Electronic Supplementary Information

Impairment of autophagy-related lysosomal degradation pathway by an anticancer rhenium(I) complex

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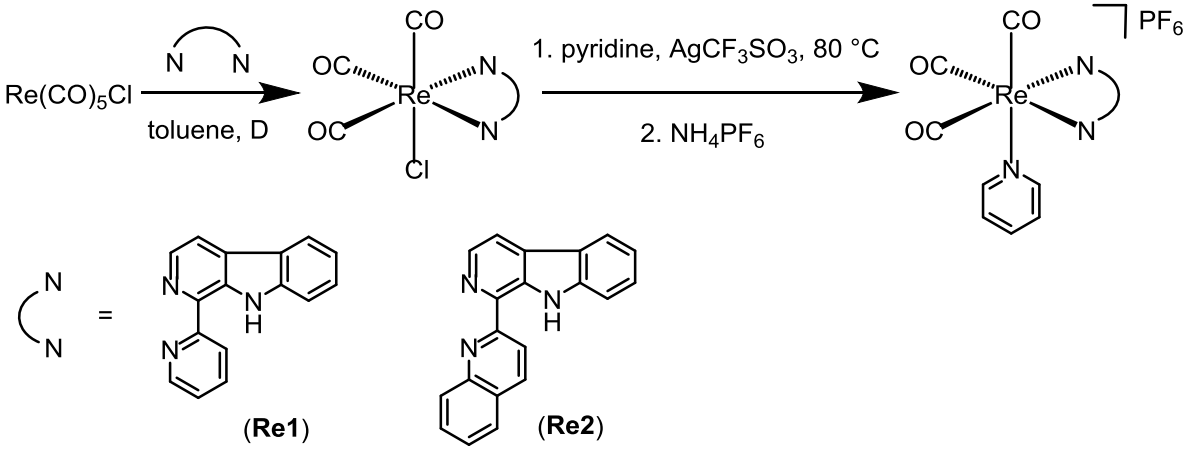
Experimental section

Materials and measurements

$Re(CO)_5Cl$ (Sigma Aldrich, USA), 1,2-bis(4-pyridyl)ethane (BPE, Sigma Aldrich), NH_4PF_6 (Alfa Aesar, USA), Silver trifluoromethanesulfonate ($AgCF_3SO_3$, Sigma Aldrich, USA), disodium hydrogen phosphate (Sigma Aldrich, USA), citric acid (Sigma Aldrich, USA), DMSO (Sigma Aldrich, USA), LysoTracker Green DND-26 (LTG, Life Technologies, USA), MitoTracker Green FM (MTG, Life Technologies, USA), PBS (phosphate buffered saline, Sigma Aldrich, USA), cisplatin (Sigma Aldrich, USA), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich, USA), AO (Sigma Aldrich, USA), Hoechst 33342 (Sigma Aldrich, USA), z-VAD-fmk (Sigma Aldrich, USA), H_2DCF-DA (Sigma Aldrich, USA), *N*-acetyl-*L*-cysteine (NAC, Sigma Aldrich, USA), MnTBAP (Santa Cruz Biotechnology, USA), catalase (Sigma Aldrich, USA), mannitol (J&K Scientific Ltd, China), KI (J&K Scientific Ltd, China), sodium azide (Sigma Aldrich, USA) and dihydroethidium (DHE, Beyotime, China) were used as received. Magic Red MR-$(RR)_2$ was purchased from Immunochemistry Tech (USA). Annexin V-FITC/PI apoptosis detection kit was purchased from Sigma Aldrich (USA). All antibodies were purchased from Cell Signaling Technology (USA) and used as recommended by the manufacturer. All the compounds tested were dissolved in DMSO just before the experiments, and the final concentration of DMSO was kept at 1% (v/v). NMR spectra were recorded on a Bruker Avance 400 spectrometer. Shifts were referenced relative to the internal solvent signals. Microanalysis (C, H, and N) was carried out using an Elemental Vario EL CHNS analyzer (Germany). UV/Vis spectra were recorded on a Varian Cary 300 spectrophotometer (USA). Steady-state emission spectra were performed on an Edinburgh FLS 920 Spectrometer (UK). ESI-MS spectra were recorded on a Thermo Finnigan LCQ DECA XP spectrometer (USA). The quoted m/z values represented the major peaks in the isotopic distribution.

Synthesis and characterization

Ligands **L1** (1-(2-pyridyl)-β-carboline) and **L2** (1-(2-quinolinyl)-β-carboline) were synthesized as previously reported.[1] $[Re(CO)_3(\mathbf{L1})Cl]$ and $[Re(CO)_3(\mathbf{L2})Cl]$ were prepared according to the literature method.[2]



Scheme S1. Synthetic procedures of **Re1** and **Re2**.

General method for Re (I) complexes Re1 and Re2.

[Re(CO)$_3$(**L1**)Cl] or [Re(CO)$_3$(**L2**)Cl] (0.2 mmol, 1 equiv) was dissolved in pyridine (Py, 15 mL), and $AgCF_3SO_3$ (0.2 mmol, 1 equiv) was added. The mixture was heated at 80 ℃ under nitrogen for 24 h in the dark. The mixture was evaporated to dryness under reduced pressure, ethanol (40 mL × 3) was added and evaporated to dryness in order to remove pyridine completely. The solid obtained was dissolved in methanol (20 mL) and the undissolved off-white AgCl was filtered off. The filtrate was added to NH_4PF_6 aqueous solution (30 mL, 1 g/10 mL) and the resulting orange or red precipitate was collected by centrifugation and washed with water and diethyl ether. The desired product was purified by recrystallization from dichloromethane/diethyl ether or acetonitrile/diethyl ether.

