Electronic Supplemental Information for

X-Ray Fluorescence Microscopy Reveals that Rhenium(I) Tricarbonyl Isonitrile Complexes Remain Intact In Vitro

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Experimental

Physical Measurements. NMR samples were prepared in DMSO-d_6 and analyzed on a 500 MHz Bruker AV 3HD spectrometer equipped with a broadband Prodigy cryoprobe. ^{1}H and ^{13}C NMR spectra were analyzed using MestReNova and referenced to the residual DMSO peak at 2.50 ppm and 39.52 ppm, respectively. Analytical RP-HPLC was performed using a Shimadzu LC20-AT HPLC equipped with an Ultra Aqueous C18 column, 100 Å, 5 µm, 250 mm x 4.6 mm (Restek, Bellefonte, PA) and an SPD-20AV UV–Vis detector monitoring at 220 and 270 nm with a flow rate of 1 mL/min. Gradient elution began with 10% MeCN in water containing 0.1% TFA for 5 min followed by a linear gradient to 100% MeCN over 20 min and then an additional 5 min of 100% MeCN. UV–Vis spectra were acquired using an Agilent Cary 8454 UV–visible spectrophotometer. Elemental analysis (CHN) was performed by Atlantic Microlab Inc. (Norcross, GA, USA). Samples were prepared for FTIR spectroscopy as KBr pellets and analyzed on a Thermo Nicolet Avatar 370 DTGS FTIR spectrometer. High-resolution electrospray mass spectrometry measurements (HR-ESI-MS) were performed on an Exactive Orbitrap mass spectrometer in positive ion mode (ThermoFisher Scientific, Waltham, MA). Absorbance signatures from cell viability assays were measured using a Biotek Synergy HT plate reader. Immunoblotting experiments were carried out using a Bio-Rad Mini PROTEAN© Tetra Cell, an Invitrogen PowerEase™ 500, and a Bio-Rad ChemiDoc MP imaging system. XFM was performed at the Advanced Photon Source (Argonne National Laboratory, Lemon, IL), and elemental maps were generated with the MAPS software package. Quantification of the data (in µg/cm^2) was performed by comparing the X-ray fluorescence intensity to those from National Bureau of Standards thin film standards NBS-1832, NBS-1833 (National Bureau of Standards, Gaithersburg, MD, USA).

Materials and Reagents. All reagents were purchased from commercial vendors. All reactions were carried out under ambient atmospheric conditions without any efforts to exclude oxygen or water. Solvents used were of ACS grade or higher. The compound *fac*-[Re(CO)$_3$(dmphen)Cl was synthesized using previously described methods,[14] and its purity was verified to be >95% pure by ^{1}H NMR spectroscopy. The compound **TRIP** was synthesized using previously described methods,[16] and its purity was verified to be >95% pure by RP-HPLC and ^{1}H NMR spectroscopy.

Synthesis of *para*-iodo isonitrile (I-ICN). I-ICN was synthesized following a modified version of previously reported procedures.[41,42] *Para*-iodoaniline (3 g, 13.7 mmol) was stirred in 9 mL of hot formic acid overnight (60 °C), yielding a purple solution. The reaction mixture was quenched in 250 mL saturated $NaHCO_3$, extracted three times with ethyl acetate (100 mL), and washed with brine (250 mL). The organic layer was dried with anhydrous Na_2SO_4, and the solvent was removed under reduced pressure, yielding a lavender colored solid. This solid and NEt_3 (3 equiv.) was stirred in 60 mL of tetrahydrofuran (THF) over an ice bath for 10 min. $POCl_3$ (1.2 equiv.) was then added dropwise to the solution, which was then stirred over an ice bath for 2 h. Na_2CO_3 (3 M, 60 mL) was added to the reaction mixture and allowed to stir overnight at room temperature. The crude product was then extracted three times with dichloromethane (DCM, 200 mL). The yellow-brown organic layer was then washed twice with brine (200 mL), dried with anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The crude was then purified using silica gel chromatography (1:1 Hexane:Et_2O), and the first band (R_f = 0.8) was dried to yield the malodorous product as a yellow-orange powder. Yield: 46.5%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.39 (d, 2H),

7.87 (d, 2H). ^{13}C NMR (500 MHz, DMSO-d_6): δ 96.50 ($C_{aromatic}$–I), 125.26 ($C_{aromatic}$–N), 128.18 ($C_{aromatic}$), 138.60 ($C_{aromatic}$), 165.48 (C≡N). Anal. Calcd for **I-ICN·0.2Et$_2$O** ($C_{7.8}H_6INO_{0.2}$): C, 38.42; H, 2.48; N, 5.72. Found: C, 38.64; H, 2.12; N, 5.49.

