Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

An organoruthenium complex overcomes ABCG2-mediated multidrug resistance via multiple mechanisms

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Contents

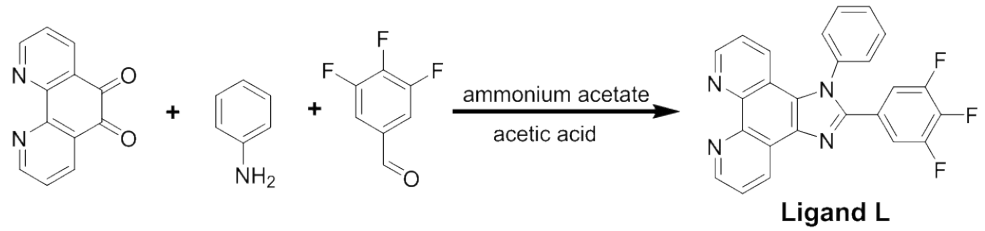
Experimental Section:

Materials

All reagents were purchased from commercial sources in reagent grade and were used as received, unless stated otherwise. The ^{1}H NMR spectrum of the complexes were recorded using a Varian Mercury Plus 400 Nuclear Magnetic Resonance Spectrometer. ^{13}C NMR spectrums were recorded using a Varian Mercury Plus 500 Nuclear Magnetic Resonance Spectrometer. Electrospray ionization mass spectra (ESI-MS) were recorded using an LCQ system (Finnigan MAT, USA), and microanalyses (C, H, and N) were conducted using an Elemental Vario EL CHNS analyzer (Germany). The z-values stated in this paper represent the major peaks in the isotope distribution. All ruthenium complexes were dissolved in 100% DMSO, and the concentration of DMSO was less 1% (v/v). Cisplatin was dissolved in a NaCl solution (nomal saline). Ru(III) chloride hydrate, mitoxantrone, 2′,7′-dichlorofluorescein diacetate, 5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), propidium iodide (PI), acridine orange (AO), ethidium bromide (EB), cisplatin, ATP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), MTR-red and Hoechest were purchased from Sigma-Aldrich and Energy Chemica. The antibodies for the proteins ABCG2, caspase-3, caspase-7, caspase-9 and β-actin (monoclonal) were purchased from GeneTex (Irvine, CA). The ribonucleic acid (RNA) extraction reagent kit (RNAiso plus) and the Prime Script™ RT reagent Kit with cDNA Eraser for Perfect Real Time were purchased from Sigma-Aldrich and Cell Signalling Technology Inc. (Beverly, MA). The Edu Kit was purchased from Ruibo Technology (Guangzhou, China).

Synthesis and characterization

Synthetic procedure of Ligand L: A mixture of 1,10-phenanthroline-5,6-dione (0.3 mmol), phenylamine (0.3 mmol), 3,4,5-trifluorobenzaldehyde (0.3 mmol) and ammonium acetate (3.0 mmol), in glacial aceticacid (40 mL), was refluxed under argon at 130 °C for 12 h to obtain a pale-yellow solid. The solid was purified by column chromatography using dichloromethane-ethyl acetate (10:1) as the eluent.



Ligand L

The synthetic protocols for ligand **L**.

Synthetic procedure for RuF: $[Ru(C_6H_6)Cl_2]_2$ (0.50 mmol) and 2-phenylpyridine (0.50 mmol) were added into a MeCN solution (15 mL) and stirred at 45 °C for 24 h. After an anion exchange with NH_4PF_6, complex **a** was obtained as a yellow solid. 2,2-bipyridine (0.50 mmol) was added to the complex **a** solution and the mixture was stirred at room temperature for 12 h, yielding complex **b**. Complex **b** was subjected to photochemical conditions in a acetonitrile solution under a 5.5 W UV lamp at room temperature for 6 h. The isomer of **b** was produced and purified by column chromatography using CH_3CN-CH_2Cl_2 (1:9) as the eluent. Finally, ligand **L** (0.50 mmol) and isomer of **b** were refluxed in 15 mL methanol for 24 h and the solvent was evaporated under vacuum, producing a dark residue. The dark residue was dissolved in dichloromethane (5 mL) and the solution was purified through aluminium trioxide using a CH_3CN/toluene (1:10 to 10:1, v/v) eluent. The purple pure product was collected. (Yield: 30%). Anal. ESI-MS (m/z). 838 $(M\text{-}PF_6)^+$. Calcd for $C_{46}H_{29}F_9N_7Ru$ (%): C, 56.14; H, 2.50; N, 9.97. Found (%): C, 56.01; H, 2.82; N, 9.63. ^{1}H NMR (400 MHz, DMSO) δ 8.98 (1 H, d, J 8.1), 8.69 – 8.60 (2 H, m), 8.31 (1 H, d, J 5.2), 8.18 (1 H, d, J8.1), 8.09 (1 H, d, J 5.0), 7.96 (2 H, d, J 8.0), 7.89 (3 H, dd, J 16.7, 9.4), 7.85 – 7.75 (4H, m), 7.71 (2 H, dd, J 14.8, 7.9), 7.50 (3 H, dd, J 10.4, 6.6), 7.41 (3 H, dd, J 14.1,5.9), 7.23 (1 H, t, J 6.6), 6.96 – 6.82 (4 H, m), 6.45 (1 H, d, J 7.1). ^{13}C NMR (126 MHz, DMSO-d_6): d = 167.19, 156.94, 156.66, 153.35, 151.36, 150.42, 150.16, 149.50, 147.70, 147.02, 145.79, 144.75, 136.66, 136.32, 135.38, 134.57, 131.82, 131.38, 129.28, 128.81, 128.37, 128.14, 127.11, 126.96, 126.53, 126.25, 125.77, 124.73, 123.83, 122.98, 121.34, 119.46, 114.66 ppm. ^{19}F NMR (400 MHz, DMSO) δ -158.25 (1 F, t), -133.84 (1 F, d), -70.11 (1 F, d).

ABCG2 ATPase assay: The ABCG2 ATPase assay was performed as previously described.[1] ABCG2 membrane vesicles were incubated in an ATPase assay buffer, with or without vanadate, for 5 min at 37 °C. The **RuF** complex was added to the assay buffer in a concentration gradient and incubated for 3 min 37 °C. Ten µL of 25 mM Mg-ATP was added to the assay buffer and the reaction mixture was incubated for another 20 min at 37 °C. The reactions were stopped by adding 100 µL of a 5% SDS solution. Two hundred µL of the detection reagent was added and the absorption at 880 µm was measured following a 10-minute incubation period.

ABCG2 induced-fit docking and molecular dynamics simulation: A human ABCG2 homology model and docking grids were prepared and defined as previously described.[2] The structures of the ruthenium complexes and mitoxantrone were constructed and prepared using the Ligprepv3.3 module (Schrödinger, Cambridge, MA, USA, 2015). The energy minimized compounds were subjected to Glide XP (extra precision) docking (Glide v6.6, Schrödinger, Cambridge, MA, USA, 2015). In order to study the interactions of **RuF** with the ABCG2 transporter, the structure of **RuF** was subjected to Glide XP docking at both of the transmembrane domains (TMD). Mitoxantrone (a substrate of

ABCG2) was docked into the TMD of the ABCG2 protein as a control. All docking simulations were performed using identical protocols.