[Re(CO)$_3$(L1)(Py)](PF$_6$) (Re1). Complex **Re1** was obtained as orange crystals. Yield: 0.119 g (72%). ^{1}H NMR (400 MHz, d_6-DMSO): δ = 12.38 (s, 1H, H_7), 9.43 (d, J = 5.2 Hz, 1H, H_1), 9.13 (d, J = 5.6 Hz, 1H, H_8), 8.87 (d, J = 8.2 Hz, 1H, H_3), 8.74 (d, J = 5.6 Hz, 1H, H_2), 8.53 (t, J = 8.2 Hz, 2H, H_{12} and H_{16}), 8.39 (d, J = 5.5 Hz, 2H, H_6 and H_{11}), 7.98–7.88 (m, 2H, H_9 and H_{10}), 7.84–7.75 (m, 2H, H_{13} and H_{15}), 7.47 (t, J = 7.2 Hz, 1H, H_{14}), 7.40 (t, J = 6.6 Hz, 2H, H_4 and H_5). IR (KBr, cm^{-1}): ν = 2029, 1928 (CO); 3437 (indolyl-*NH*). ESI-MS (CH_2Cl_2): m/z calcd for [M−PF$_6$]$^+$, 594.62; found: 594.7. Elemental analysis calcd (%) for $C_{24}H_{16}F_6N_4O_3PRe \cdot CH_2Cl_2$: C, 36.42; H, 2.20; N, 6.80; found: C, 36.51; H, 2.12; N, 6.81.

[Re(CO)$_3$(L2)(Py)](PF$_6$) (Re2). Complex **Re2** was obtained as red crystals. Yield: 0.120 g (76%). ^{1}H NMR (400 MHz, d_6-DMSO): δ = 12.51 (s, 1H, H_7), 9.25 (d, J = 5.6 Hz, 1H, H_1), 9.15 (d, J = 8.7

Hz, 1H, H_8), 8.90–8.74 (m, 3H, H_3, H_{12} and H_{16}), 8.54 (d, *J* = 7.9 Hz, 1H, H_2), 8.43 (d, *J* = 8.1 Hz, 1H, H_6), 8.29 (t, *J* = 7.8 Hz, 1H, H_{11}), 8.03 (t, *J* = 7.5 Hz, 1H, H_9), 7.83 (dt, *J* = 19.4, 6.4 Hz, 5H, H_{10}, H_{13}, H_{15}, H_{17} and H_{18}), 7.48 (t, *J* = 7.2 Hz, 1H, H_{14}), 7.26 (t, *J* = 6.7 Hz, 2H, H_4 and H_5). IR (KBr, cm^{-1}): ν = 2028, 1917, 1899 (CO); 3414 (indolyl-*NH*). ESI-MS (CH_2Cl_2): m/z calcd for $[M-PF_6]^+$, 644.67; found: 644.8. Elemental analysis calcd (%) for $C_{28}H_{18}F_6N_4O_3PRe$: C, 42.59; H, 2.30; N, 7.10; found: C, 42.56; H, 2.18; N, 7.16.

X-ray Crystallography[3]

Single crystals of **Re2** were grown by diffusion of diethyl ether into a dilute dichloromethane solution of the complex. X-ray diffraction data were collected on a Bruker Smart 1000 CCD diffractometer with Mo Kα radiation (0.71073 Å) at 298 K. The structure of **Re2** was solved by direct methods with program SHELXS and refined using the full-matrix least-squares program SHELXL. The structure was plotted using the xp package in SHELXTL at a 50% thermal ellipsoids probability level.

Cell lines and culture conditions

A549, A549cisR, HeLa, MCF-7 and HLF cells were obtained from Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Cells were maintained in DMEM (Dulbecco's modified Eagle's medium, Gibco BRL) or RPMI 1640 (Roswell Park Memorial Institute 1640, Gibco BRL) medium, which contained 10% FBS (fetal bovine serum, Gibco BRL), 100 μg/mL streptomycin (Gibco BRL), and 100 U/mL penicillin (Gibco BRL). The cells were cultured in a humidified incubator, which provided an atmosphere of 5% CO_2 and 95% air at a constant temperature of 37 ℃. In each experiment, cells treated with vehicle control (1% DMSO) were kept as the reference group.

Cellular uptake and colocalization assay

Cellular uptake: A549 cells were treated with **Re1** (10 μM) or **Re2** (5 μM) at 37 ℃ for 1 h, then washed three times with ice-cold PBS and visualised by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 580 ± 30 nm (**Re1**) or 630 ± 30 nm (**Re2**) upon excitation at 405 nm.

Colocalization assay: A549 cells were incubated with **Re1** (10 μM) or **Re2** (5 μM) at 37 ℃ for 0.5 h

and further co-incubated with LTG (150 nM) or MTG (150 nM) for another 0.5 h. Cells were washed three times with PBS and visualized by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany) with a 63× oil-immersion objective lens immmediately. The exicitaiton wavelength for Re(I) complexes is 405 nm. The exicitaiton wavelength of LTG and MTG is 488 nm. Emission was collected at 580 ± 30 nm (**Re1**), 630 ± 30 nm (**Re2**), 511 ± 20 nm (LTG) and 516 ± 20 nm (MTG).

Cytotoxicity[3]

The cytotoxicity of the tested compounds toward A549, A549cisR, HeLa, MCF-7, HepG2, LO2 and HLF cells was determined by MTT assay. Cells cultured in 96-well plates were grown to confluence. The compounds were dissolved in DMSO (1%, v/v), and diluted with fresh media immediately. the cells were incubated with a series of concentrations of the tested compounds for 44 h at 37 ℃. 20 μL of MTT solution was then added to each well, and the plates were incubated for an additional 4 h. The media was carefully removed, and DMSO was added (150 μL per well) and incubated for 10 min with shaking. The absorbance at 595 nm was measured using a microplate reader (Infinite M200 Pro, Tecan, Männedorf, Switzerland).

AO staining

A549 cells seeded into 35 mm dishes (Corning) were treated with **Re2** for 4 h. The cells were then washed twice with PBS and incubated with AO (5 μM) at 37 ℃ for 15 min. The cells were washed twice with PBS and visualized by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 510 ± 20 nm (green) and 625 ± 20 nm (red) upon excitation at 488 nm.

Cathepsin B activity detection[4]

Cathepsin B activity was detected using the fluorogenic susbtrate Magic Red MR-$(RR)_2$ (Immunochemistry Tech, Bloomington, USA) according to the manufacturer's instructions. Briefly, A549 cells seeded into 35 mm dishes (Corning) were treated with **Re2** for 4 h. The media was removed and the cells were washed twice with PBS and then incubated with cathepsin B substrate at 37 ℃ for 1 h. The media was removed and the cells were washed twice with PBS and visualized by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 630 ± 20 nm upon excitation at 543 nm.

