Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

Photodegradation of Carbonic Anhydrase IX via a Binding-Enhanced Ruthenium Photosensitizer

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Methods

Materials

Ruthenium trichloride (J&K Chemical, China), 1,10-phenanthroline (phen, J&K Chemical, China), 5,6-diamino-1,10-phenanthroline (J&K Chemical, China), (9,10-anthracenediyl-bis(methylene)-dimalonic acid (ABDA, J&K Chemical, China), ammonium hexafluorophosphate (J&K Chemical, China), 4-bromo-1,2-benzenediamine (J&K Chemical, China), 4-ethynylbenzenesulfonamide (J&K Chemical, China), Acetazolamide (AAZ, J&K Chemical, China), Carbonic Anhydrase (CA, YuanYe, China), dimethyl sulfoxide (DMSO, Sigma Aldrich, USA), glycerol (Gly, J&K Chemical, China), trypsin (Hyclone Laboratoreis Inc, USA), Roswell Park Memorial Institute Medium (RPMI1640 Medium, Hyclone Laboratoreis Inc, USA), phosphate buffered saline (PBS, Sigma Aldrich, USA), fetal bovine serum (FBS, Hyclone Laboratoreis Inc), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, J&K Chemical, China), were used as received. Other materials and chemicals were purchased from the commercial sources. All the tested compounds were dissolved in DMSO as mother liquor before diluted into the experimental concentration with 1% (v/v) DMSO in the solvents.

Instrumentation

^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer (Germany). Shifts were referenced relative to the internal solvent signals. ESI-MS were recorded on a Thermo Finnigan LCQ DECA XP spectrometer (USA). The quoted m/z values represented the major peaks in the isotopic distribution. Microanalysis (C, H, and N) was carried out using an Elemental Vario EL CHNS analyzer (Germany). HPLC spectra were carried out with a Hewlett Packard High Performance Liquid Chromatograph (USA). UV/Vis spectra were recorded on a Varian Cary 300 spectrophotometer (USA). Emission measurements were conducted on an FLS 920 combined fluorescence lifetime and steady state spectrometer (Japan). Tecan Infinite M200 Pro microplate

reader (Switzerland) was used in MTT assay and enzyme activity assay. FluorChem M (Protein Simple, USA) was used for chemiluminescence detection in western blot. HPLC was carried out using Hewlett Packrd (USA). Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) was carried out using Bruker ultrafleXtreme (Germany).

Supplementary experimental section

Synthetic protocols and characterizations

11-Bromodipyrido[3,2-a:2′,3′-c]phenazine (**1**)[1] and $Ru(phen)_2Cl_2$[2] were synthesized by literature methods.

$[Ru(phen)_2(dppz\text{-}CAi)](PF_6)_2$ (Ru-dppz-CAi): $Ru(phen)_2Cl_2$ (532 mg, 1 mmol) and **1** (397 mg, 1.1 mmol) were dissolved in mixed solvent of ethanol and water, refluxed for 8 h. The solvent was evaporated under low pressure and diluted with saturated aqueous solution of NH_4PF_6. The residue was separated by flash chromatography to obtained **2**. Lastly, **2** (112 mg, 0.1 mmol) and 4-Ethynylbenzenesulfonamide (19.9 mg 0.11 mmol) were dissolved in DMF and tetrakistriphenylphosphine palladium and cuprous chloride were added. The solution was stirred in room temperature overnight before saturated aqueous solution of NH_4PF_6 was added. The residue was separated by flash chromatography to obtain **Ru-dppz-CAi**. Yield: 61 mg (50.3%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.60 (t, J = 7.2 Hz, 2H), 8.87 – 8.71 (m, 5H), 8.57 (d, J = 9.0 Hz, 1H), 8.42 (s, 4H), 8.34 – 8.25 (m, 3H), 8.21 (d, J = 5.3 Hz, 2H), 8.07 (d, J = 5.0 Hz, 2H), 7.93 (q, J = 7.4 Hz, 5H), 7.88 – 7.70 (m, 5H), 7.55 (s, 2H). ^{13}C NMR (101 MHz, DMSO-d_6)) δ 154.68, 153.76, 153.17, 151.40, 151.36, 147.62, 147.60, 145.06, 142.24, 142.09, 141.60, 141.17, 137.47, 134.84, 133.76, 132.91, 132.79, 130.98, 130.95, 130.66, 130.42, 130.39, 128.55, 128.12, 126.85, 126.73, 126.64, 126.02, 125.30, 93.06, 91.02, 40.63, 40.42, 40.21, 40.01, 39.80, 39.59, 39.38. ESI-MS m/z (CH_3OH): calculated for $[M\text{-}2PF_6]^{2+}$ 461.57, found 461.79. FT-IR (4000-400 cm^{-1}): 3637 (m), 3388 (s), 3324 (s), 3083 (s), 2954 (s), 2923 (s), 2852 (s), 2210 (w)1619 (s), 1598 (s),

1496(s), 1429(s), 1411(s), 1351(s), 1162(s), 1120(s), 1093(s), 1079(s), 835 (vs), 775 (s), 748 (s), 721 (s), 698 (s), 642 (s), 620 (s), 588 (s), 557 (s), 528 (s), 470 (s), 431 (s), 406 (s). Purity (HPLC): 98.38%. Mobile phase：KH_2PO_4 aqueous solution (10 mM) : CH_3CN = 4:6 (0~6 min), 2:8 (6~10 min).

Photophysical properties

The UV-Vis spectra, emission and lifetimes of **Ru-dppz-CAi** in CH_3OH, Gly, CH_3CN, CH_2Cl_2 and H_2O at 298K were obtained on a Varian Cary 300 spectrophotometer (USA) and an FLS 920 combined fluorescence lifetime and steady spectrometer (Japan). The resulting data were processed with Origin Pro v8.0. All media contain minimum DMSO (1% v/v) for better solvency.

CA Binding Photophysical Properties

The fluorescence emission spectra and lifetimes of the **Ru-dppz-CAi** (20 μM) Minteracting with CA (20 μM) in PBS (pH 7.4) at 298 K were carried out on a FLS920 steady-state transient fluorescence spectrometer, using Acetazolamide (AAZ, 100 μM) as competitive inhibitor.

Determination of binding constants between Ru-dppz-CAi and CA

The binding constants between **Ru-dppz-CAi** and CA was determined by UV spectrum titration. CA was titrated into the aqueous solution of **Ru-dppz-CAi** (5 μM) until CA was saturated. The binding constant *K* value of the complex and CA was obtained by fitting equations:

$$\frac{[\mathrm{CA}]}{\varepsilon_a - \varepsilon_f} = \frac{[\mathrm{CA}]}{\varepsilon_b - \varepsilon_f} + \frac{1}{K_\mathrm{b}(\varepsilon_b - \varepsilon_f)}$$

ε_f, ε_a and ε_b are the molar absorption coefficient of **Ru-dppz-CAi** in the free state, with different CA concentrations and saturated CA, respectively. [CA] represents the concentration of CA. K_b represents the binding constant between complex and CA.

MALDI-TOF-MS Analysis

Molecular weight changes of mixed solutions of **Ru-dppz-CAi** and CA were determined using a Bruker ultrafleXtreme Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS). **Ru-dppz-CAi** (20 μM) and CA (20 μM) were mixed in water for 10 min before experiment.

Molecular Docking

Molecular docking was performed by AutoDock 4.2 using Lamarckian genetic algorithm method. We used the PDB structure of the cocrystallized CAIX-AAZ complex (PDB code: 3IAI) as the starting point. The search space was defined as following: center x = 67.419, center y = 55.704, center z = 14.134; size x = 72, size y = 74, size z = 72; with the spacing set to 0.375 Å and the exhaustiveness set to 25 iterations. The structure of Ru was optimized using Gaussian09 at B3LYP/genecp (SDD basis for Re and 6-31G** for other atoms) computation level

Inhibition of CAIX Enzyme

36 μL protein solvent was added to 96-well plate, then 4 μL stock solution of **Ru-dppz-CAi** or Acetazolamide (AAZ) was added. After shaking for 15 min, 40 μL of 4-NPA solution was added. The total amount of DMSO was 1 %. After incubation at 37 °C for 5 h, the absorption at 400 nm was determined by microplate reader.