Cell line and culture conditions: The human non-small lung cancer cell line H460 and H460/MX20cells were kindly provided by Dr. Susan Bates (NIH, Bethesda, MD). The S1-M1-80 cells, derived from colon carcinoma S1 cells, are mitoxantrone-selected, ABCG2-overexpressing cells. HEK/ABCG2-482R, HEK/ABCG2-R482G and HEK/ABCG2-R482T were established by transfecting HEK293/pcDNA3.1 cells with either an empty pcDNA3.1 vector by inserting the ABCG2 gene. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) or Roswell Park Memorial Institute 1640 (RPMI 1640, Gibco BRL) medium, supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and 1% PS (100 U/mL penicillin, 100 μg/mL streptomycin, Gibco BRL), in a humidified incubator under 5% CO_2 and 20% O_2 at 37 °C. To maintain the resistant phenotype, H460/MX20, S1-M1-80, HEK/ABCG2-482R, HEK/ABCG2-R482G and HEK/ABCG2-R482T cells were incubated with different concentrations of mitoxantrone and cultured in drug-free medium for at least two weeks before use.

Cytotoxicity assay: The cytotoxicity of the drugs was determined using the MTT assay. Briefly, 10000 cells were seeded into each well in a 96-well plate and grown overnight. Subsequently, the cell culture medium, containing different concentrations of drugs, was added to the wells. The plates were incubated for 48 h. The stock MTT dye solution (5 mg/mL, 20 μL) was added to each well. After 4 h of incubation, the cultures were removed and 150 μL of DMSO was added to each well. The optical density of each well was measured on a microplate spectrophotometer at a wavelength of 595 nm.

Generation and analysis of MCTSs: MCTSs were cultured using the liquid overlay method. Two hundred μL of culture media, containing 3000 diluted H460/MX20 or H460 cells, was transferred to 1.5% agarose-coated transparent 96-well plates. The single cells formed MCTS aggregates approximately 400 μm in diameter after three days of incubation. The MCTSs were treated by replacing 50% of the old medium with drug-supplemented standard medium or drug-free medium. MCTSs in the 96-well plate were imaged with a phase contrast microscope using a 10×objective to evaluate their integrity, diameter and volume. H460/MX20MCTSs were incubated with drugs, and incubated in the dark for 1 day. In parallel, the medium containing the solvent was replaced with a solvent-free medium for the unincubated MCTSs. The culture media was refreshed every two days with the same concentration of the test drug. The images and diameter data of 3D MCTSs were collected every 24 h using an inverted fluorescence microscope (Zeiss Axio Observer D1, Germany).

Cell uptake studies: The cells were plated at a density of 10^6 cells/mL in 10 mL of DMEM for 24 h, and the **RuF** complex (0.5 μM) was added to the culture medium and incubated for 2 h. The culture was removed and the cells were

washed three times with cold PBS. The cells were collected in the tube and digested in a solution of 20% HNO_3 (1 mL) and 10% H_2O_2 (1 mL) for 48 h. Each sample was then diluted with Milli-Q water to obtain 2% HNO_3 sample solutions. The ruthenium content was determined using inductively coupled plasma mass spectrometry (ICP-MS Thermo Elemental Co., Ltd.). In order to evaluate **RuF** efflux in H460/MX20 cells, the cells were incubated sequentially with the **RuF**-containing medium for 2 h (accumulation phase) and then the cells were incubated with drug-free culture medium for another 2 h (efflux phase). Samples from different time points (0, 30, 60 and 120 min) in the efflux phase were taken and washed with cold PBS three times. The samples were analyzed as previously described in accumulation assay.

[^{3}H]-mitoxantrone intracellular accumulation and efflux assay Cells were trypsinized, washed and resuspended at 10^6 cells/mL in complete culture medium. Cells were incubated in the complete culture medium containing 0.5 μM [^{3}H]-mitoxantrone for 2 h. After washing three times with cold PBS, the cells were pelleted by centrifugation (3000 rpms/min) and lysed using lysis buffer (pH 7.4, containing 1% Trition X-100 and 0.2% SDS). The sample was placed in scintillation fluid in respective vials. The cell lysates were placed in 5 mL of liquid scintillation cocktail and the radioactivity was determined using Packard TRI-CARB 1900CA liquid scintillation analyzer (Packard Instrument, Downers Grove, IL) as previously described.[1] For the determination of [^{3}H]-mitoxantrone efflux, the cells were incubated sequentially with [^{3}H]-mitoxantrone - containing medium for 2 h (accumulation phase), and then incubated in drug-free culture medium for another 2 h (efflux phase). Samples from different time points (0, 30, 60 and 120 min) in the efflux phase were taken and washed with cold PBS three times. The samples were analyzed as previously described in accumulation assay.

Subcellular distribution studies: The cells were plated at a density of 1×10^5 cells/mL in 10 mL of culture medium. The **RuF** complex (2 μM) was added to the culture medium and incubated for 8 h and then washed three times with cold PBS. The cells were collected in a centrifuge tube for counting using a trypsin-EDTA solution. Subsequently, the nuclei, mitochondria and cytoplasm of the cells were extracted using a nuclear, mitochondria and cytoplasmic protein Extraction Kit (Beyotime Biotechnology). All of the samples were digested in a solution of 20% HNO_3 (1 mL) and 10% H_2O_2 (1 mL) at room temperature for 48 h. Each sample was then diluted with Milli-Q water to obtain 2% HNO_3 sample solutions. The ruthenium content was determined using ICP-MS.

Mitochondria luminescence imaging: H460/MX20 cells were incubated at a density of approximately 10,000 cells/mL in a confocal dish. After overnight incubation, the cells were incubated with **RuF** (0.5 μM and 1 μM) for 8 h. After removing the DMEM and washing three times with pre-warmed PBS, the cells

were stained with MTR (100 nM) for 30 min. The cells were washed three times with PBS and the cell images were captured using a monochromatic CoolSNAP FX camera (Roper Scientific, USA).

Hoechst 33324 intracellular accumulation study: H460/MX20 cells were seeded on coverslips in confocal dishes and incubated overnight. Cells were washed with PBS and incubated with **RuF** (0.5, 2 or 4 μM) for 8 h. After removing the culture medium and washing three times with pre-warmed PBS, Hoechst 33324 solution (5 μg/ml) was added to the dishes for 1 h incubation. After the cells were washed with ice-cold PBS, images were obtained using confocal luminescence microscopy.

Measurement of intracellular reactive oxygen species: H460/MX20 cells were incubated with **RuF** at different concentrations (0.5, 1 or 2 μM) in a 6-well plate and the unincubated cells were maintained as a control. After 48 h of incubation, the cells were washed twice with PBS. DCFH-DA (10 mM) was dissolved in the DMEM and added to each well of the plate for 30 min in the dark. The cells were washed three times with PBS before images were taken using an inverted fluorescence microscope (Zeiss AxioObserver D1). The excitation wavelength was 485 nm and the fluorescence were measured at 530 nm.