Synthesis of I-TRIP. $Re(CO)_3$(dmphen)Cl (50 mg, 0.10 mmol) and AgOTf (25 mg, 0.10 mmol) were stirred in refluxing THF in the dark for 3 h, and the resulting solution was filtered to remove AgCl. The bright yellow filtrate was then heated at reflux with **I-ICN** (95 mg, 0.40 mmol) overnight, yielding a pale, yellow solution. The solvent was removed under reduced pressure, and 5 mL of toluene was added to dissolve unreacted starting materials. The suspension was sonicated, and the pure product was isolated by filtration and washing with plenty of Et_2O. Yield: 77.2%.^{1}H NMR (500 MHz, DMSO-d_6): δ 3.30 (s, 6 H), 7.13 (d, 2H), 7.77 (d, 2H), 8.18 (d, 2H), 8.24 (s, 2H), 8.85 (d, 2H). FTIR (KBr, ν, cm^{-1}), 1906, 1960, 2050, 2165. Anal. Calcd for [**I-TRIP]OTf·0.2Et$_2$O** ($C_{25.8}H_{18}IN_3F_3O_{6.2}ReS$): C, 35.56; H, 2.08; N, 4.82. Found: C, 35.80; H, 2.08; N, 4.69. HR-ESI-MS (positive ion mode, MeCN): *m/z* 707.9812, calcd 707.9794 for $[M]^+$.

General Cell Culture. Cells were cultured as monolayers in a humidified incubator at 37 °C with 5% CO_2. The human cervical cancer, HeLa, cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. Cells were routinely passed at 80–90% confluency using 0.05% trypsin, 0.53 mM EDTA, 1× solution. Cell lines were tested monthly for contamination using the InvivoGen mycoplasma detection kit, PlasmoTest™.

Cytotoxicity. Dose-escalation studies were performed for **TRIP** and **I-TRIP**. Stock solutions were prepared as 20 mM solutions in DMSO. Solutions for dose-dependent studies were prepared by first serially diluting the stock solution 1:4 in DMSO, and then each was further diluted 1:100 into growth media. Cells were seeded in a 96-well plate at a density of 8000 cells/well and allowed to adhere for 24 h. Growth media was removed, and the cells were dosed with 200 µL of compounds in a dose-dependent manner for 48 h, then analyzed using an MTT assay described below.

The effect of the apoptosis inhibition was analyzed for **I-TRIP** by first seeding HeLa cells in a 96-well plate at a density of 8000 cells/well, which were allowed to adhere for 24 h. The growth media was then replaced with Z-VAD-FMK in growth media (100 µL, 20 µM) and incubated at 37 ˚C for 1 h. After incubation, an additional 100 µL of serially diluted **I-TRIP** was added to the Z-VAD-FMK solutions, incubated at 37 ˚C for another 48 h, and analyzed using an MTT assay described below.

The effect of the ER stress modulator, salubrinal, was analyzed for **I-TRIP** by first seeding HeLa cells in a 96-well plate at a density of 8000 cells/well, which were allowed to adhere for 24 h. The growth media was then replaced with serially diluted **I-TRIP** in growth media containing 25 µM salubrinal, incubated at 37 ˚C for 48 h, and analyzed using an MTT assay described below.

MTT assays were performed following compound treatment by first replacing growth media with 200 µL MTT (1 mg/mL). Cells were incubated with the dye for 4 h, and growth media was removed to yield purple formazan crystals. The crystals were dissolved in 200 µL of DMSO containing 12% glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The absorbance was measured at 570 nm for each well using a Biotek Synergy HT plate reader. Each plate contained six replicates per concentration, and at least three independent experiments were conducted with the same procedures. Data points were fit to the equation:

$$y = E_{inf} + \frac{E_0 - E_{inf}}{1 + (\frac{D}{IC_{50}})^{HS}}$$

where y is cell viability, E_{inf} is the viability at infinite drug concentration, E_0 is the viability at zero drug concentration, D is the drug concentration, IC_{50} is the 50% growth inhibitory concentration, and HS is the Hill slope.[43] E_0 was constrained to lie between 0.9 and 1. The compounds' IC_{50} values were determined by fitting the data and solving for these variables using the curve-fitting program, MagicPlot Pro.