GFP-LC3 analysis[5]

A549 cells were transfected with GFP-LC3 vector using Lipofectamine 2000 (Life Technologies, USA). After 12 h, cells were treated with **Re2** (4 μM) for 12 h. Cells were fixed with 4% paraformalclehyde for 30 min at room temperature, washed twice with ice-cold PBS, and then visualized by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 510 ± 20 nm upon excitation at 488 nm.

Western blotting

The assays were performed according to similar procedures previously reported.[6]

Transmission electron microscopy[5]

A549 cells were treated with different concentrations of **Re2** for 24 h. Cells were collected and fixed overnight at 4 ℃ in phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. The cells were then treated with osmium tetroxide, stained with uranyl acetate and lead citrate, and visualized under a transmission electron microscope (JEM 100 CX, JEOL, Tokyo, Japan). Images were photographed using the Eversmart Jazz program (Scitex).

Hoechst 33342 staining[3]

A549 cells were treated with different concentrations of **Re2** for 24 h. The cells were washed twice with PBS gently and fixed with 4% paraformalclehyde for 30 min at room temprature. The cells were washed with PBS twice, and then Hoechst 33342 (5 μg/mL) in PBS was added to the medium by gently shaking in the dark for 10 min. The cells were washed with PBS twice and visualized by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 460 ± 20 nm upon excitation at 405 nm.

Annexin V/PI double staining assay

The assay was performed according to the manufacturer's (Sigma Aldrich, USA) protocol. After treatment with **Re2** for 24 h, the cells were washed twice with PBS, and then incubated with 500 μL annexin-binding buffer supplemented with 5 μL annexin V and 10 μL propidium iodide at room temperature for 15 min in the dark. The samples were visualized immediately by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 530 ± 20 nm

(annexin V) and 620 ± 20 nm (propidium iodide) upon excitation at 488 nm.

Cellular ROS detection[3]

Flow cytometry. A549 cells seeded into 6-well plates were treated with different concentrations of **Re2** at 37 ℃ for 4 h. The cells were collected and incubated with 10 μM H_2DCF-DA for 20 min at 37 ℃ in the dark. The cells were washed twice with PBS, and the fluorescence intensity of DCF in A549 cells was measured by flow cytometry (FACSCalibur™, Becton Dickinson, NJ, USA) with excitation at 488 nm and emission at 530 nm. Data were analysed by FlowJo software (Tree Star, OR, USA). Ten thousand events were acquired for each sample.

Confocal microscopy. A549 cells seeded in 35-mm tissue culture dishes (Corning, USA) were treated with different concentrations of **Re2** at 37 ℃ for 4 h. The cells were washed twice with PBS and then incubated with 10 μM H_2DCF-DA in serum-free RPMI 1640 for 20 min at 37 ℃ in the dark. The cells were washed twice with PBS and visualized immediately by confocal microscopy (LSM 710, Carl Zeiss, Göttingen, Germany). Emission was collected at 520 ± 20 nm upon excitation at 488 nm. To measure superoxide production, cells were loaded with 5 μM dihydroethidium (DHE) for 10 min in the dark, washed with PBS, and visualized immediately by confocal microscopy. Emission was collected at 570−600 nm upon excitation at 514 nm.

***In vivo* antitumor evaluation of Re2**

The assays were performed according to the same procedures previously reported.[3] All animal experiments were conducted under the guidelines approved by the Sun Yat-Sen University Animal Care and Use Committee. The nude mice were treated with solvent control or **Re2** (5 mg/kg) (n = 4) every 3 days for 21 days by intraperitoneal injection.

Histological analysis (H&E staining) and Immunohistochemical Staining

The assays were performed according to similar procedures previously reported.[3]

Statistical analysis

All biological experiments were performed at least twice with triplicates in each experiment. Representative results were depicted in this report and data were presented as means ± standard deviations (SD).

Supporting figures and tables

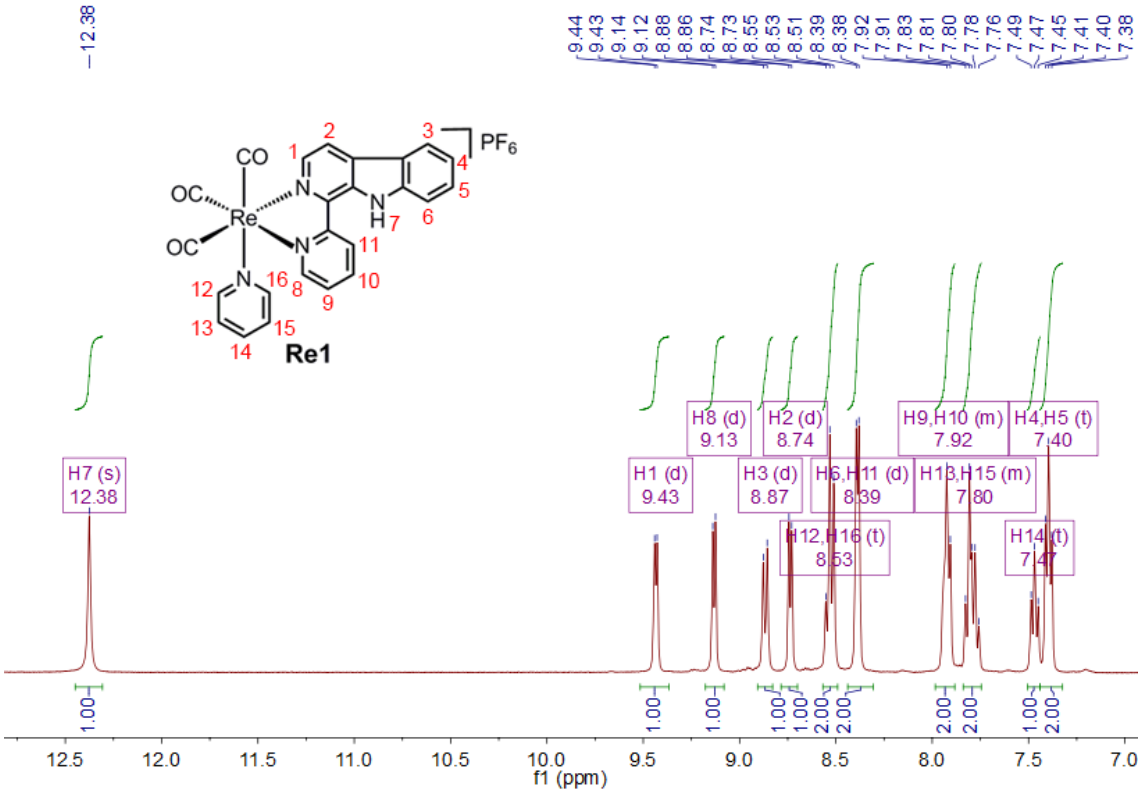


Fig. S1 ^{1}H NMR spectrum of **Re1** in d_6-DMSO at 298 K.