Cell cycle analysis: H460/MX20 cells were incubated with **RuF** (0.5, 1 and 2 μM) for 48 h. The cells were collected and fixed in 2 mL of 70% cold aqueous ethanol (v/v). The cells were centrifuged (3000 rpms/min) and washed three times with PBS and resuspended in PBS (0.5 mL). Fifty μM of RNAase (1 mg/mL) was added to the resuspended cell sample and incubated for 30 min at 37 °C. Subsequently, 50 μM of propidium iodide (500 mg/mL) solution was added after gentle mixing for 5 min at 37 °C in the dark. The samples were resuspended and 10,000 cells were analyzed for each sample by a BD FACS Calibur cytometer (Becton Dickinson, Heidelberg, Germany). The data were acquired and analyzed with ModFit LT 3.2.

Analysis of MMP: H460/MX20 cells were cultured in 6-well plate overnight and incubated with **RuF** (0.5, 1 and 2μM) for 24 h. Following incubation, the cells were washed and incubated in a pre-warmed staining working solution containing JC-1 (5 μg/mL) for 15 min at 37 °C. Subsequently, the cells were washed twice with pre-warmed PBS and visualized under an inverted fluorescence microscope (Zeiss Axio Observer D1). JC-1 fluorescence was measured with a single excitation (488 nm) and dual emission (shift from green 530 nm to red 590 nm).

Acridine orange/ethidium bromide (AO/EB) staining: A monolayer of H460/MX20 cells was incubated in the absence or presence of **RuF**, at different concentrations, at 37 °C and 5% CO_2 for 48 h. After washing, cells were stained with AO/EB solution (AO: 100 μg/ml, EB: 100 μg/ml) for 10 min. The cells were

washed three times with PBS and then observed under an inverted fluorescence microscope (Zeiss Axio Observer D1).

RT-PCR analysis: The H460/MX20 cells were incubated in 6-well plate overnight, and then were incubated with **RuF** for 24 h. Subsequently, the cells were subjected to RT-PCR analysis. The total mRNA of the H460/MX20 cells was extracted using the Trizol reagent, according to the manufacturer's instructions and was resuspended in RNAase water. The content and purity of total RNA of the sample was measured using a spectrophotometer (260 nm), and the integrity of the RNA was determined using a Bioanalyser 2100. One μg of total RNA was reverse transcribed (RT) to cDNA by using the Prime Script and RT Primer Mix (including Oligo dt Primer and Random 6 mers). Two μL of cDNA liquid was then added to 23 μL of PCR reaction media for cDNA amplification.

h-ABCG2-F: ATTGAAGGCAAAGGCAGATG;

h-ABCG2-R: TGAGTCCTGGGCAGAAGTTT;

h-GAPDH-F541: AGAAGGCTGGGGCTCATTT;

h-GAPDH-R744: AGTGATGGCATGGACTGTGG;

Polymerase chain reactions were conducted as follows: initial denaturation (95 °C for 30 s), followed by 40 cycles of denaturation (95 °C for 5 s), and hybridization-extension (60 °C for 31 s) using an Applied Biosystems7300 Real Time PCR system. The target gene was the ABCG2 gene and the house keeping gene was GAPDH. In this study, the transcription level of the ABCG2 was normalized to that of GAPDH to compensate for difference in efficiency of reverse transcription and input RNA amounts. The expression of the genes was relative to the control. The relative quantitation of ABCG2 expression was calculated using the comparative ΔΔCT method.

Western blot analysis: H460/MX20 cells were seeded into 10 cm tissue culture dishes (Corning), incubated for 24 h and incubated with **RuF** for 24 h. The cells were washed with ice-cold PBS and lysed by incubation in a radio immune precipitation assay buffer (RIPA) containing a protease inhibitor and a phosphatase inhibitor cocktail for 30 min on ice. The lysates were centrifuged at 15,000 rpm at 4 °C for 15 min and the concentrations of the protein samples were determined using a BCA protein assay reagent kit. The proteins were separated on precast NuPAGE 4% to 12% polyacrylamide gradient Bis-Tris gels and then transferred to PVDF membranes and subjected to western blot analyses. The membrane was then submerged in 5% nonfat powdered milk for 2 h. The primary antibodies were diluted (1:1000, respectively) in 5% nonfat powdered milk TBST solution and incubated with the membrane overnight at 4 °C. After washing with TBST, the membrane was incubated with the horseradish peroxidase conjugated secondary antibody. The signal of the

protein was detected using enhanced chemiluminescence and exposure of X-ray film.

Animal study: Female athymic BALB/c nude mice, aged 4–6 weeks, were purchased from Guangdong Medicinal Laboratory Animal Center (Guangzhou, China) and were used in accordance with the regulations of the Animal Ethical and Welfare Committee (AEWC) of Sun Yat-Sen University. To establish a subcutaneous xenograft model, 5×10^6 human H460/MX20 cancer cells were subcutaneously injected into the right flank of 6−7 weeks old athymic nude mice. During the experiment, the animals were monitored daily or once on alternate days for any clinically relevant abnormalities. If any animal was debilitated, or unable to eat due to factors unrelated to the treatment, the animals was removed from the study If any animal was moribund due to treatment-associated toxicity or tumor over-growth (tumor length $\geq$ 20 mm), it was euthanized by an overdose of carbon dioxide. This implementation of the protocol was approved by the AEWC. The mice bearing H460/MX20 tumors were allocated into four groups randomly when the tumor volumes of the mice reached approximately 75 mm^3. The group 1) PBS; 2) 0.5 mg/kg **RuF**; 3) 1.5 mg/kg **RuF**; 4) 3 mg/kg **RuF**. Then the tumors of the mice were injected with 200 μL of the various treatments, the lengths and widths of the tumors were measured every 2 days using of a digital caliper for a period of 16 days. The tumor volume was calculated according to the following formula: volume = $width^2 \times (length/2)$.

Ex vivo **histological staining:** H460/MX20 tumor-bearing mice were euthanized using carbon dioxide at 16 days after intratumoral injection, and the tumors and major organs of the mice in groups 1-4 were collected and fixed with paraformaldehyde or cryosectioned for hematoxylin-eosin (H&E) staining.

References

1. Y.-K. Zhang, G.-N. Zhang, Y.-J. Wang, B. A. Patel, T. T. Talele, D.-H. Yang and Z.-S. Chen, *Sci. Rep.* **2016**, *6*, 25694.
2. Y.-K. Zhang, X.-Y. Zhang, G.-N. Zhang, Y.-J. Wang, H. Xu, D. Zhang, S. Shukla, L. Liu, D.-H. Yang, S. V. Ambudkar, Z.-S. Chen, *Biochem. Pharmacol.* **2017**, *132*, 29-37.