For light-condition inhibition experiments, the 96-well plate was irradiated by 450 nm laser (20 mW cm^{-2}) for 15 min (18 J cm^{-2}) after shaking, and then 40 μL of 4-NPA solution was added.

Singlet Oxygen Detection

The singlet oxygen capture agent 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) was used to measure the singlet oxygen generated by **Ru-dppz-Cai** and **Ru-dppz-CAi**-CA using Varian Cary 300 UV-Vis Spectrophotometer.

Cell cultures

MDA-MB-231 (human triple negative breast cancer cells) cell lines were obtained from the animal experimental Centre of Sun Yat-Sen University (Guangzhou, China). Cells were maintained in DMEM media supplemented with 10 % FBS, 100 μg/mL streptomycin, and 100 μg/mL penicillin in a humidified atmosphere with 5% CO_2 and 95% air at 37 °C. 1% DMSO (v/v) was used to treat the control group in each experiment.

Cytotoxicity

The cytotoxicity of **Ru-dppz-CAi** towards MBA-MD-231 cell lines was determined by MTT assay. Cells cultured in 96-well plates were grown to confluence before incubated with **Ru-dppz-CAi** for 20 h. The media containing **Ru-dppz-CAi** was removed and fresh media without the complexes was added. Then the cells were irradiated with a 450 nm light array (20 mW cm^{-2}) for 15 min (18 J cm^{-2}) and further incubated for 24 h. 20 μL of MTT solution (5 mg/mL) was then added to each well. The plates were incubated for an additional 4 h before the media was carefully removed, and DMSO was added (150 μL per well). The plate was shaken for 3 min. The absorbance at 595 nm was measured using a microplate reader (Infinite M200 Pro, Tecan, Switzerland).

Western blotting

MDA-MB-231 cells were seeded in 6 cm dishes (Corning) and cultured for 48 h. the old medium were replaced by fresh medium containing **Ru-dppz-CAi** (1 μM) and dimethyl sulfoxide (1 %, v/v). after culturing for 1 h, the cells were irradiated with 450 nm laser (20 mW cm^{-2}) for 15 min (18 J cm^{-2}), and continued to incubate for 6 h. Afterwards, cells were collected and lysed, and intracellular proteins were extracted by differential centrifugation (Cell lysis buffer, PMSF, 1 mM). The extracted protein was quantified with BCA protein detection kit, electrophoresed in 10 % polyacrylamide gel and transferred to PVDF membrane. PVDF membranes were blocked with QuickBlock™ and incubated with primary antibodies against CAIX (goat anti rabbit),

HIF-1α (goat anti rabbit) or GAPDH (goat anti rabbit) overnight at 4 °C. After washing with PBST 3 times, PVDF membranes were incubated with secondary antibody Goat Anti-Rabbit IgG H&L (HRP) for 2 h. Subsequent staining was performed with DAB horseradish peroxidase chromogenic kit, and chemiluminescence was detected with Fluochem M.

Proteomics

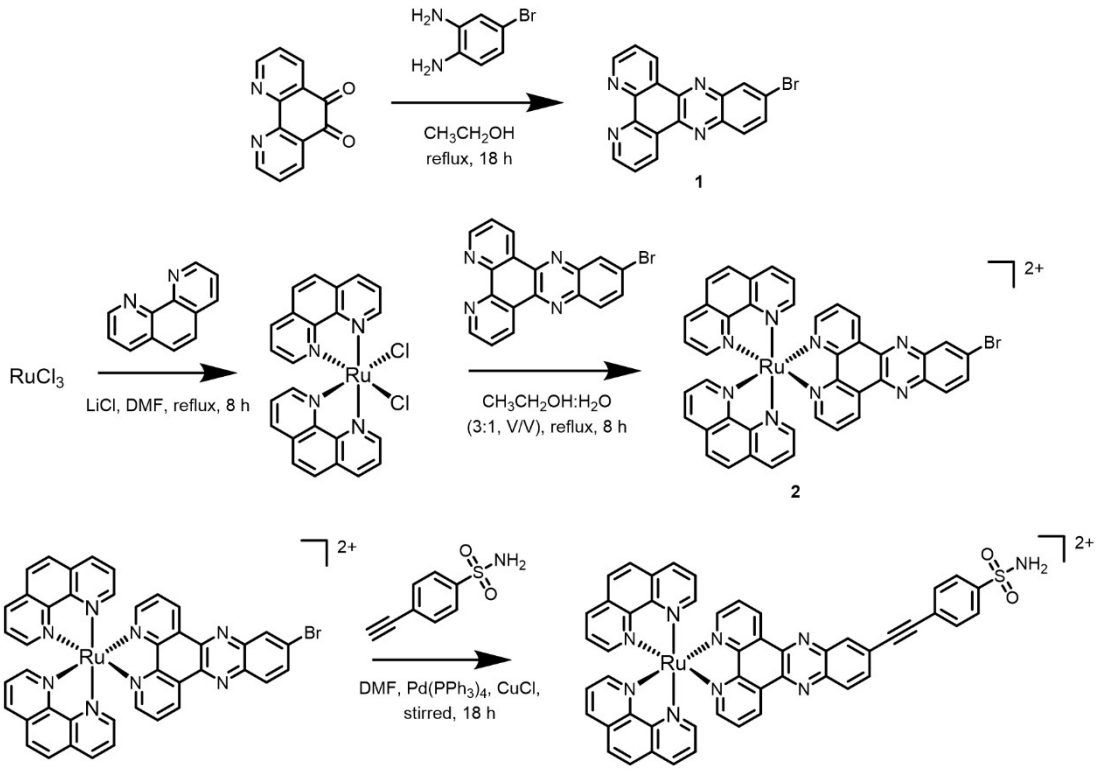
MDA-MB-231 cells were seeded in 6 cm dishes (Corning) and cultured for 48 h. the old medium were replaced by fresh medium containing **Ru-dppz-CAi** (1 μM) and dimethyl sulfoxide (1 %, v/v). after culturing for 1 h, the cells were irradiated with 450 nm laser (20 mW cm^{-2}) for 15 min (18 J cm^{-2}), and continued to incubate for 6 h. Afterwards, cells were collected and 200 μL PBS was added. After transferred to 1.5 ml EP tube and centrifuged at 20000 g, 4 ° C for 5 min, 100 μL lysate [1% SDS, 50 mM NaCl, 50 mM HEPES (pH 8.5), Roche Complete Edta-Free Protease Inhibitor, and 5 mM DTT, 25 U/mL Benzonase, 1% (w/v) glycerol] was added before vortex mixing and lysered at room temperature for 10 min. Then the cells were centrifuged at 20000 g for 10 min after incubation for half an hour at room temperature. After that, protein concentration was diluted to a minimum concentration of 0.67 mg / mL via a Bradford assay kit.

Take 40 μg protein per sample, add DTT of 10 mM, vortex. The samples were then heated to 90 ° C for 15 min and dry to room temperature. IAA was added with a final concentration at 25 mM, and the reaction was taken at room temperature for 30 min. After that, DTT (25 mM) was added and reacted at room temperature for 15 min.

Two hydrophilic carboxy magnetic beads was mixed at 1∶1 and washed twice with water and volumed to 25 μg/μL with water. 400 μg magnetic beads (16 μL) was added to the end volume of 67.5 μL and mixed. Then, 67.5 μL ethanol was added, gently mixed and centrifuged at 1000 rpm, 24 ° C for 10 min.After magnetic separation for 2 min, 180 μL 80% ethanol solution was added to the precipitated portion and mixed, repeat for 3 times.50 μL ammonium hydrogencarbonate solution (100 mM) and

LYSC (1: 50, wt: wt) were added, ultrasounded for 30 s and reacted for 14-16 h at 37 ° C, 1500 rpm. Then, Trypsin (1: 50, wt: wt) was added, ultrasounded for 30 s, and reacted for 6 h at 37 ° C, 1500 rpm.1 mL of acetonitrile was added to the reaction mixture, shuffled for 10 min and washed with 1 ml acetonitrile for three times. The magnetic beads were added to 100 μL 2% DMSO aqueous solution and reacted at 37 ° C for 30 min. After centrifuged at 20000g, room temperature for 1 min and magnetic separation, the supernatant was removed. 200 μL 80% aqueous solution was added and centrifuged at 2000 g for 10 min. The supernatant was then removed and the beads were vacuum coated at 45 ℃。The sapmles were then analysed with LC-MS (Easy-nLC 1200 and Orbitrap Q Exactive HF-X).