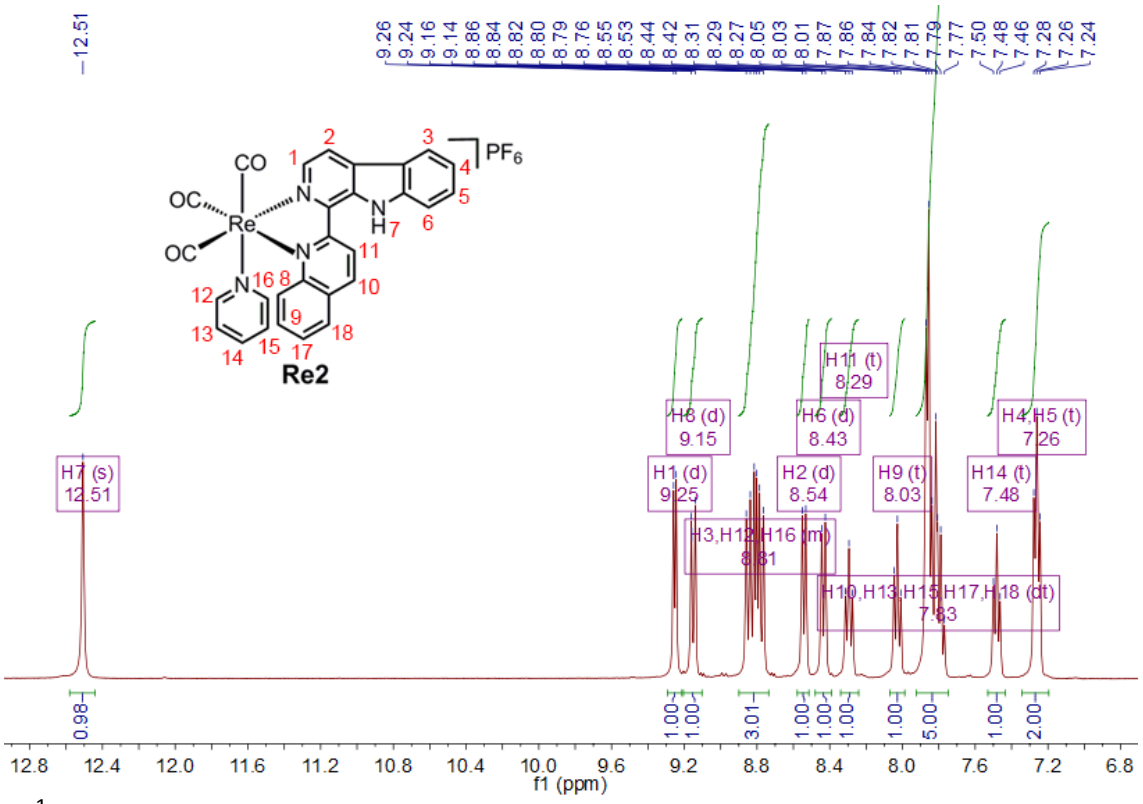


Fig. S2 ^{1}H NMR spectrum of **Re2** in d_6-DMSO at 298 K.

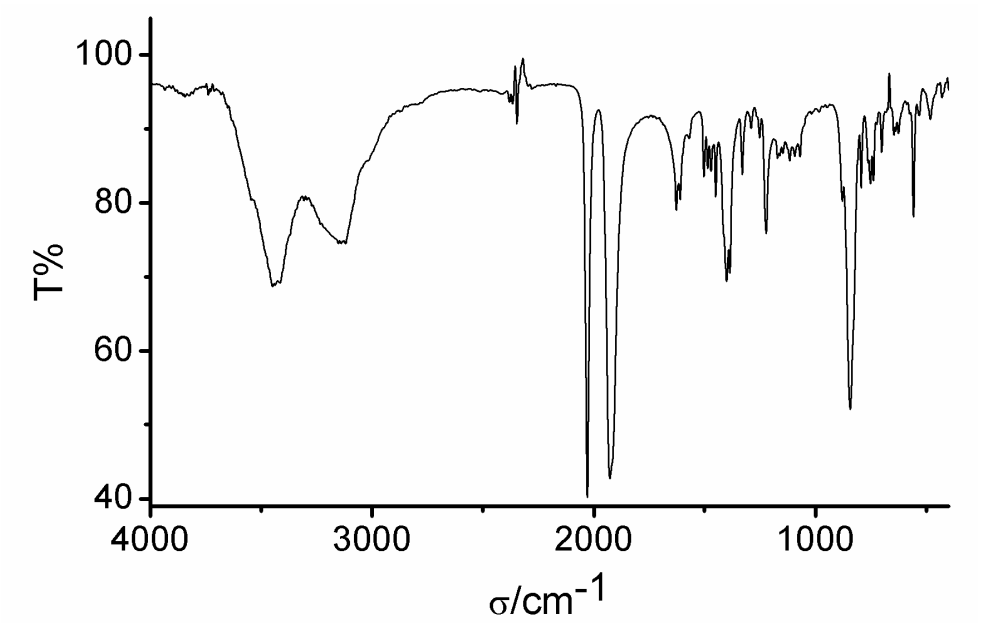


Fig. S3 Infrared spectrum of **Re1**.