Supporting Tables and Figures:

Table S1 Toxicity of the complexes to the LO2, HEK293/pcDNA3.1 and the ABCG2 transfected cells.[a]

Treatments	IC_{50}±SD (μM, RF[b])				
	HEK293/pcDNA3.1	HEK/ABCG2-R482	HEK/ABCG2-R482G	HEK/ABCG2-R482T	LO2
MX[c]	0.17±0.04	5.95±0.55(35)	5.27±0.63(31)	5.78±0.37(34)	0.37±0.06
RuF	4.34±0.41	5.05±0.43 (1.2)	4.81±0.36 (1.1)	4.76±0.44(1.1)	6.82±0.76
Cisplatin	9.62±0.35	9.82±0. 57(1.0)	10.16±0.61 (1.1)	10.41±0.28 (1.1)	19.86±2.12

[a] Cells were incubated with various concentrations of compounds for 48 h. Each value represents the mean ± SD of three independent experiments. [b] The values represent the resistance - fold (RF) obtained by dividing IC_{50} value of drugs in HEK293/pcDNA3.1 and the transfected cells. [c] MX = Mitoxantrone.

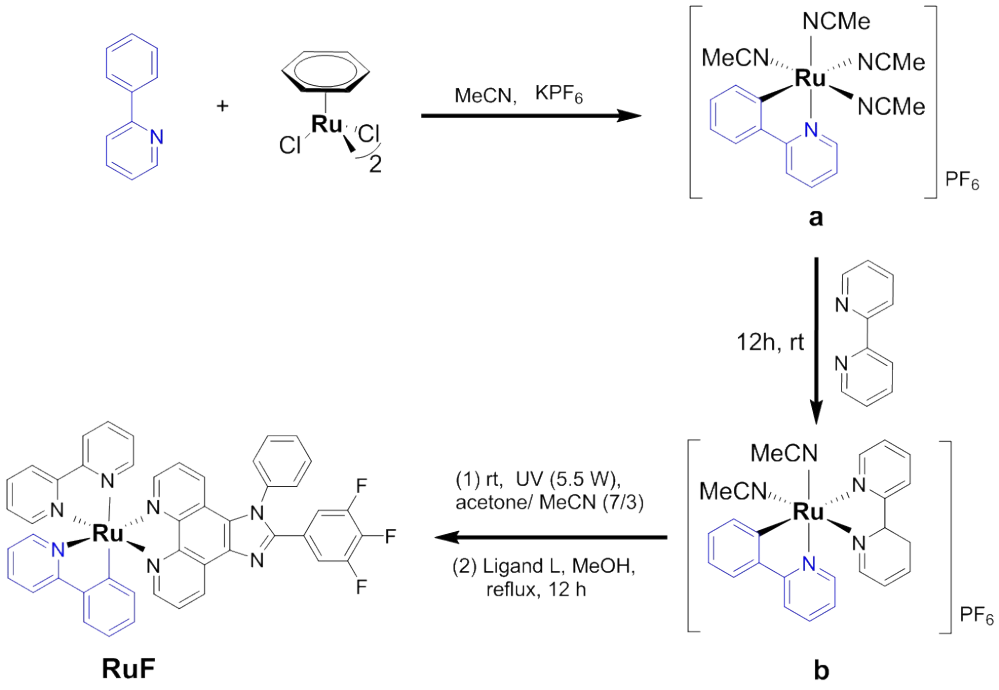


Fig. S1 The synthetic protocol for **RuF**.

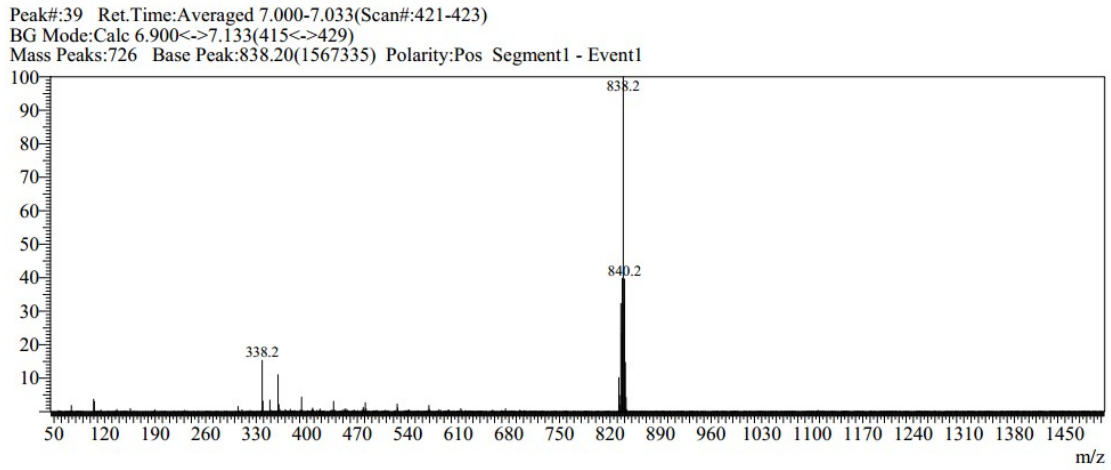


Fig. S2 ESI-MS spectrum of **RuF** in CH_3OH.

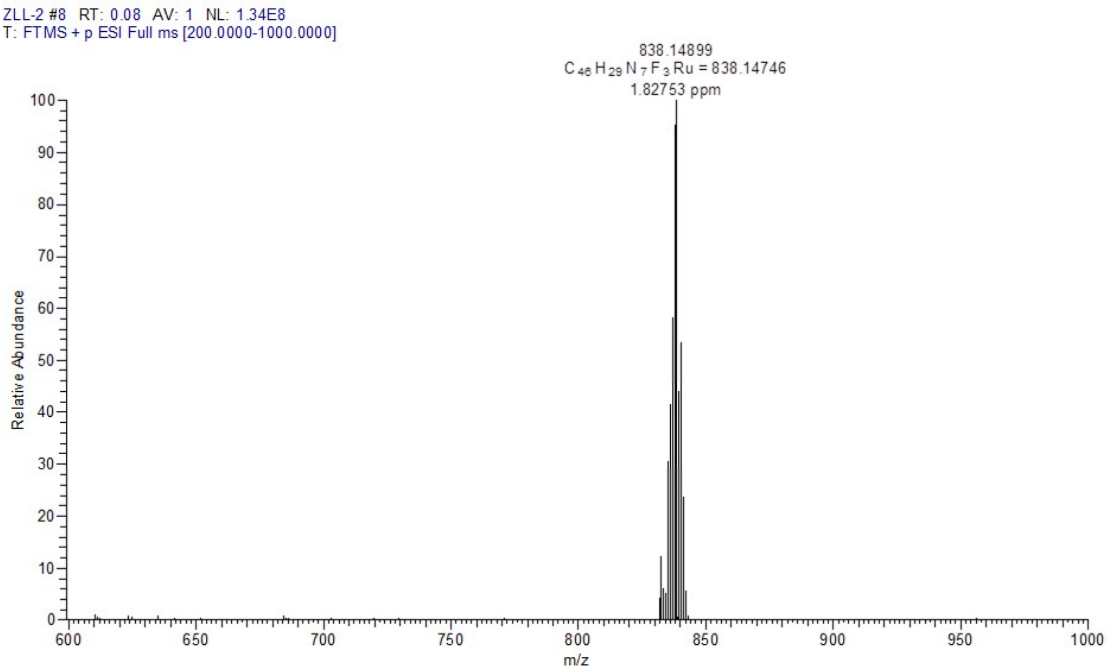


Fig. S3 The HRMS of the **RuF**.