Western Blotting. HeLa cervical cancer cells were plated in T-75 tissue culture flasks and allowed to grow to 80% confluency. For puromycin blotting, growth media was then replaced with 6 mL of **TRIP** (5 or 10 μM), **I-TRIP** (5 or 10 μM), or 0.3% v/v DMSO as a negative control and incubated at 37 ˚C for 24 h. During the last 10 min of the 24 h incubation, 10 μL of puromycin (20 mg/mL) was added to each sample and allowed to incubate for another 10 min. For CHOP blotting, growth media was then replaced with 6 mL of **TRIP** (10 μM), **I-TRIP** (10 μM), or 0.3% v/v DMSO as a negative control and incubated at 37 ˚C for 24 h. After incubation, cells were washed with 3 mL of DPBS and harvested. The cell suspension was then centrifuged, washed with 1 mL of DPBS, and lysed in 1× Cell Lysis Buffer (Cell Signaling). The lysates were vortexed on the highest setting for 10 s, centrifuged, and the supernatants were analyzed for protein content using the bicinchoninic acid (BCA) assay. The procedure for measuring protein content was previously described by the manufacturer.[44] The lysates were diluted 4:5 with 5× SDS-PAGE sample loading dye (0.4 M SDS, 0.9 mM bromophenol blue, 47% glycerol, 0.6 M dithiothreitol, 60 mM Tris buffer, pH 6.8), and the sample mixture was heated at 95 ˚C for 10 min. Proteins were separated on 10% acrylamide SDS-PAGE and transferred to PVDF membranes (Fisher). Membranes were blocked in tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and 5% non-fat milk for 1 h. Immunoblotting for quantifying the amount of puromycin incorporated was performed by incubating membranes with anti-puromycin, or anti-β-actin as the loading control, at 4 ˚C overnight. Immunoblotting for CHOP expression was performed by incubating membranes with anti-CHOP, or anti-β-actin as the loading control, at 4 ˚C overnight. After incubation, membranes were washed three times with TBST buffer (5 mL, 8 min), incubated with secondary horseradish peroxidase-coupled antibody solution for 1 h, and washed again three times with TBST buffer (5 mL, 8 min). Immunoblots were then visualized using enhanced chemiluminescence detector reagent (Fisher) via a Bio-Rad ChemiDoc MP imaging system.

Sample Preparation for XFM. HeLa cervical cancer cells were plated on 1.5 mm × 1.5 mm × 500 nm silicon nitride windows (Norcada, Canada) attached to 100 mm × 20 mm tissue culture dishes and allowed to grow to 70–80% confluency. Growth media was then replaced with 6 mL of 2 μM **TRIP**, 3 μM **I-TRIP**, or 0.06% v/v DMSO in media. After 4 h, the cells were washed with 6 mL of DPBS twice and then fixed with 6 mL of 4% paraformaldehyde for 15 min in the dark at room temperature. The fixative was removed, and cells were then incubated with 6 mL of 100 mM ammonium acetate for 2 min twice. The cells were washed with 6 mL of MilliQ water three times, and the silicon nitride window was detached from the tissue culture dish, dried using lens paper, and stored in a plastic capsule. The capsule was kept in a 1.5 mL microcentrifuge tube filled with Drierite and cotton until XFM analysis.

XFM Analysis. The elemental distributions in HeLa cells incubated with **TRIP** and **I-TRIP** were mapped at the 2-ID-D beamline at the Advanced Photon Source (Argonne National Laboratory,

Lemont, IL). Cells of interest were selected by optical microscopy. Five cells that were treated with **TRIP** were imaged across 5 scans. Five cells that were treated with **I-TRIP** were imaged across 4 scans. Five untreated HeLa control cells were imaged across 2 scans. The incident X-ray beam was tuned to an energy of 13.1 keV using a double multilayer monochromator and focused to a spot size of ~0.35 μm on the sample using with "a gold "high flux" zone plate setup. An energy-dispersive silicon drift detector (Vortex EX, SII Nanotechnology, Northridge, California, USA) was used to collect the X-ray fluorescence spectra from the sample, which was placed in a He environment at 75° to the incident beam. All elemental maps were recorded using the fly-scan mode, with a 0.5 μm step-size and a 200 ms dwell time. Elemental maps were generated with the MAPS software package by Gaussian fitting of the raw emission spectra for each image pixel. The Gaussian peaks were matched to characteristic X-ray emission lines to determine the fluorescence signal for each element. Quantification of the data (in $\mu g/cm^2$) was performed by comparing the X-ray fluorescence intensity to those from National Bureau of Standards thin film standards NBS-1832, NBS-1833 (National Bureau of Standards, Gaithersburg, MD, USA). Correlation coefficients for the colocalization of Re and I were determined using the Coloc 2 plug-in on ImageJ. Regions of interest comprising full cells were drawn using the Re elemental maps as a template for the total cell area.

Table S1 IC_{50} Values (μM) for **TRIP** and **I-TRIP** in HeLa cervical cancer cells. The errors represent the standard deviation from three independent experiments.

	HeLa
TRIP	1.8 ± 0.1
I-TRIP	3.3 ± 0.8

Table S2 Pearson's correlation coefficients for XFM elemental distributions within HeLa cells treated with 3 μM **I-TRIP**. These values were calculated using ImageJ.