Supporting Scheme, Figures and Tables



Scheme S1. Synthetic protocols of **Ru-dppz-CAi**.

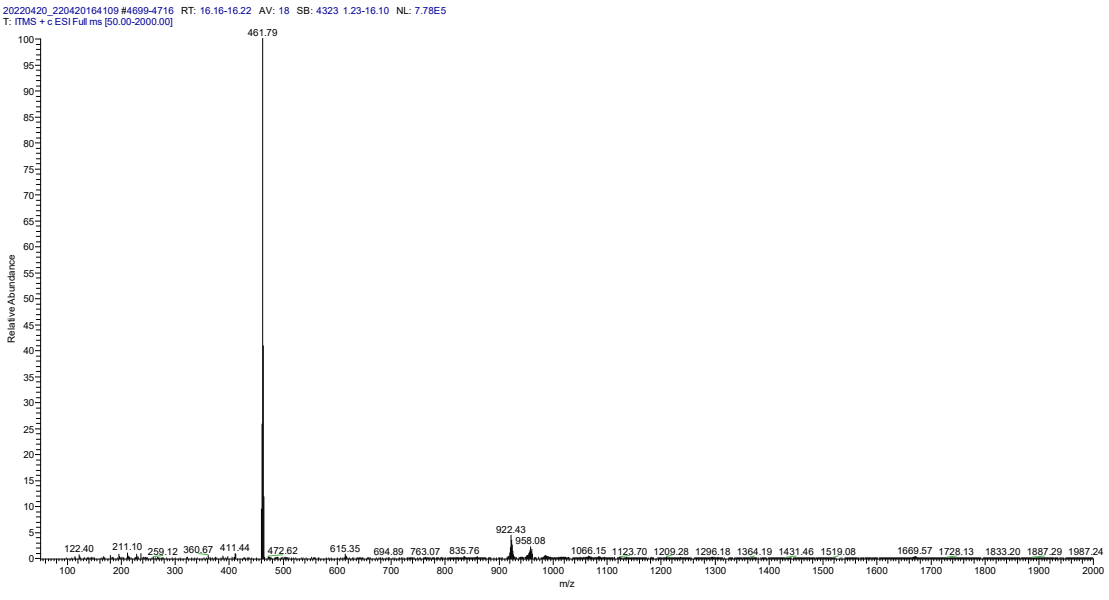


Figure S1. ESI-MS spectrum of **Ru-dppz-CAi** in CH_3OH.

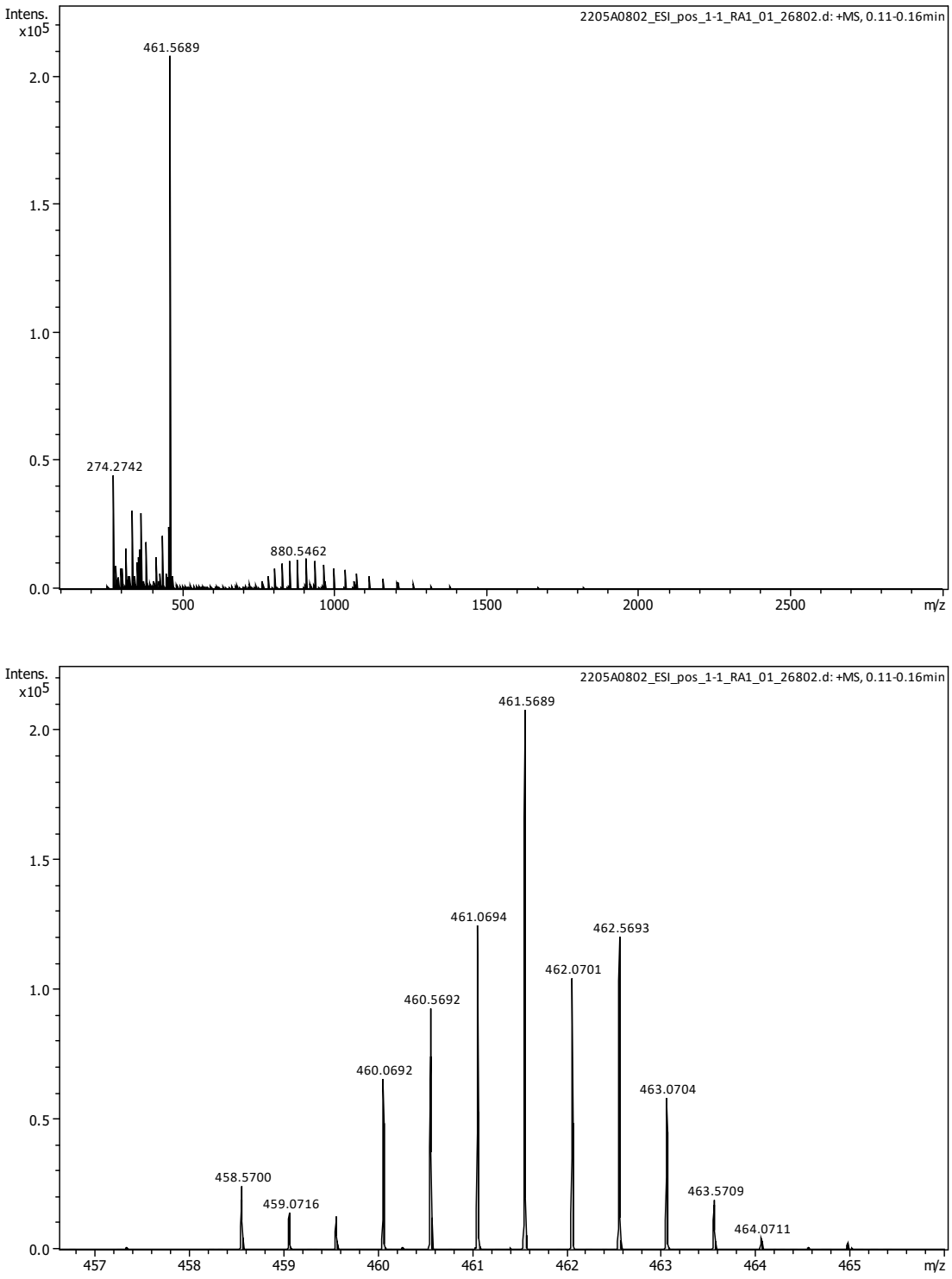


Figure S2. High resolution ESI-MS spectrum of **Ru-dppz-CAi** in CH_3OH.

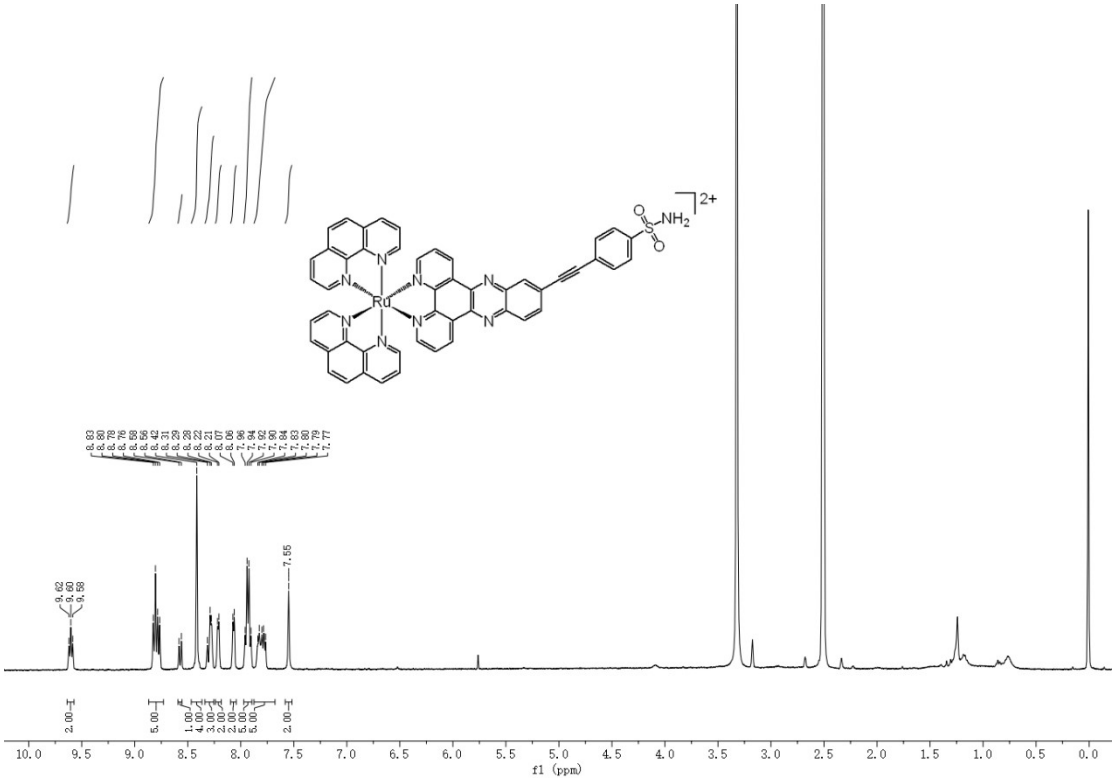


Figure S3. ^{1}H NMR spectrum of **Ru-dppz-CAi** in DMSO-*d6*.