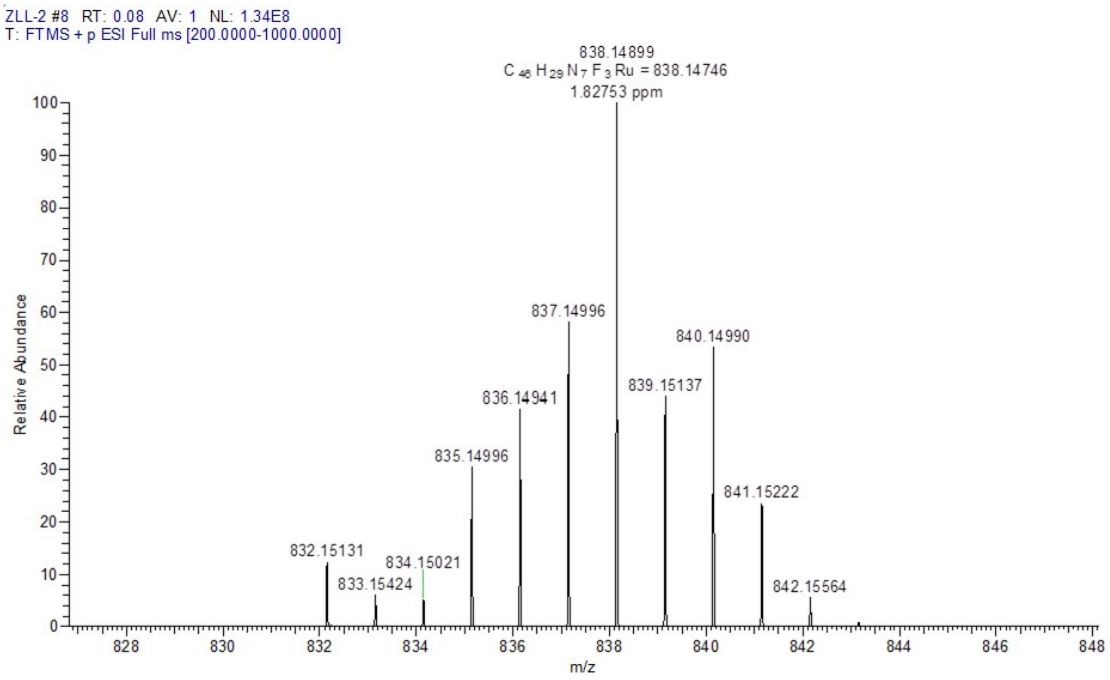


Fig. S4. The isotope peaks of the **RuF**.