Cell Sample	Re/I
1	0.68
2	0.67
3	0.69
4	0.64
5	0.74
Average	0.68 ± 0.02

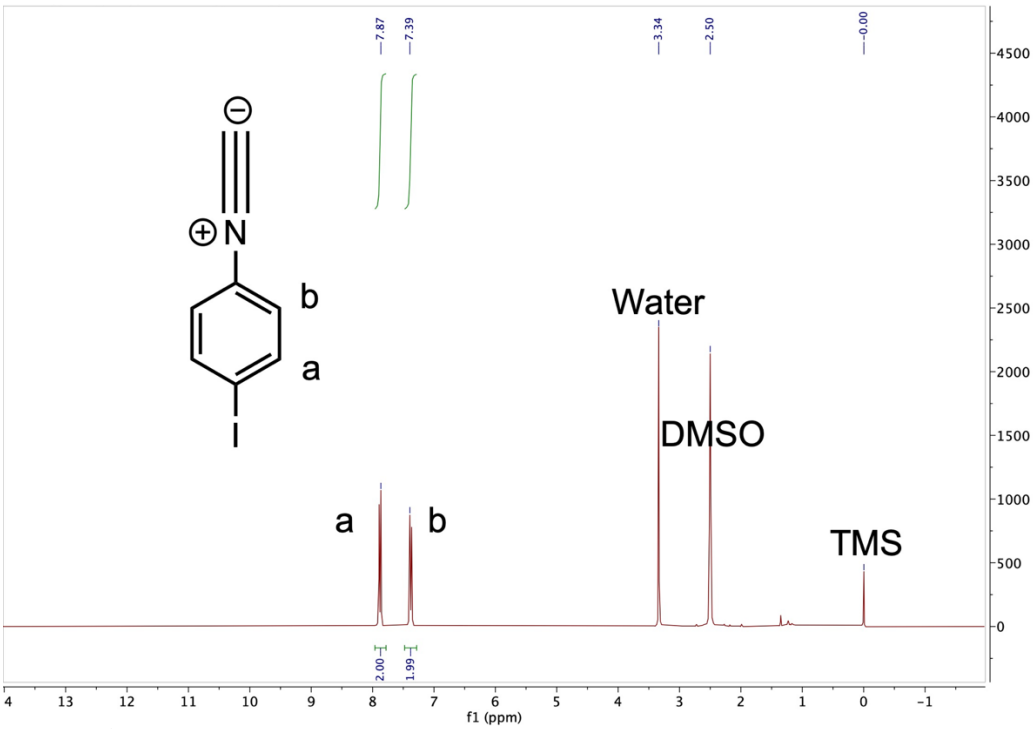

Fig. S1 ^{1}H NMR spectrum of **I-ICN** in DMSO-d_6 (500 MHz, 298 K).

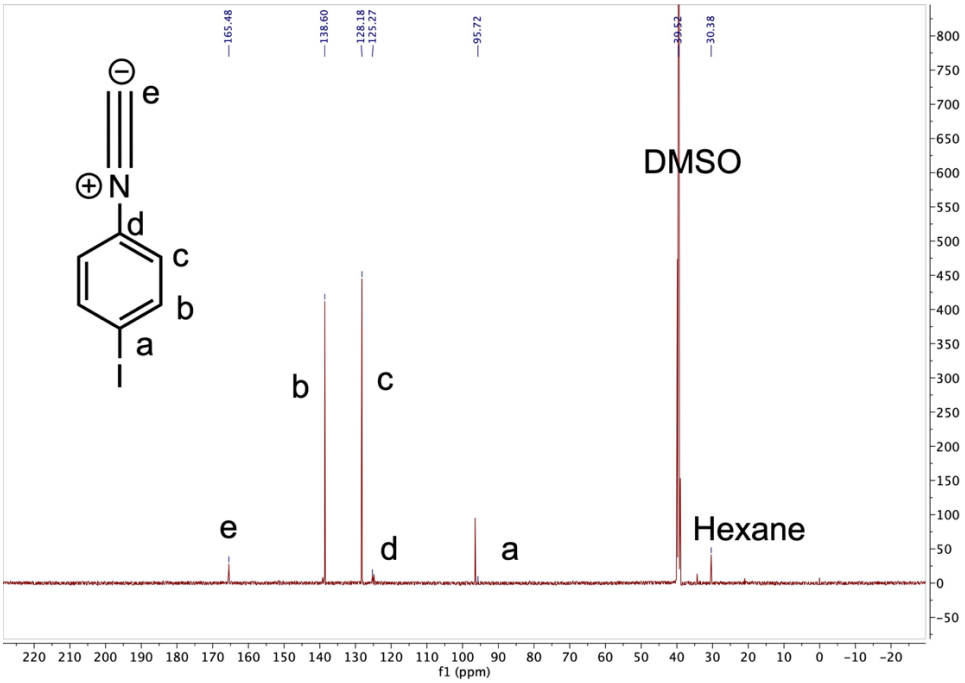

Fig. S2 ^{13}C{^{1}H} NMR spectrum of **I-ICN** in DMSO-d_6 (125 MHz, 298 K).