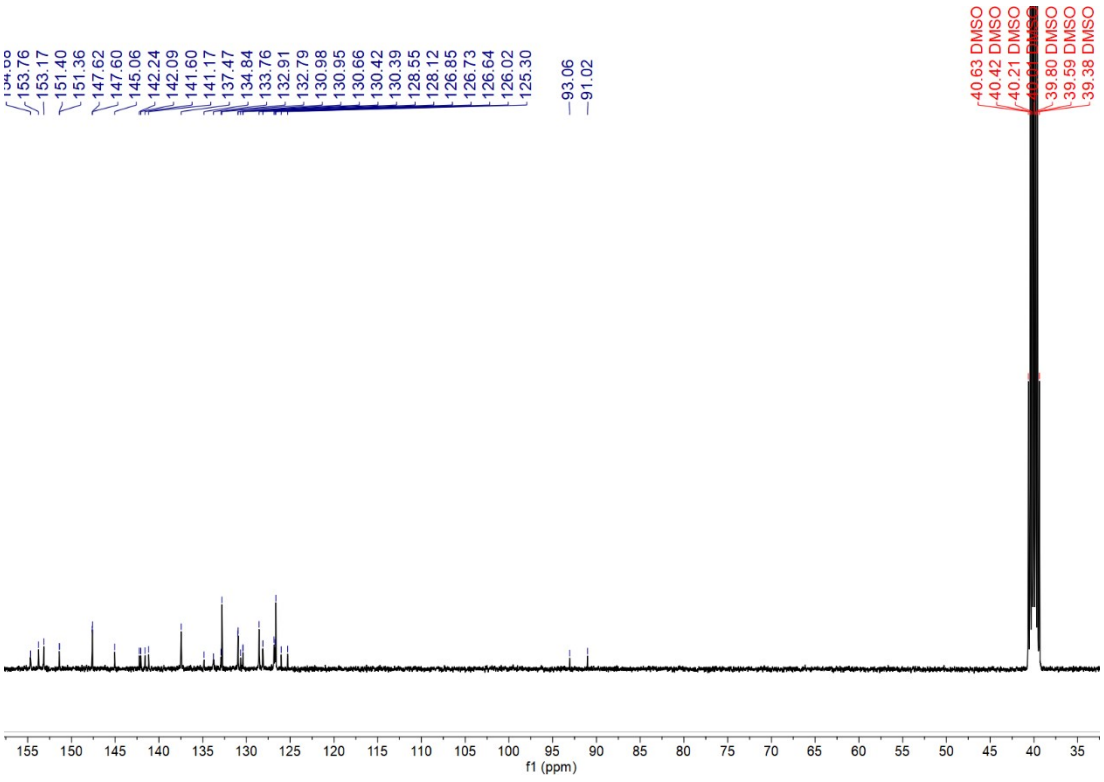


Figure S4. ^{13}C NMR spectrum of **Ru-dppz-CAi** in DMSO-*d6*.

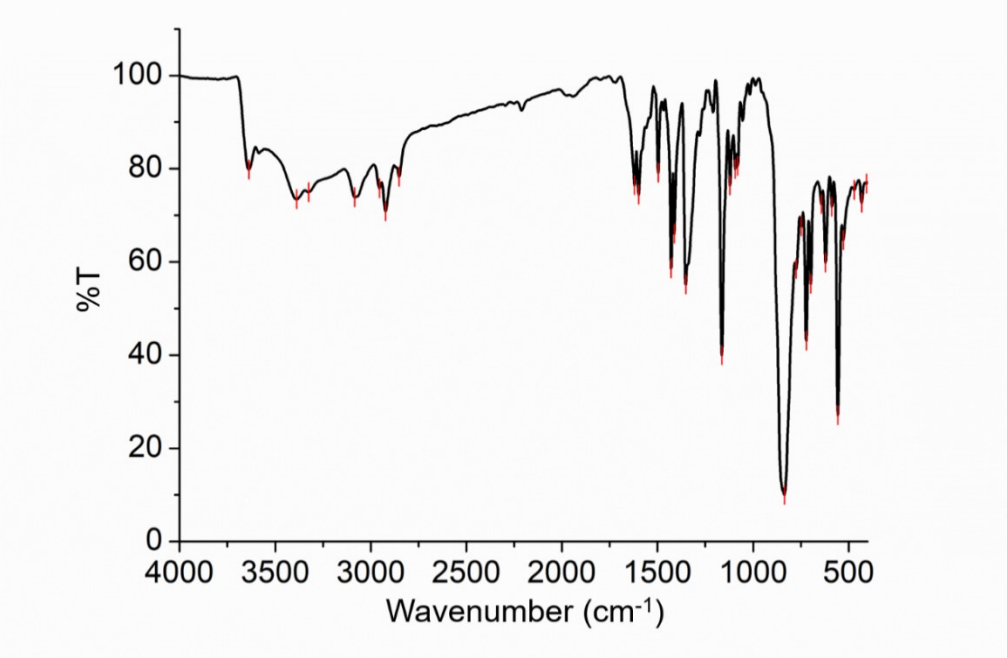


Figure S5. FT-IR spectrum of **Ru-dppz-CAi**.

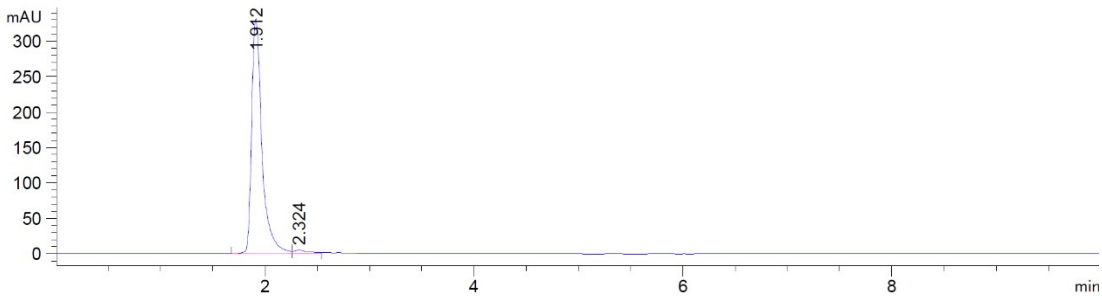


Figure S6. HPLC spectrum of **Ru-dppz-CAi**.

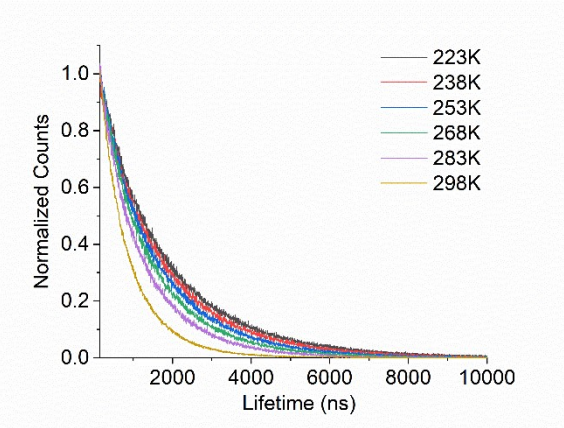


Figure S7. Phosphorescent lifetime of **Ru-dppz-CAi** (20 μM) in CH_3OH/Gly (1:1, v/v) at different temperature.