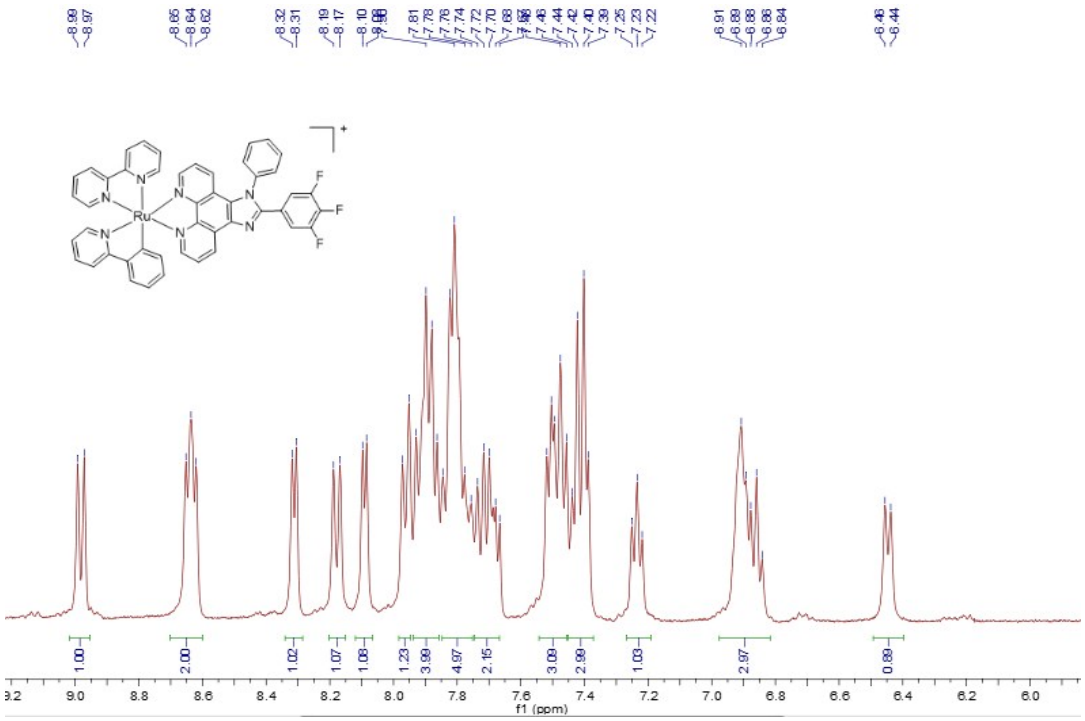


Fig. S5 ^{1}H NMR spectrum of **RuF** in d_6-DMSO**.**

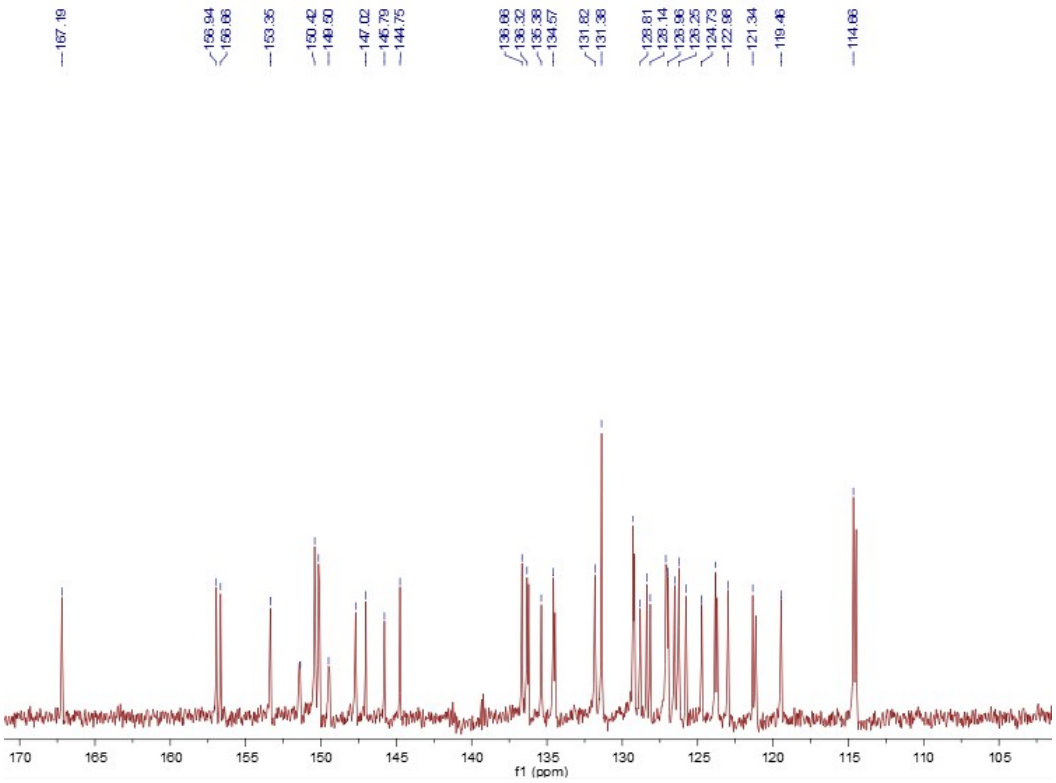


Fig. S6 ^{13}C NMR spectrum of **RuF** in d_6-DMSO.

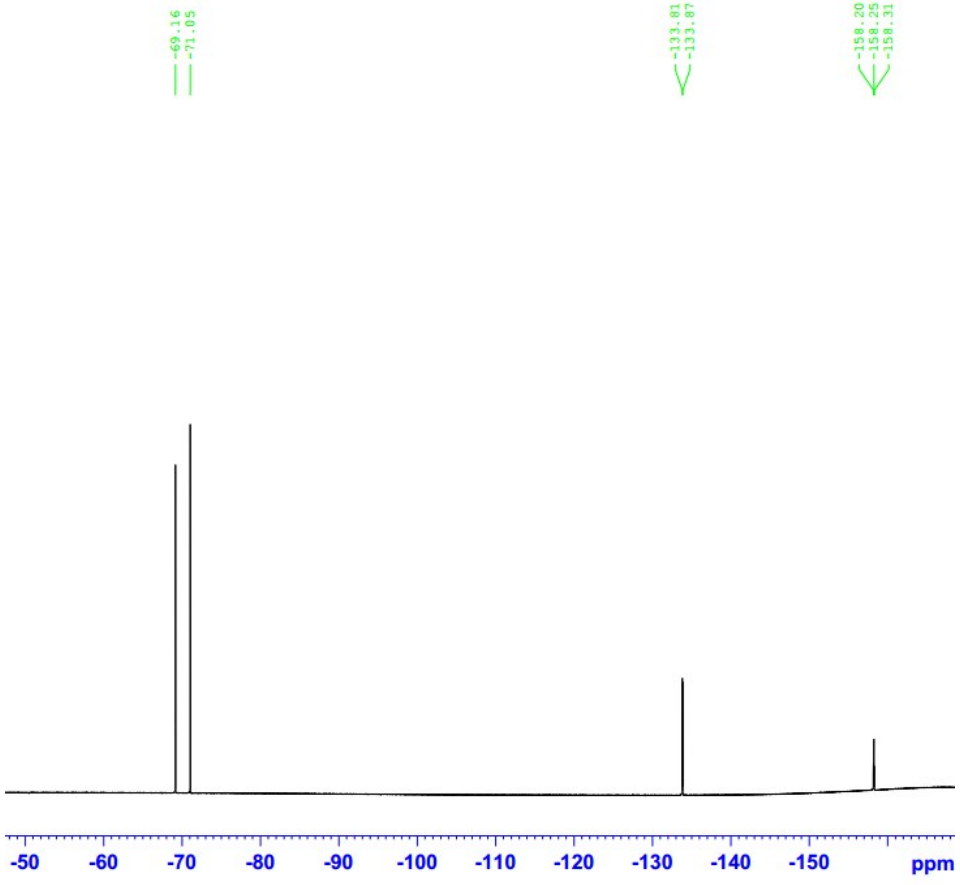


Fig. S7 ^{19}F NMR spectrum of **RuF** in d_6-DMSO**.**

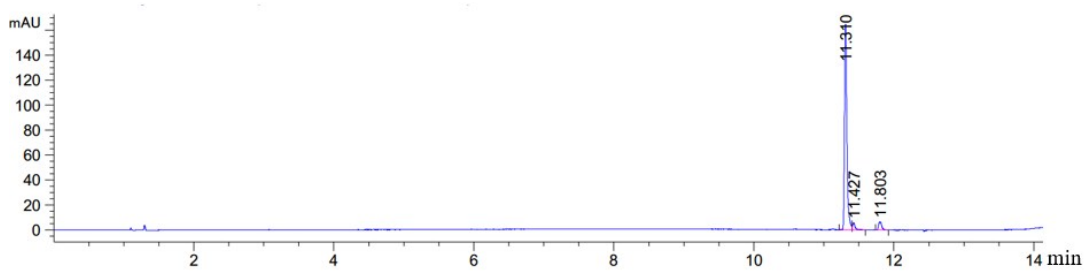


Fig. S8 The HPLC spectrum of the **RuF**.

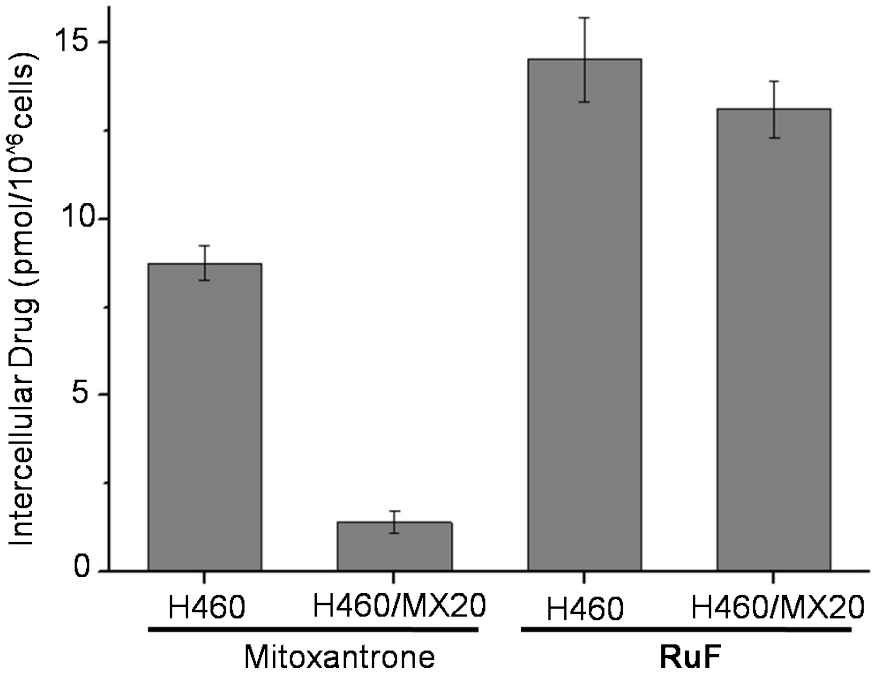


Fig. S9 The accumulation of 0.5 uM of mitoxantrone and **RuF** in H460 and H460/XM20 cells following incubation for 2 h.