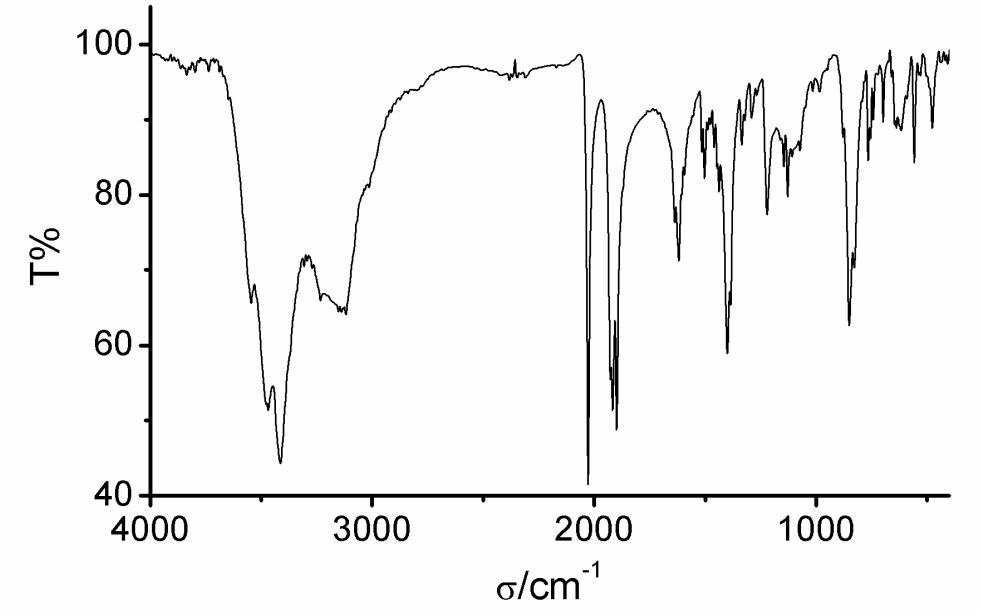


Fig. S4 Infrared spectrum of **Re2**.

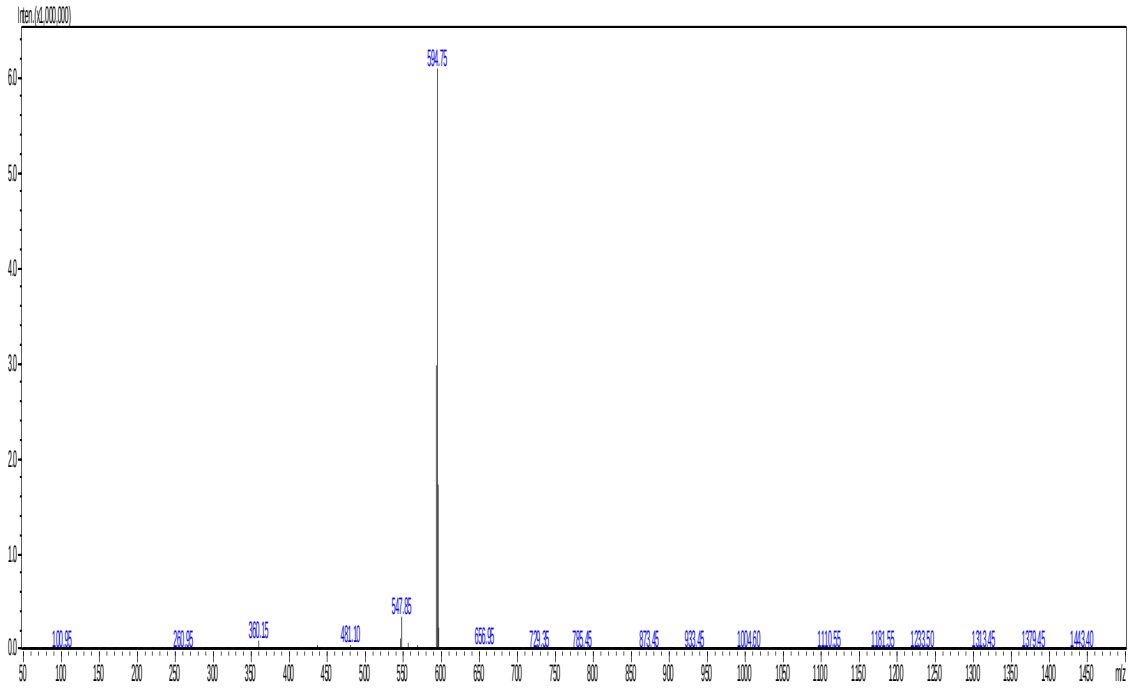


Fig. S5 ESI-MS spectrum of **Re1** in CH_2Cl_2.

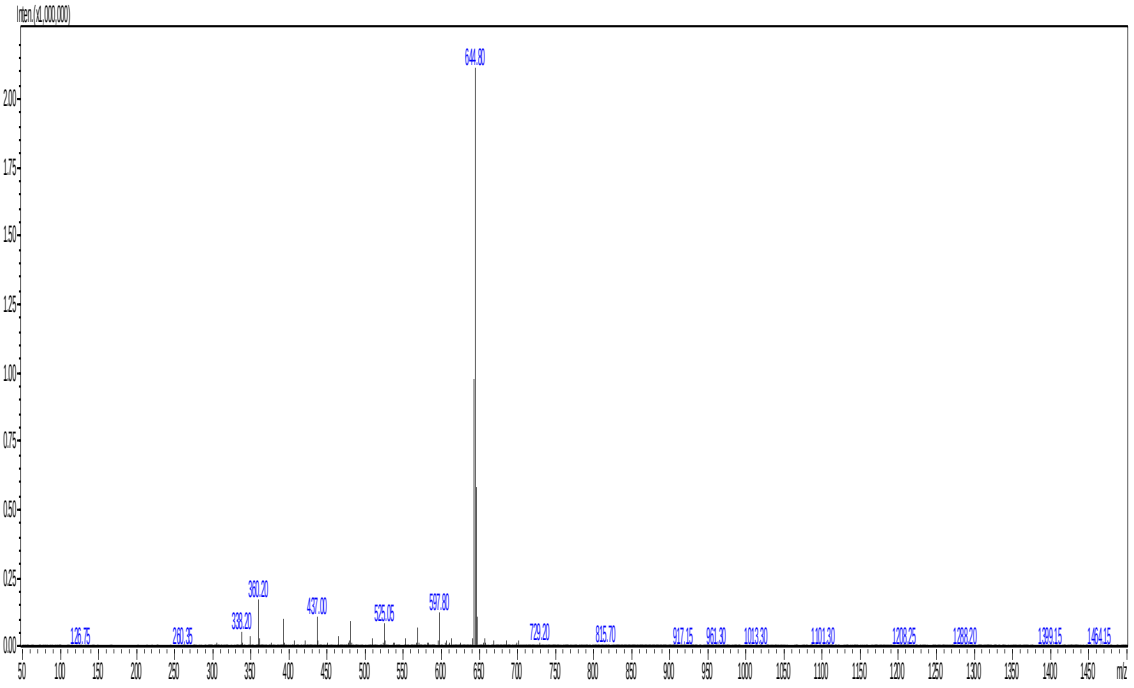


Fig. S6 ESI-MS spectrum of **Re2** in CH_2Cl_2.