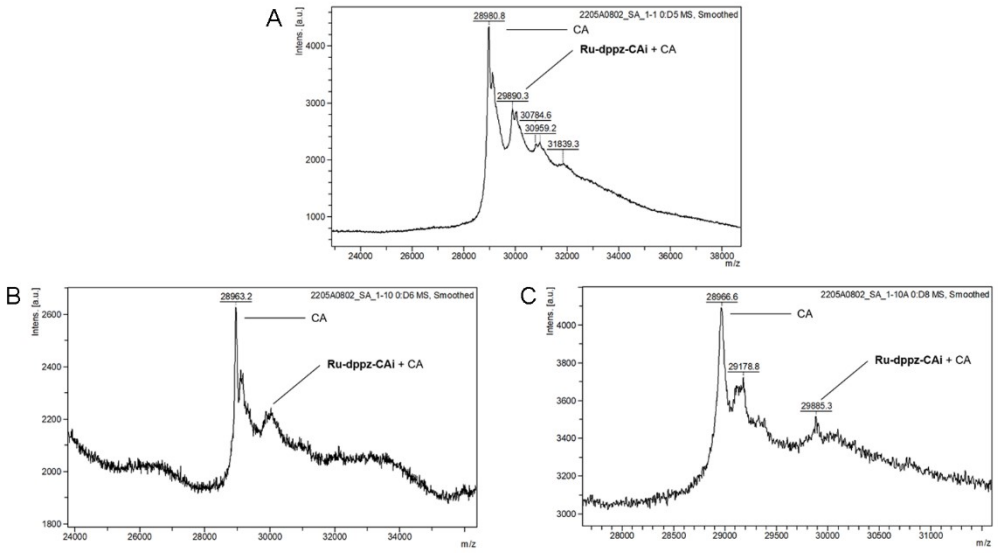


Figure S8. MALDI-TOF spectra of **Ru-dppz-CAi** (A: 50 μM; B and C: 500 μM) binding with CA (50 μM). AAZ (1 mM) was used as a competitor (C).

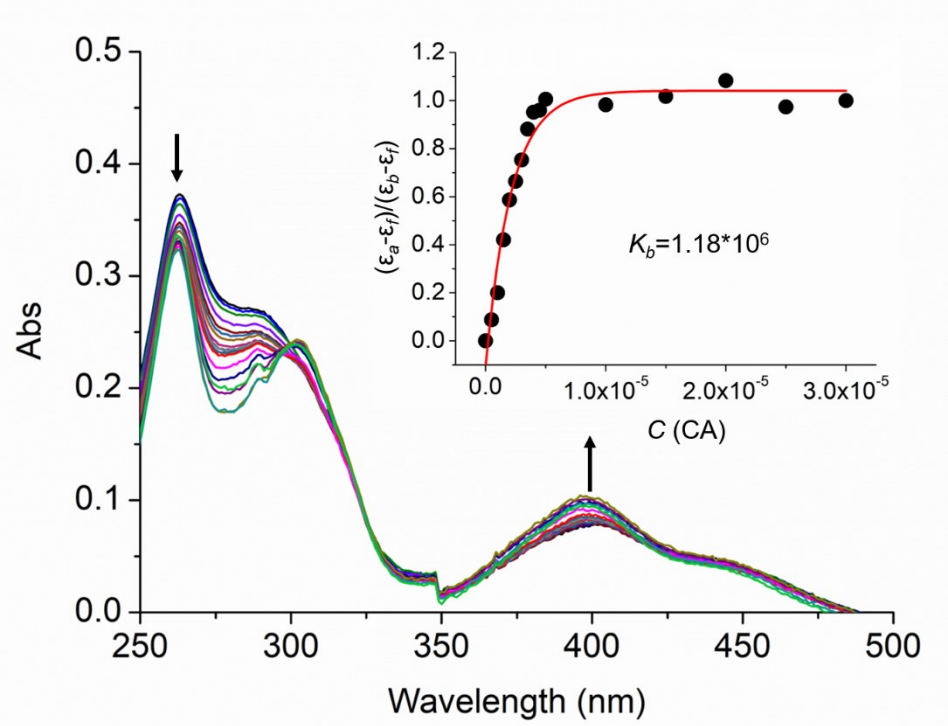


Figure S9. UV-Vis titration of **Ru-dppz-CAi** (5 μM) with CA (0–30 μM) in H_2O. The arrows show the changes in the absorption upon increasing amounts of CA. Insets: plot of $(\varepsilon_a-\varepsilon_f]/(\varepsilon_b-\varepsilon_f)$ vs [CA].

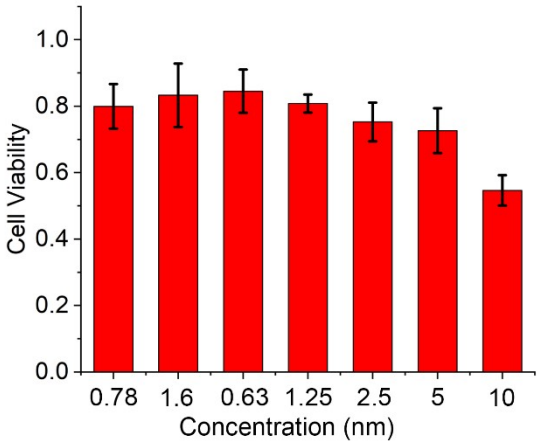


Figure S10. Cytotoxicity of **Ru-dppz-CAi** against MDA-MB-231 cells over 48 h. Cells were irradiated with a 450 nm light array (20 mW cm^{-2}) for 15 min (18 J cm^{-2})

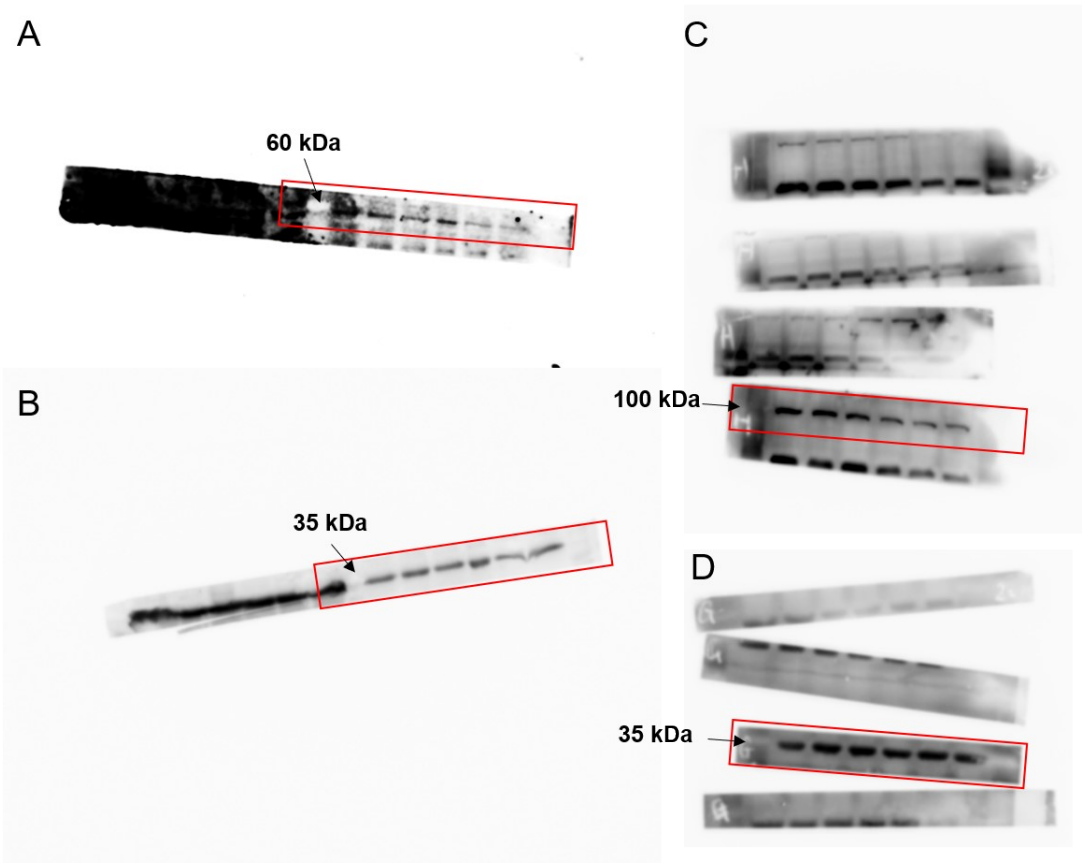


Figure S11. Raw images of the western blot. Blots of CAIX (A) with the internal reference GAPDH (B) and HIF-1α(C) with the internal reference GAPDH (D) was circled with red boxes.