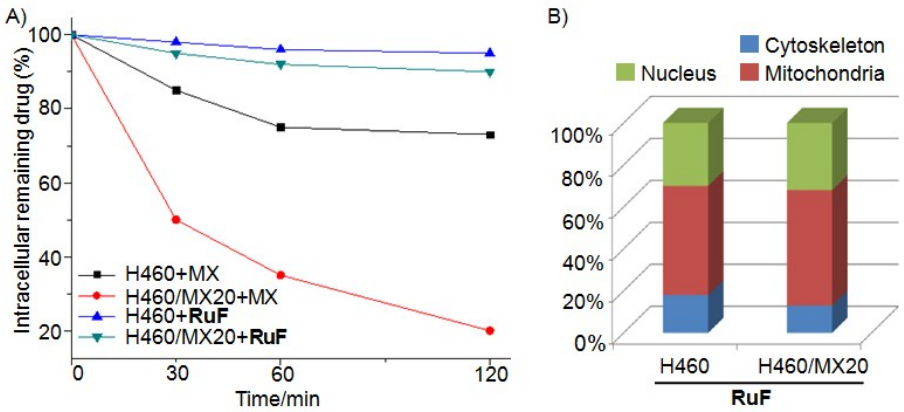


Fig. S10 A) The efflux of drugs in H460 and H460/XM20 cells after incubation with 0.5 μM of mitoxantrone (MX) or **RuF**. B) The subcellular distribution of ruthenium in cells after incubation with **RuF** (2 μM) for 8 h based on inductively coupled plasma mass spectrometry (ICP-MS).

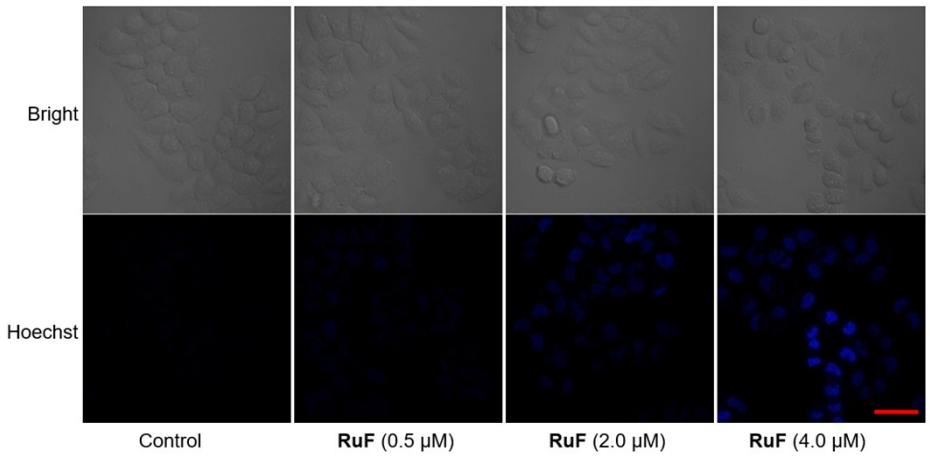


Fig. S11 The effect of **RuF** on the accumulation of Hoechest 33342 in H460/MX20 cells. The cells were pre-incubated with **RuF** for 8 h and were incubated with Hoechst 33342 (5 μg/ml) for 2 h. Hoechest (λ_{ex} = 350 nm, λ_{em} = 460 ± 20 nm). Scale bar = 50 μm.

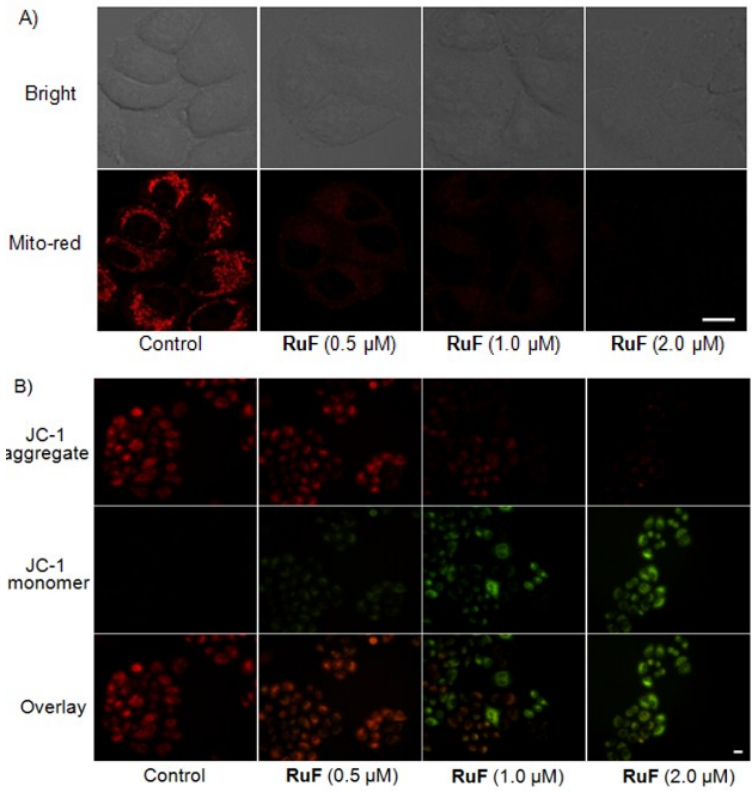


Fig. S12 A) The effect of **RuF** on the staining of MTR in the mitochondria of H460/MX20 cells. H460/MX20 cells were incubated with **RuF** for 8 h and then co-stained with 100 nM of MTR-red for 30 min. (λ_{ex} = 580 nm, λ_{em} = 620 ± 20 nm) Scale bar = 20 μm. B) The induction of mitochondrial dysfunction caused by **RuF** after incubation for 24 h in JC-1 labeled cells and observed under a fluorescence microscope. (λ_{ex} = 488 nm, $\lambda_{em/green}$ = 530 nm, $\lambda_{em/red}$ = 590 nm.) Scale bar = 20 μm.