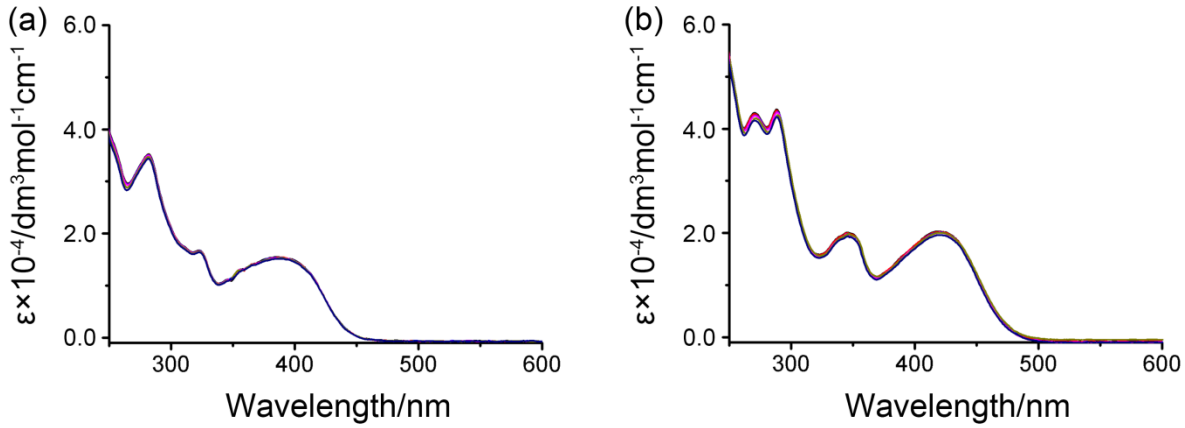


Fig. S7 Time-dependent UV/vis absorption spectra of (a) **Re1** and (b) **Re2** (10 μM) measured in PBS/DMSO (4:1, v/v) at 298 K. Time: 0 h, 3 h, 6 h, 12 h, 24 h.

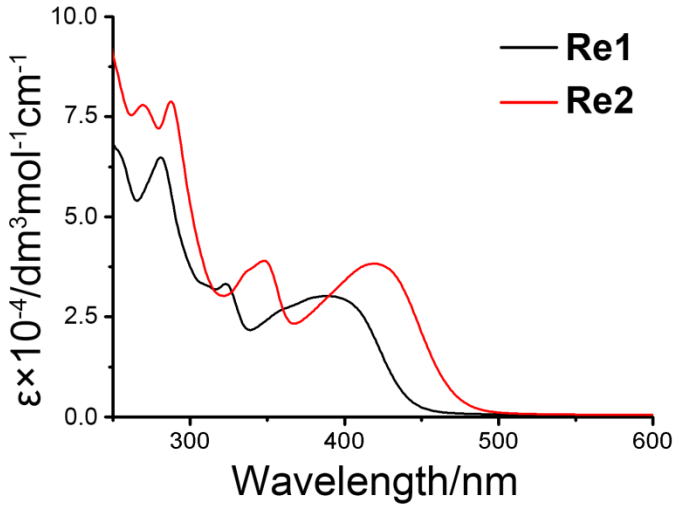


Fig. S8 UV/Vis spectra of **Re1** and **Re2** (10 μM) measured in CH_3CN at 298 K.

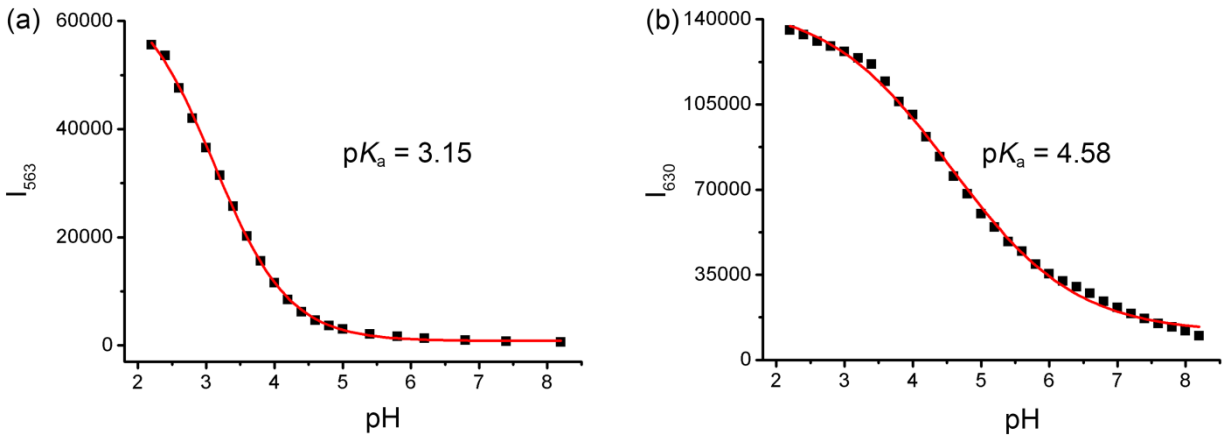


Fig. S9 Plots of emission intensity of (a) **Re1** and (b) **Re2** at 563 and 630 nm versus different pH values, respectively.

