D. M. Dattelbaum, J. R. Schoonover and T. J. Meyer, *J. Org. Chem.*, 2006, **71**, 5186-5190.

6 W. S. Perry, S. J. A. Pope, C. Allain, B. J. Coe, A. M. Kenwright and S. Faulkner, *Dalton Trans.*, 2010, **39**, 10974-10983.

7 S. Yu, X. Gong and W. Chan, *Macromolecules*, 1998, **31**, 5639-5646