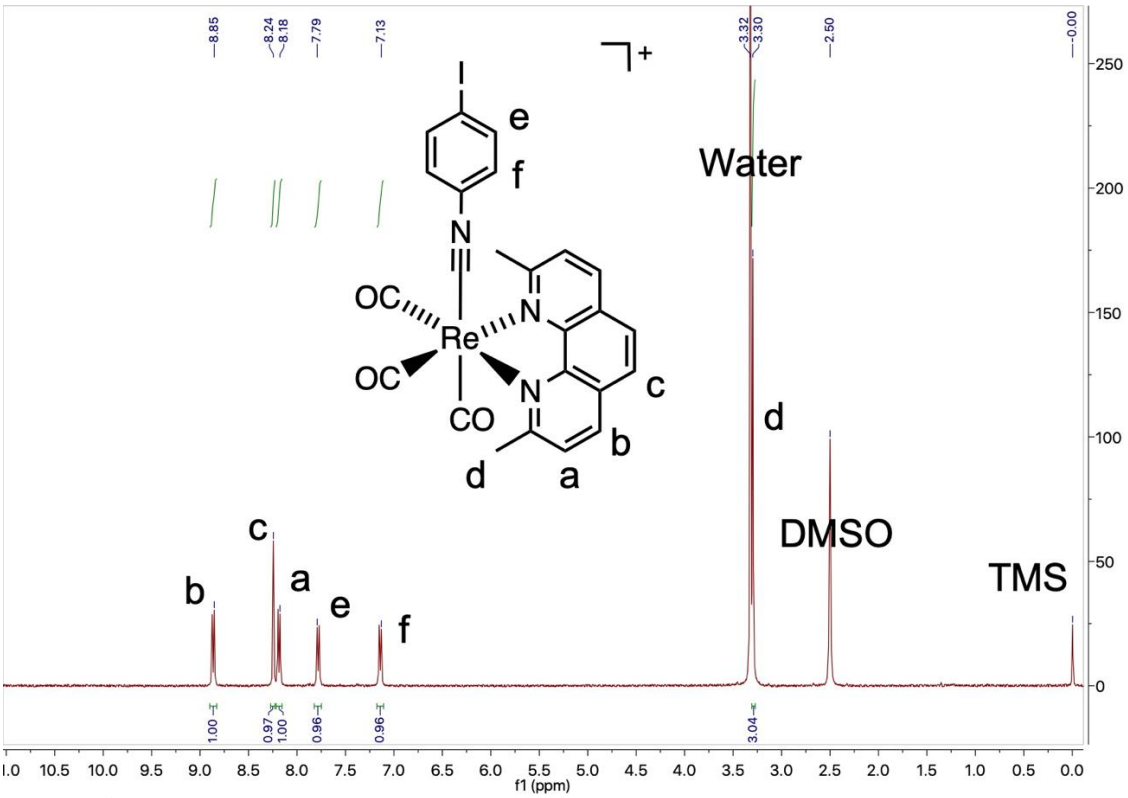

Fig. S3 ^{1}H NMR spectrum of **I-TRIP** in DMSO-d_6 (500 MHz, 298 K).

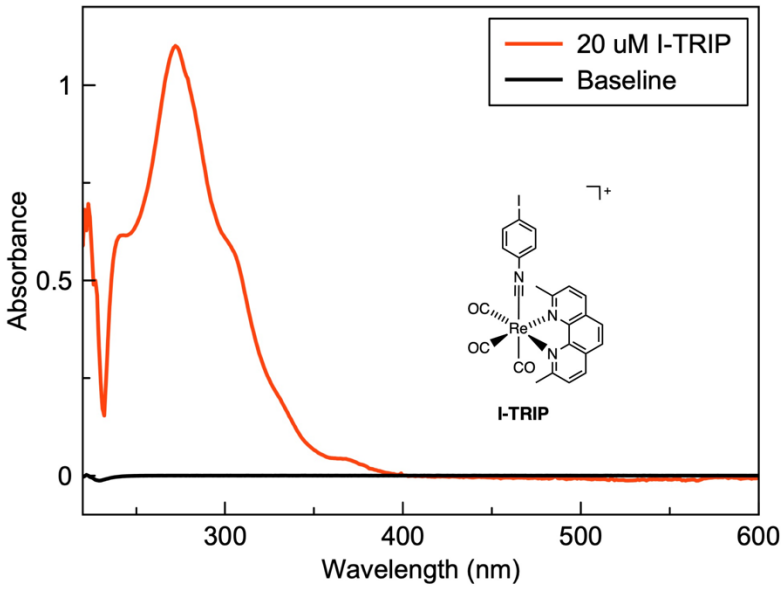

Fig. S4 UV–Vis spectrum of 20 µM **I-TRIP** in 1% DMSO in MOPS buffer, pH 7.3.