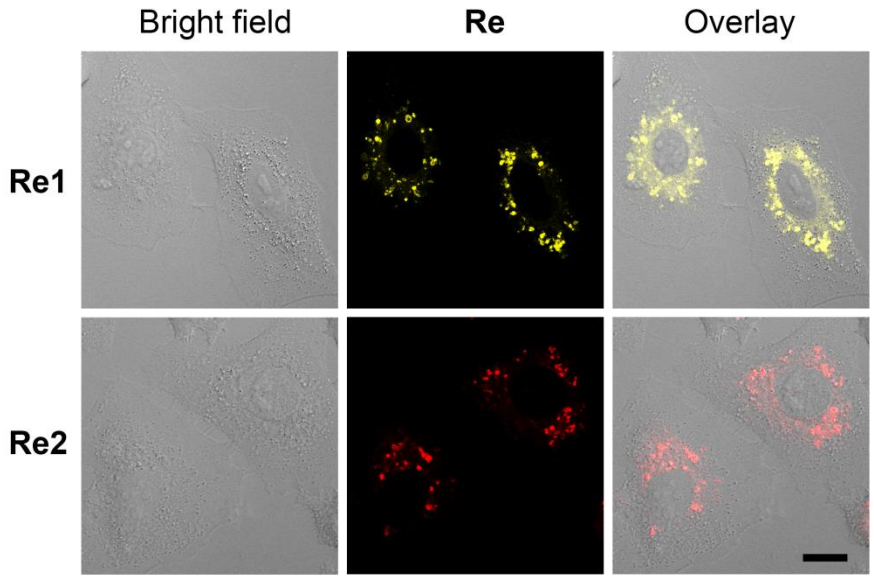


Fig. S10 Cellular uptake of **Re1** (10 μM, 1 h) and **Re2** (5 μM, 1 h) analyzed by confocal microscopy in A549 cells. Scale bar: 10 μm.

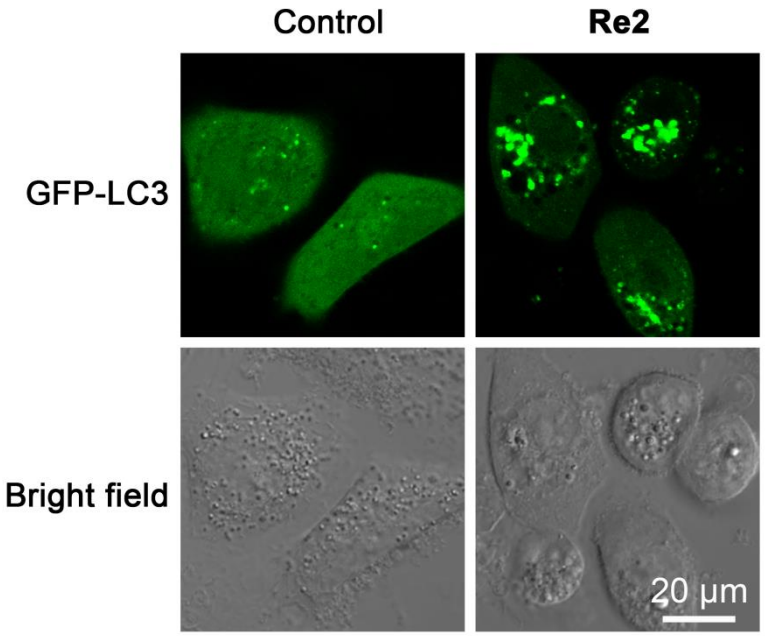


Fig. S11 A549 cells transfected with GFP-LC3 were treated with control or **Re2** (4 μM) for 12 h. The cells were examined by confocal microscopy using filters for GFP fluorescence.

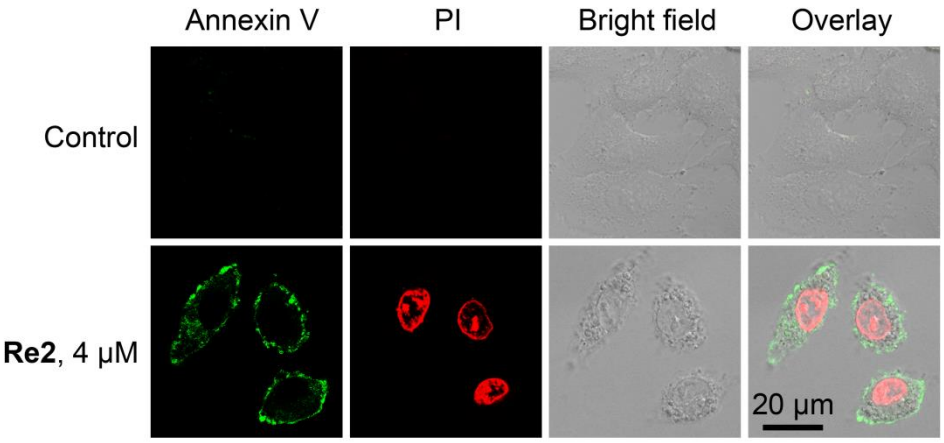


Fig. S12 Detection of apoptosis in A549 cells stained with annexin V and PI by confocal microscopy after A549 cells were incubated with **Re2** for 24 h.

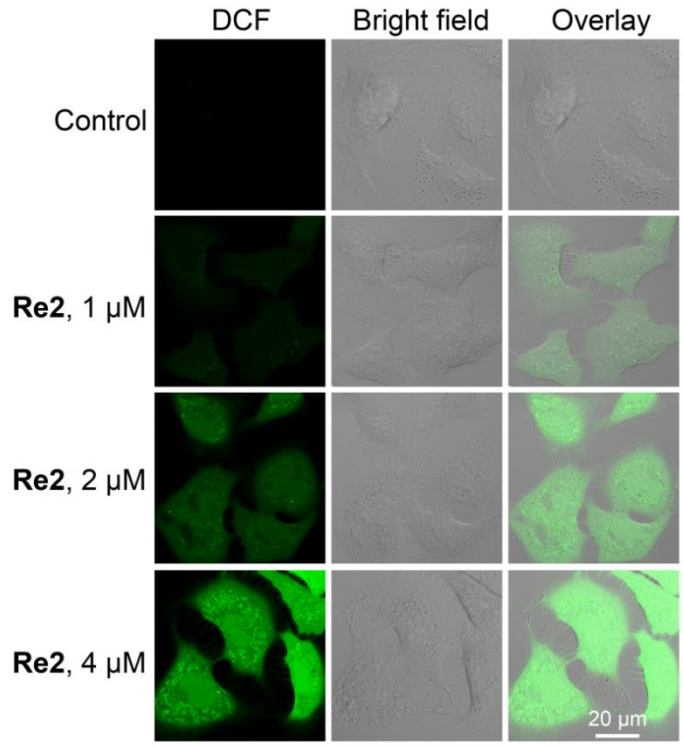


Fig. S13 Analysis of ROS generation by confocal microscopy with a ROS probe, H_2DCF-DA. A549 cells were incubated with **Re2** at the indicated concentrations for 4 h.