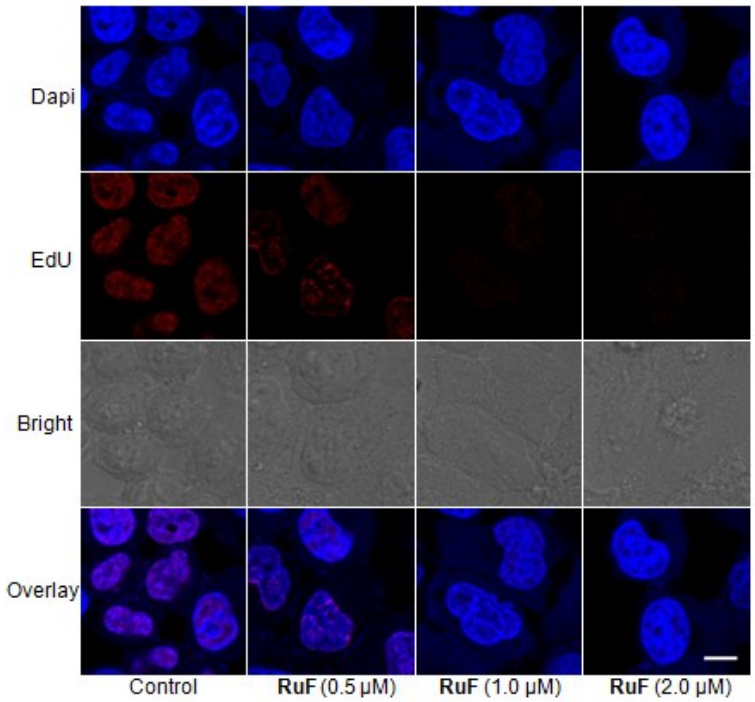


Fig. S13 The effect of **RuF** on DNA replication in H460/MX20 cells using the EdU assay. H460/MX20 cells were incubated with **RuF** for 8 h and then were incubated with Edu for 12 h. After fixing with 3.7% formaldehyde in DPBS, the cells were stained with Dapi (1 μg/mL) for 30 min. EdU (λ_{ex} = 488 nm, λ_{em} = 580-620 nm); Dapi (λ_{ex} = 405 nm, λ_{em} = 430-455 nm). Scale bar = 20 μm.

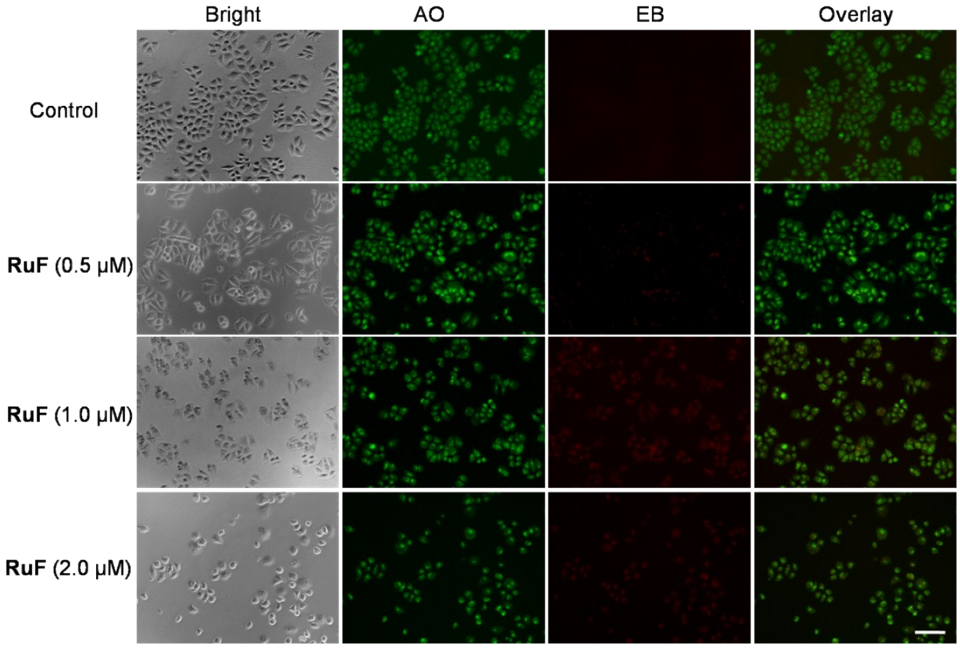


Fig. S14 Cell apoptosis assay in H460/MX20 cells after incubation with **RuF** for 48 h using AO/EB dyes under a fluorescence microscope. AO: λ_{ex} = 488 nm, λ_{em} = 530 ± 20 nm, EB: AO: λ_{ex} = 500 nm, $\lambda_{em/red}$ = 590 ± 20 nm. Scale bar 100 μm.

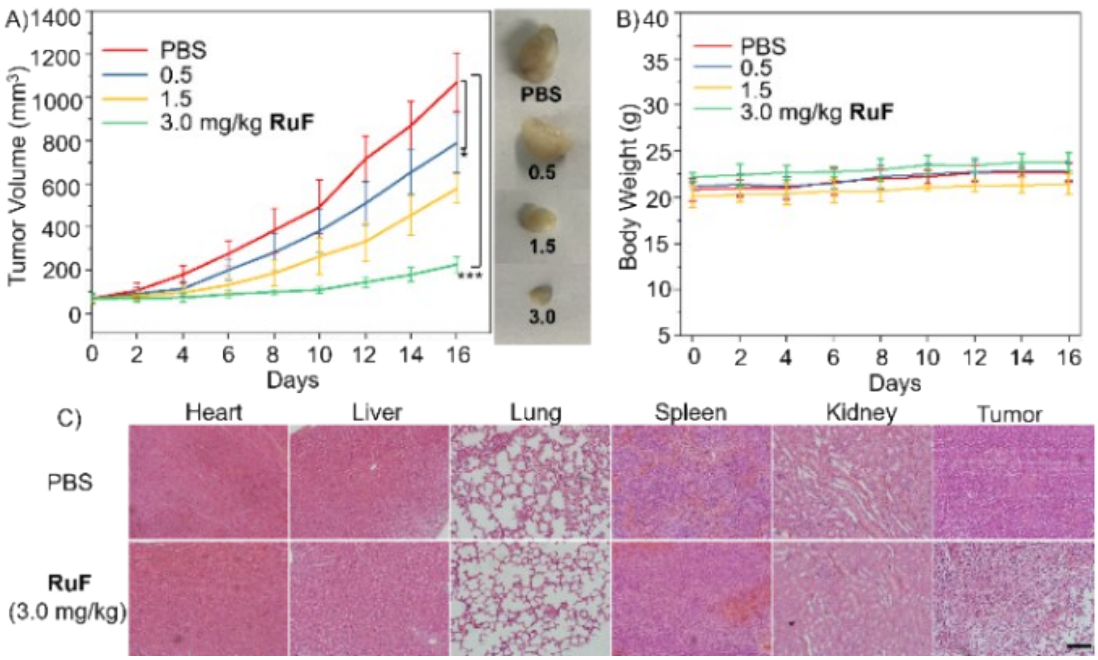


Fig. S15 A) Tumor volumes of mice after intratumoral injection with PBS or **RuF** (0.5, 1.5 and 3 mg/kg); the data are presented as the mean ± SD (n = 4). *p < 0.01, ***p < 0.001). B) Mean body weights of mice after treatment with PBS or **RuF**. C) Representative images of hematoxylin and eosin (H&E) staining from tumor sections and other major organs including the heart, liver, lung, spleen, and kidney after the mice were sacrificed on the 16th day post intratumoral injection with PBS or **RuF** (3 mg/kg). Scale bar = 100 μm.