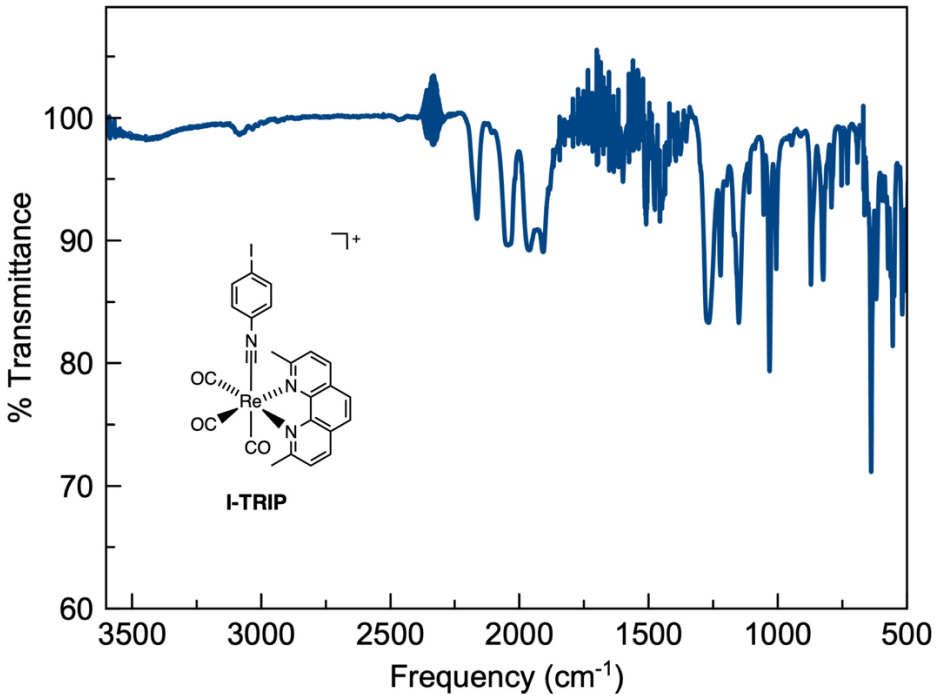

Fig. S5 FTIR spectrum of **I-TRIP** (KBr pellet).

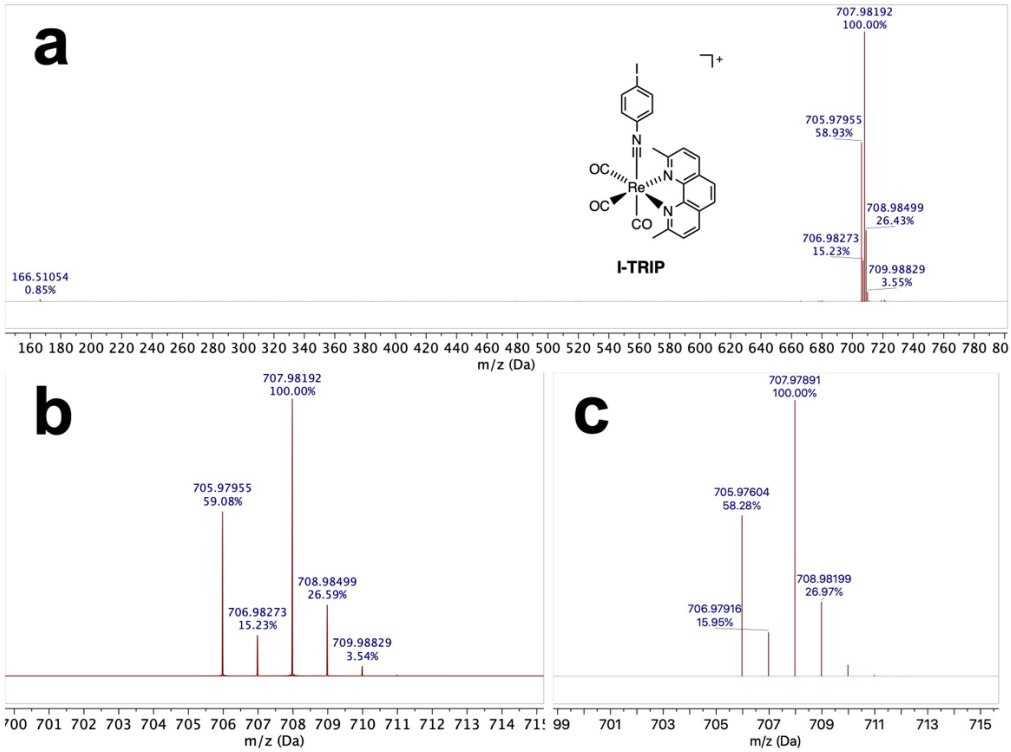

Fig. S6 (a) and (b) ESI-MS of **I-TRIP** (mobile phase: 1% formic acid in MeCN), and its (c) simulated ESI-MS using MestReNova.