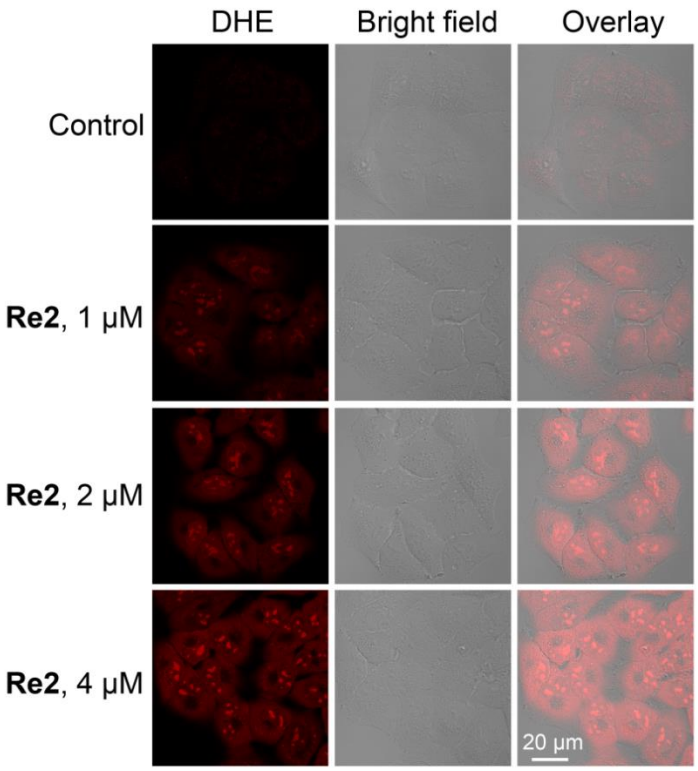


Fig. S14 Detection of superoxide after A549 cells were incubated with control or **Re2** for 1 h by staining with dihydroethidium (DHE).

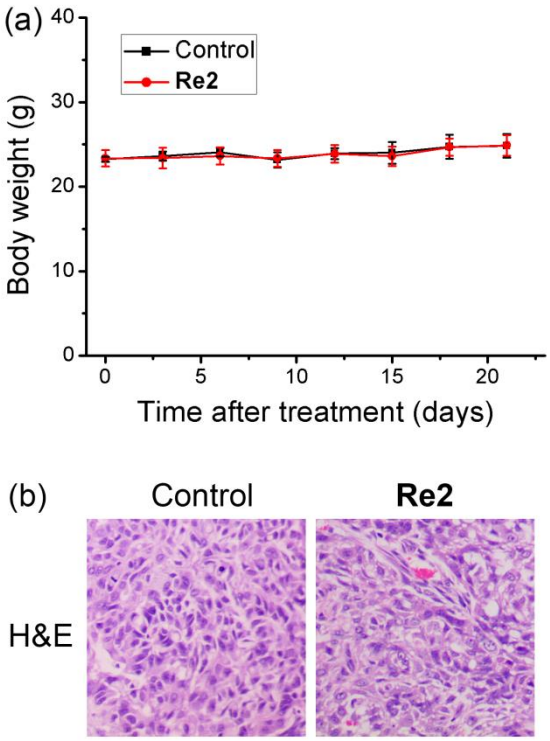


Fig. S15 (a) Body weights of mice after treatment with solvent control or **Re2** (5 mg kg^{-1}) through intraperitoneal injection. Each group contained four mice and the results are reported as the values of the mean ± SEM. (b) Representative H&E images of tumor tissues in different treatment groups.

Table S1 Crystallographic data of **Re2**

Complex	**Re2**
CCDC no.	1812912
Empirical formula	$C_{28}H_{18}N_4O_3PF_6Re$
Molecular weight	789.63
Description	Block, yellow
Temperature (K)	298 K
λ (Å)	0.71073
Crystal system	monoclinic
Space group	$P2_1/c$
a (Å)	15.2907(12)
b (Å)	11.2852(13)
c (Å)	19.3363(15)
α (^{o})	90
β (^{o})	125.149(5)
γ (^{o})	90
Volume, Å^3	2728.2(4)
Z	4
μ/mm^{-1}	4.593
F(000)	1528.0
θ_{max} (deg)	27.480
Completeness to θ_{max}	0.998
ρ_{calc} (g/cm^3)	1.922
[R_{int}]	0.0615
R1[a][I > 2σ(I)]	0.0495
wR2[a]	0.1474
GOF[b]	1.090

[a] $R1 = \sum \| F_0 | - | F_c \| / \sum | F_0 |, wR2 = \left\{ \sum \left[w \left(F_0^2 - F_c^2 \right)^2 \right] / \sum \left[w \left(F_0^2 \right)^2 \right] \right\}^{1/2}$ [b] $GOF = \left\{ \sum \left[w \left(F_0^2 - F_c^2 \right)^2 / \left(n - p \right) \right] \right\}^{1/2}$

where *n* is the number of data and *p* is the number of parameters refined.

Table S2 Selected bond lengths (Å) and bond angles (deg) of **Re2**

Complex	**Re2**	
bond lengths (Å)	Re1–N1	2.209(4)
	Re1–N2	2.163(6)
	Re1–N3	2.231(5)
	Re1–C26	1.899(6)
	Re1–C27	1.939(7)
	Re1–C28	1.935(6)
bond angles (deg)	N1–Re1–N2	74.89(16)
	N1–Re1–N3	80.39(17)
	N2–Re1–N3	84.43(19)
	C26–Re1–C27	84.7(3)
	C26–Re1–C28	87.9(3)
	C26–Re1–N2	96.8(2)

Supporting references

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