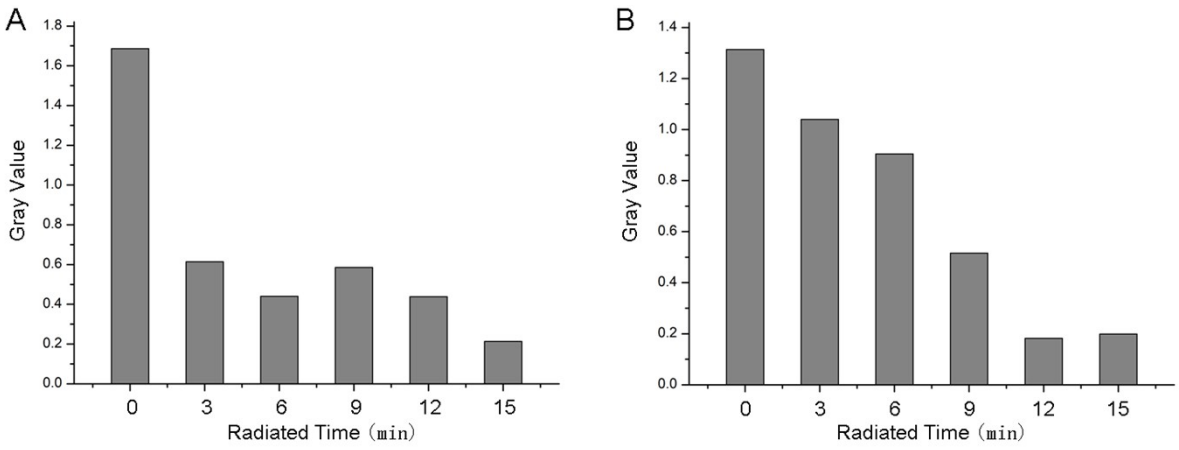


Figure S12. Gray value of the western blot of CAIX (A) and HIF-1α (B) against GAPDH.

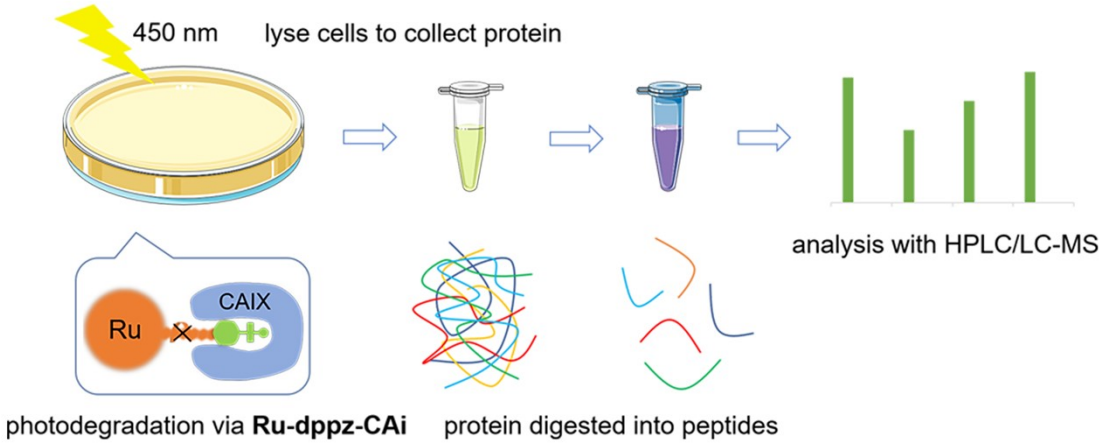


Figure S13. A simplified experimental flow of proteomics.

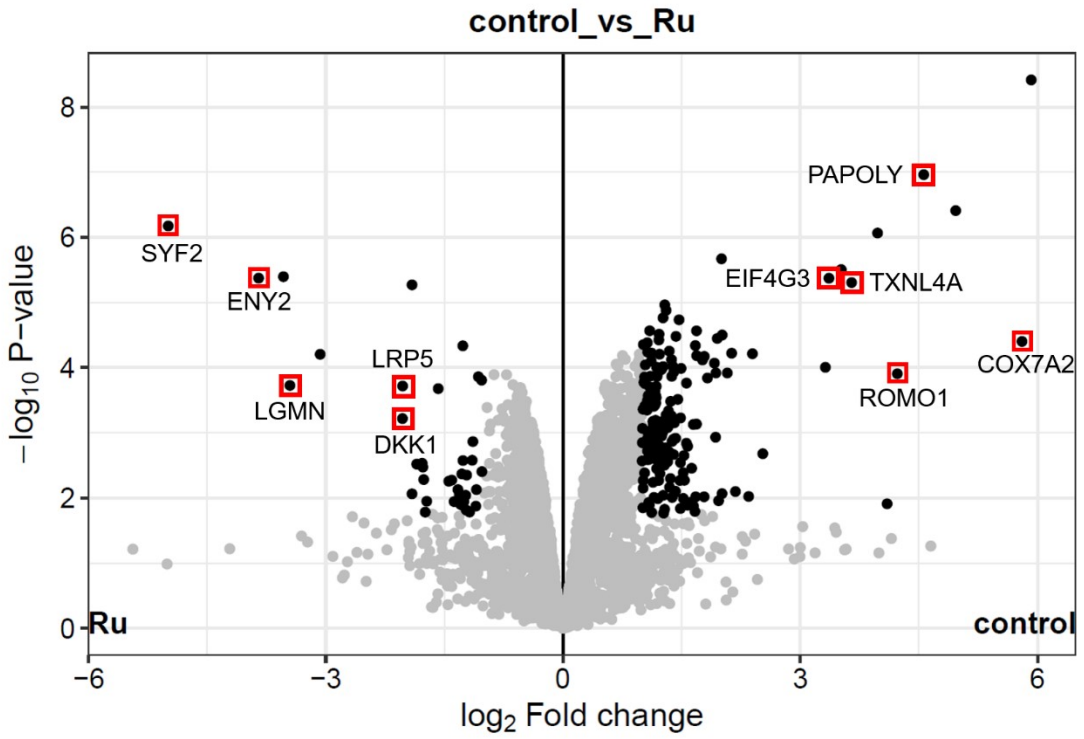


Figure S14. Volcano plot of differential proteins in **Ru-dppz-CAi**-treated cells compared with the control group. MDA-MB-231 cells were treated with **Ru-dppz-CAi** (1 mM, 1 h), irradiated with 450 nm for indicated time intervals and further incubated for 6 h.

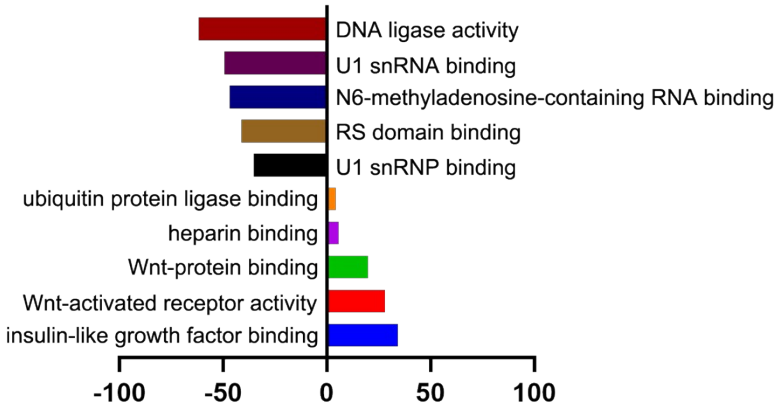


Figure S15. GO of molecular functions in **Ru-dppz-CAi**-treated cells. MDA-MB-231 cells were treated with **Ru-dppz-CAi** (1 mM, 1 h), irradiated with 450 nm for indicated time intervals and further incubated for 6 h.