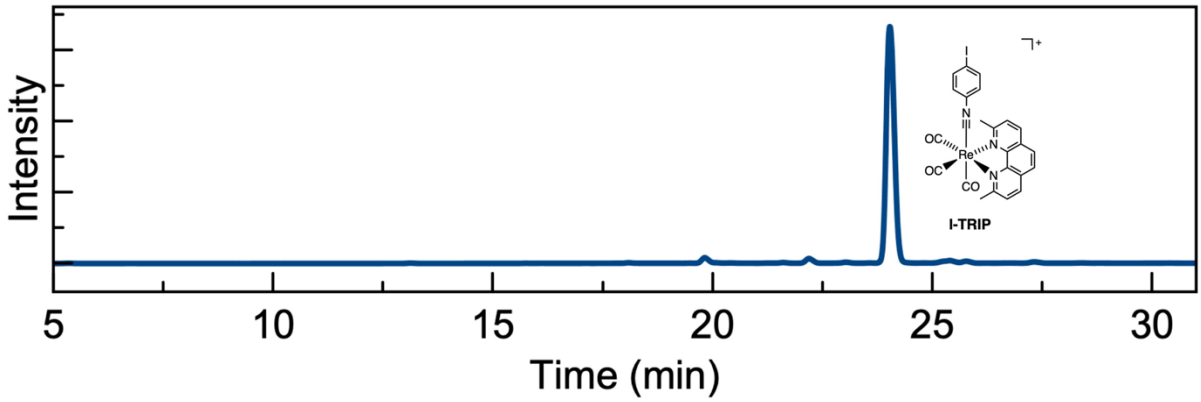

Fig. S7 RP-HPLC chromatogram of **I-TRIP** using the MeCN gradient method previously described.

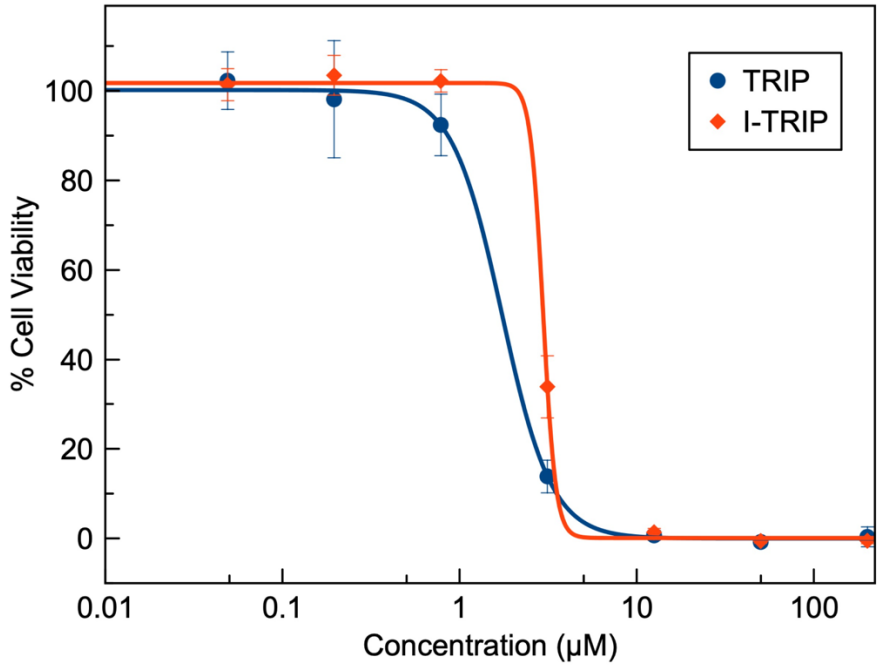

Fig. S8 Dose-response curves of **TRIP** and **I-TRIP** in HeLa cells. The error bars represent the standard deviation from six replicates.

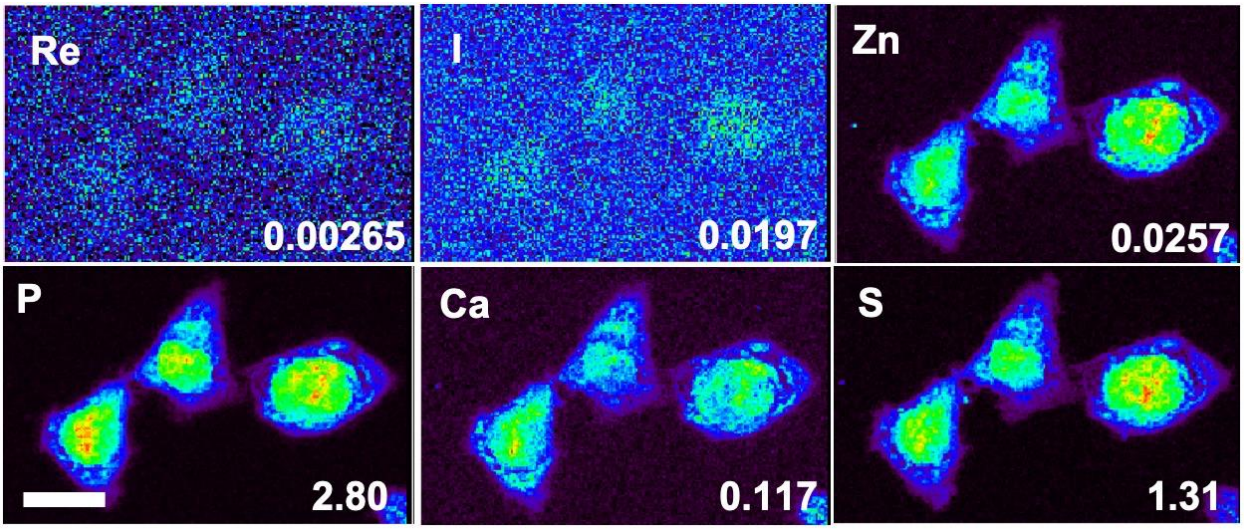

Fig. S9 XFM elemental distribution maps of the elements, Re, I, Zn, Cl, P, Ca, and S for HeLa cells treated with DMSO (0.3% v/v) for 4 h. Scale bar = 20 µm.