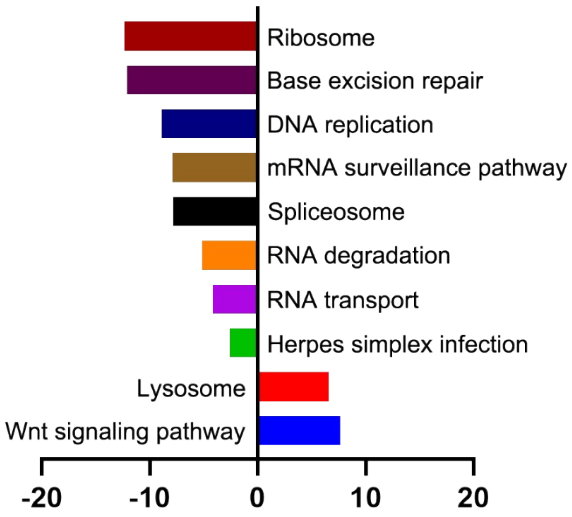


Figure S16. KEGG pathway of Ru-dppz-CAi-treated cells compared with the control group. MDA-MB-231 cells were treated with **Ru-dppz-CAi** (1 mM, 1 h), irradiated with 450 nm for indicated time intervals and further incubated for 6 h.

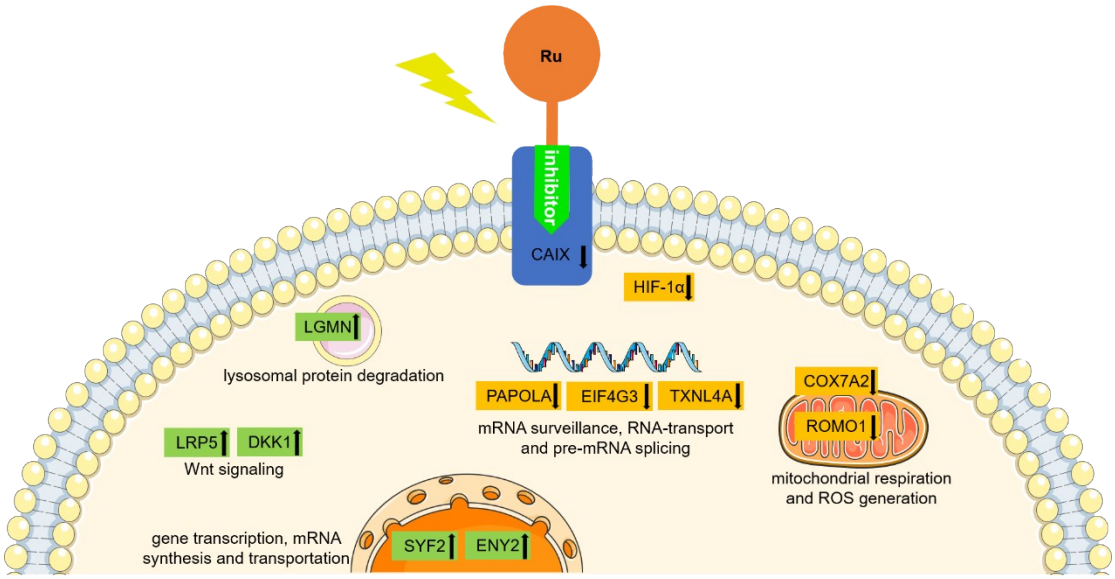


Figure S17. Possible regulatory mechanisms of **Ru-dppz-CAi** by degrading CAIX.

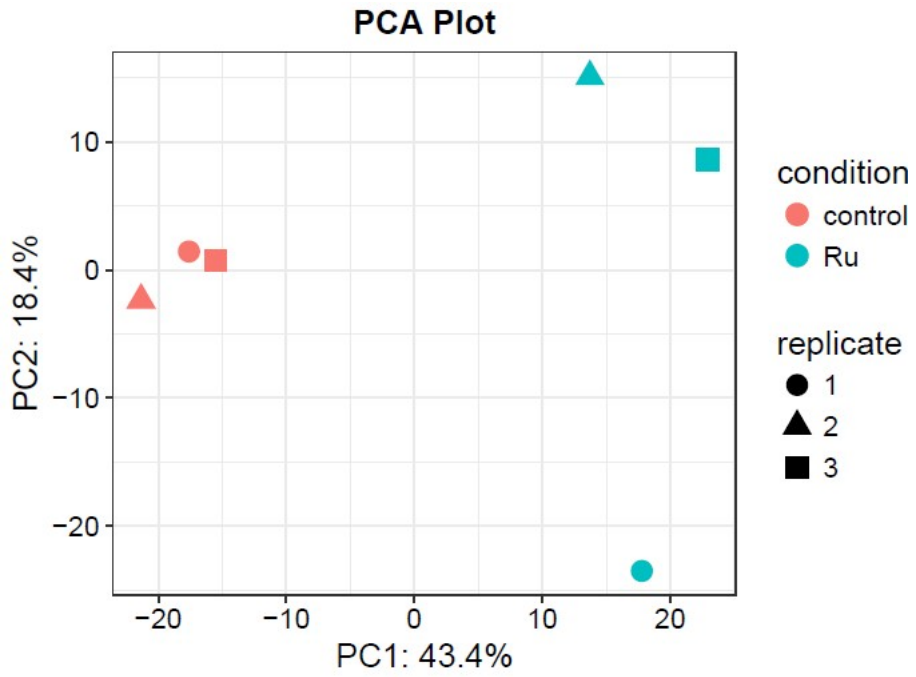


Figure S18. Principle Component Analysis (PCA) plot of proteomics.

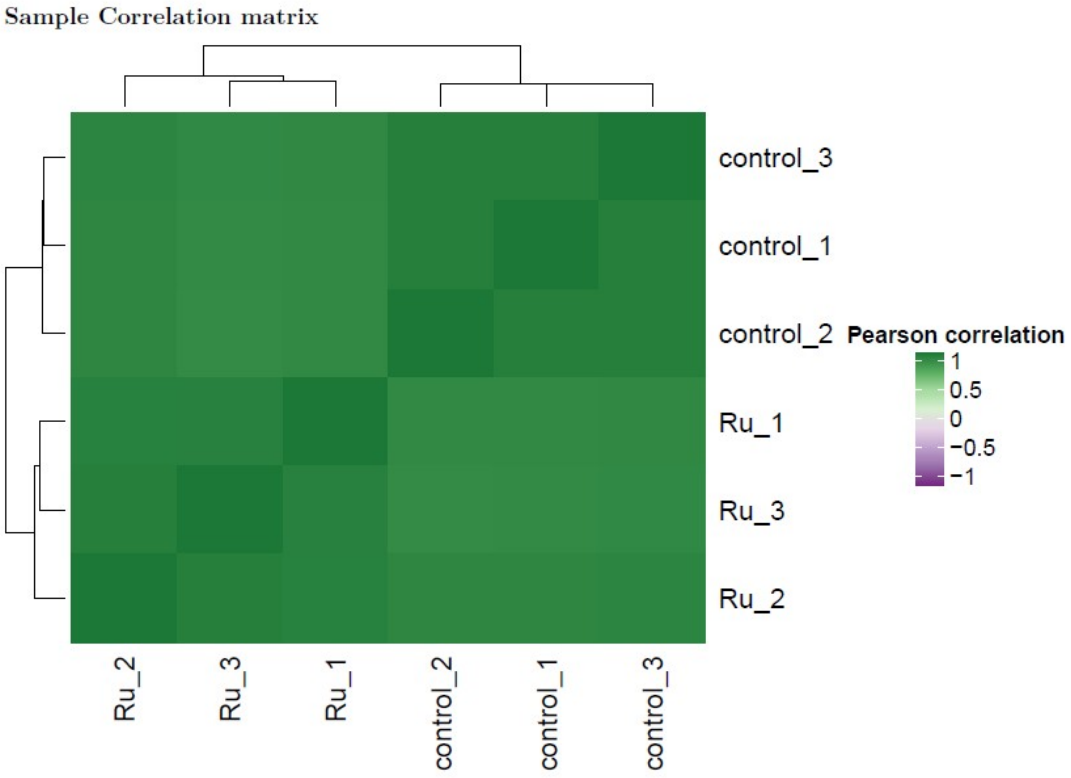


Figure S19. Sample correlation matrix of proteomics.

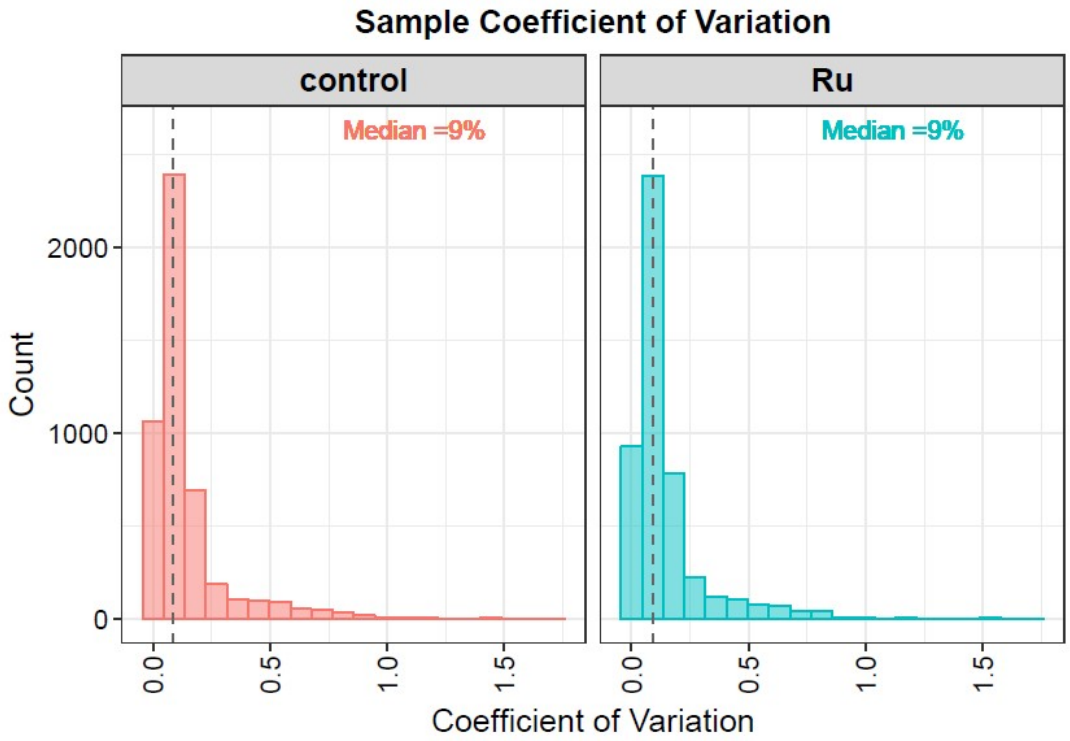


Figure S20. Sample Coefficient of variation (CVs) of proteomics.

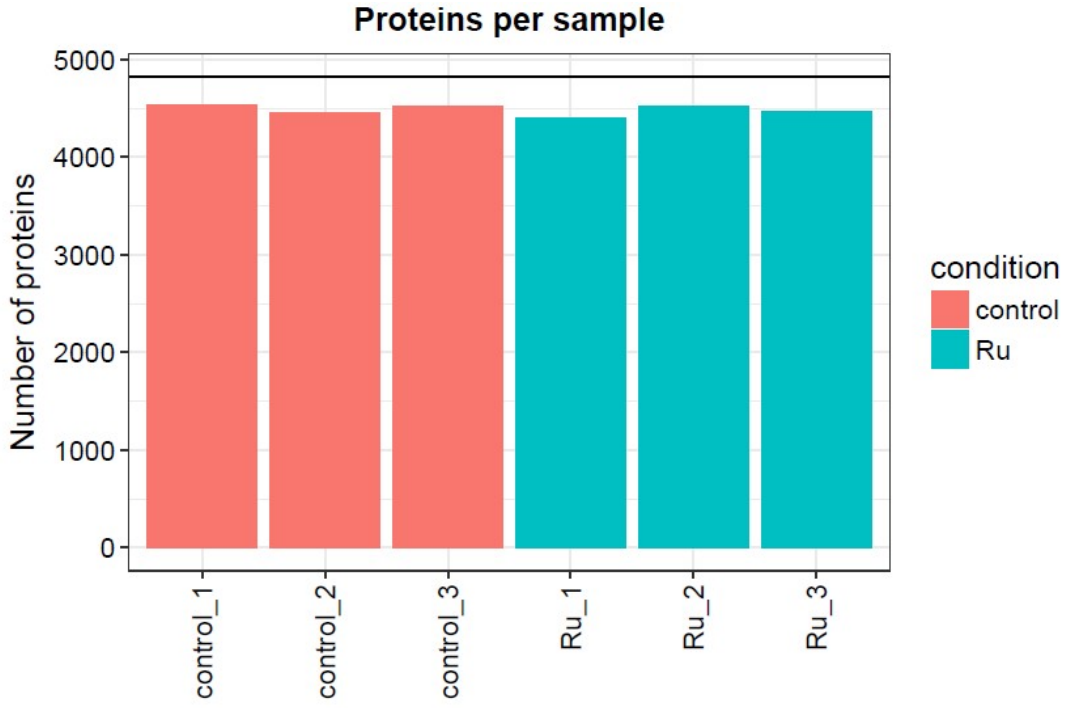


Figure S21. Protein quantified per sample (after pre-processing) in proteomics

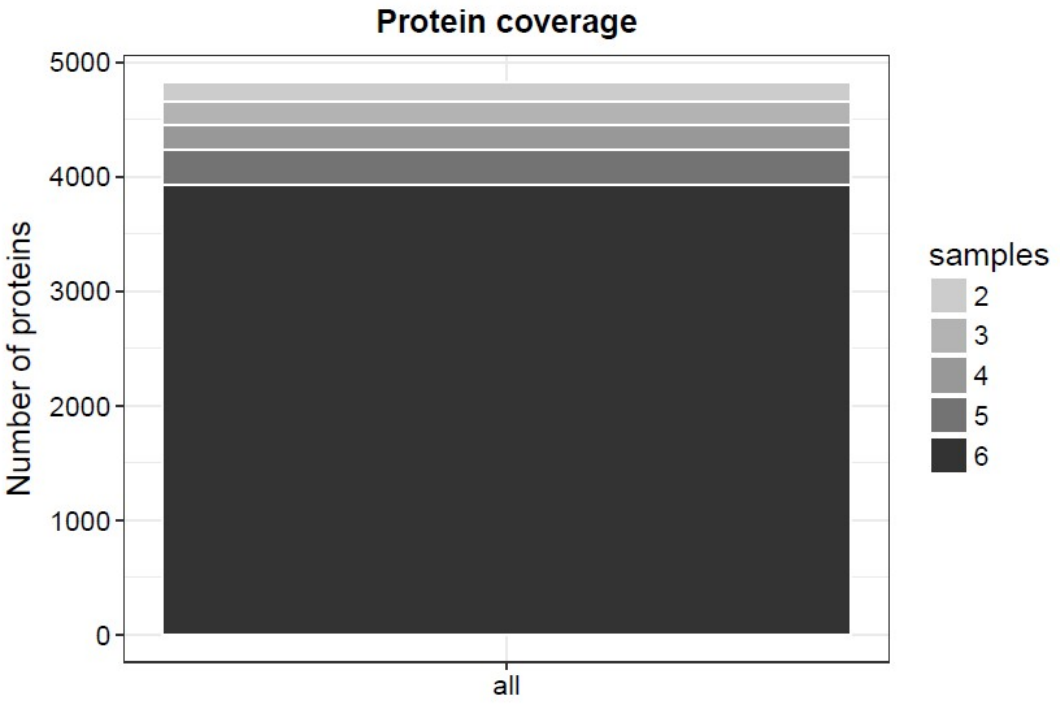


Figure S22. Protein overlap in all samples of proteomics.

References

[1] N. J. Lundin, P. J. Walsh, S. L. Howell, J. J. McGarvey, A. G. Blackman, K. C. Gordon, *Inorg. Chem.* 2005, **44**, 3551-3560.

[2] B. P. Sullivan, D. J. Salmon, T. J. Meyer, *Inorg. Chem.* 1978, **17**, 3334-3341.