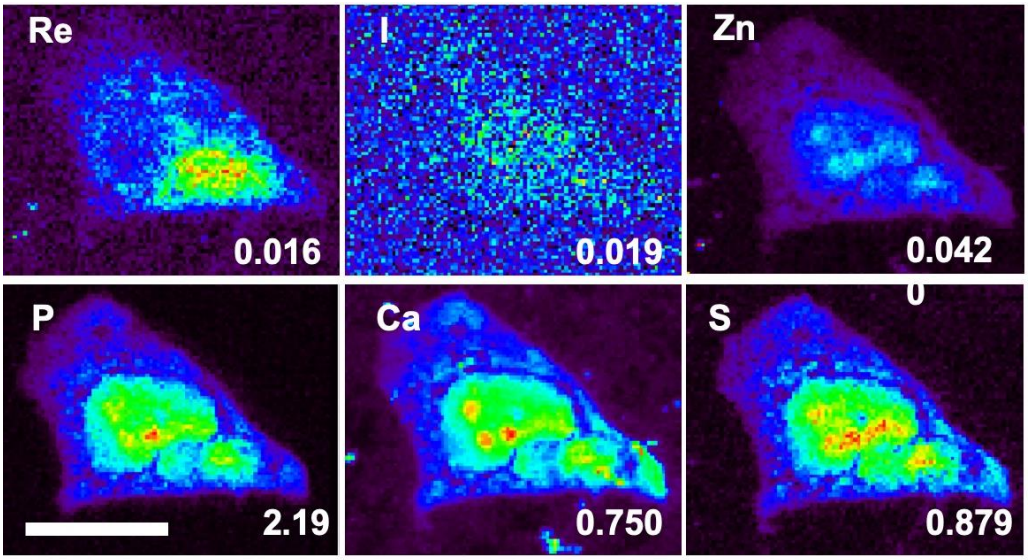

Fig. S10 XFM elemental distribution maps of the elements, Re, I, Zn, Cl, P, Ca, and S for HeLa cells treated with 2 µM **TRIP** for 4 h. Scale bar = 20 µm.

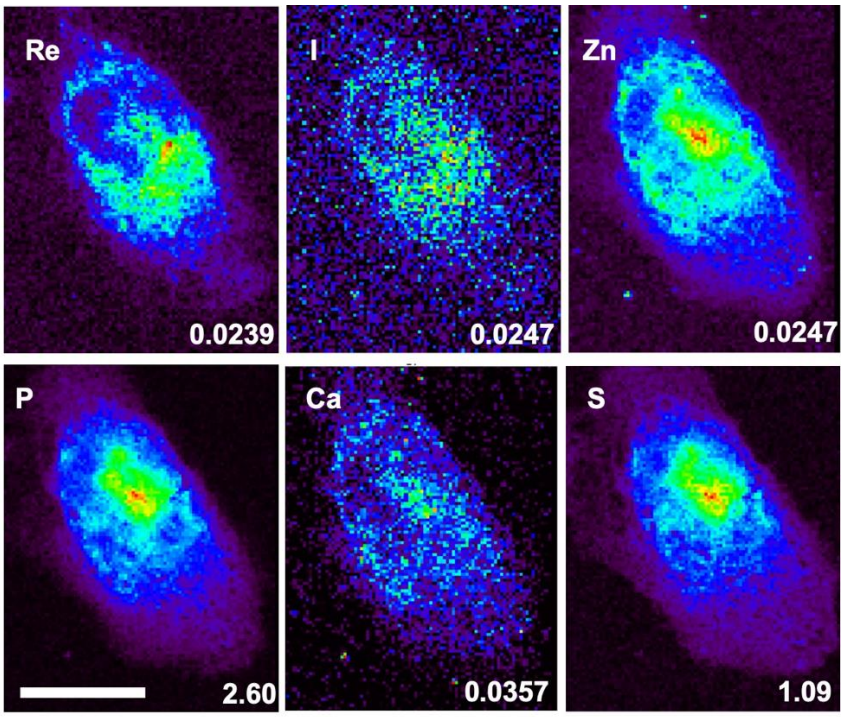

Fig. S11 XFM elemental distribution maps of the elements, Re, I, Zn, Cl, P, Ca, and S for HeLa cells treated with 3 μM **I-TRIP** for 4 h. Scale bar = 20 μm.