Supporting Information

Thiol-activated HNO release from a ruthenium anti-angiogenesis complex and HIF-1α inhibition for cancer therapy

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Experimental Details

Myoglobin as a probe for HNO production

To detect HNO production, ferric-myoglobin at 7-8 μM final concentration in 0.1 M phosphate buffer pH 6.3 at 25 ^{o}C was employed. To detect HNO production, ferric-myoglobin at 7-8 μM final concentration in 0.1 M phosphate buffer pH 6.3 at 25 ^{o}C was employed. The concentration of myoglobin was determined using ε_{408nm} = 188 mmol^{-1} L cm^{-1} in a quartz 1 cm cuvette.[1] Angeli's salt and DEA-NO were used as standard HNO and NO donors, respectively. Reduced glutathione and N-acetyl-L-cysteine were used alone with myoglobin to evaluate if any reaction could take place directly with them and also in reaction with $[Ru(bpy)_2(SO_3)(NO)]^+$ at mol ratios of 2:1 up to 20:1 thiol:complex. UV-Vis spectra were taken at defined intervals as evidence for HNO production as described elsewhere.[2]

Carboxy-PTIO reaction to identify HNO

Carboxy-PTIO was used to probe HNO and NO formation as characterised by characteristic changes in their UV-Vis and EPR spectra. This

organic spin-trap was used at 100-200 μM in 0.1 M phosphate buffer pH 6.3 along with reduced gluthathione and N-acetyl-L-cysteine alone and in reaction with $[Ru(bpy)_2(SO_3)(NO)]^+$ as well as upon light irradiation. Electronic spectroscopy measurements were carried out in a quartz 1-cm pathlength cuvette on a Cary 4000 (Varian) at 298 K. All EPR spectra were recorded at ambient temperature (ca. 291 K) on a Bruker EMX (X-band) spectrometer. A cylindrical Tm110 mode cavity (Bruker 4103TM) was used. The samples were prepared and transferred to spectrosil quartz tubes with inner diameter (I.D.) of 1.0 mm and outer diameter (O.D.) of 1.2 mm (Wilmad Labglass) filled with the sample and sealed with T-Blu Tack®. This tube was placed inside a larger quartz tube (O.D. 2.0 mm) so that the sample could be accurately and reproducibly positioned inside the resonator. The shortest measurement was taken within 9 min after the reaction mixture was prepared. The following EPR instrument settings were used: modulation amplitude 2.0 G, microwave power 0.63 mW, attenuation of 25 dB, receiver gain 1.0 $\times 10^4$, sweep gain 41.94 s, with repeated number of 5 X-scans and resolution in Y of 24.

Hydroxylamine Detection

This measurement was conducted as described elsewhere[3] with minor modifications[4]. Briefly, the Ru complex was prepared in 100 mM phosphate buffer pH=7.4 and mixed with L-glutathione (10-fold molar excess). After 30 min at 310 K, 100 μL of 10% trichloroacetic acid and 1 mL of 1% 8-hydroxyquinoline (in 50% ethanol) were quickly added, followed by 1 mL of 1 M sodium carbonate, and heating to 368 K for 5 min. This solution was allowed to cool and after 1 h its absorbance measured at 750 nm. A standard

curve was built using hydroxylamine ($NH_2OH.HCl$) along with 2 mM of glutathione to take into account any interference caused by this thiol. Angeli's salt was also used as a positive control for HNO release in the presence of 2 mM glutathione.

HIF-1α inhibition assay

MCF-7 or MB-231 breast cancer cells ($2x10^6$ cells) were plated into 60 mm cell culture dishes in RPMI complete medium supplemented with 10% HI FBS and penicillin-streptomycin and incubated overnight. The next day, the cells were pretreated with the metallonitrosyl **1** $[Ru(bpy)_2(SO_3)(NO)](PF_6)$ or control **2** compound ($[Ru(bpy)_2(SO_3)(H_2O)]$) for 30 min in serum-free medium, then treated with 100 μM SPER/NO for 4 h or flushed with N_2 gas for 1.5 h. The cells were then rinsed with cold PBS, scrape harvested, centrifuged, and the pellet was resuspended and sonicated in lysis buffer [1% Nonidet P-40 0.5% sodium deoxycholate 0.1% SDS and protease inhibitor mixture (Calbiochem)]. After a 10-min incubation on ice, the samples were centrifuged at 10,000xg, and the supernatant protein concentration was determined by the bichoncinic acid method (Pierce).

Automated Capillary Western Blot (WES)

Western blots were performed using WES, an automated capillary-based size sorting system (ProteinSimple, San Jose CA).[5] All procedures were performed with manufacturer's reagents according to their user manual. Briefly, 8 μL of diluted protein lysate was mixed with 2 μL of 5x fluorescent master mix and heated at 368 K for 5 min. The samples (1 μg), blocking

reagent, wash buffer, primary antibodies, secondary antibodies, and chemiluminescent substrate were dispensed into designated wells in a manufacturer provided microplate. The plate was loaded into the instrument and protein was drawn into individual capillaries on a 25 capillary cassette provided by the manufacturer. Protein separation and immuno detection were performed automatically on the individual capillaries using default settings. The data were analyzed using Compass software (ProteinSimple, San Jose CA).[5] Primary antibodies used were HIF-1α (BD Bioscience, San Jose CA diluted 1:100) and HPRT loading control (Santa Cruz Biotech, Santa Cruz CA diluted 1:100). Results obtained from WES are consistent with those using PAGE. Data are representative of n = 3 individual experiments.

In vitro angiogenesis assay

Anti-angiogenic effects were examined using an in vitro angiogenesis assay kit (EMD Millipore, Billerica MA) according to the manufacturer's recommendations. Briefly, while on ice ECMatrix was mixed with 10X diluent buffer and 50 µl aliquotted per well into a cold 96-well plate. The plate was incubated at 310 K to allow the matrix to solidify. HUVEC cells (15,000) at passage 3 were prepared in the presence and absence of drug and gently layered on top of the matrix. The cells were incubated at 310 K for 17 h and endothelial cell tube formation was monitored and quantified by the number of fully or partially enclosed networks.

Note 1: As expected, based on the literature, the standard HNO donor (Angeli's salt) caused a red shift of the Soret band along with changes in the alpha and beta bands in the Q-band region, whereas the NO donor (DEANO NOATE) did not cause any changes (Supporting Figure S2). Depending on the conditions and molar excess of GSH used, slow reduction of met-myoglobin can occur. We noted that use of phosphate buffer at pH 6.3 can slow down dramatically GSH reactivity toward met-Mb, while it maintains full reactivity with the metallonitrosyl complex, based on spectroscopic changes. On the other hand, N-acetyl-L-cysteine (NAC), did not show any significant reaction with met-myoglobin even after much longer incubation times, or even at pH 7.4.

Note 2: One potential interference for cPTIO detection is a reaction with thiols itself that could lead to EPR-silent product. However, NAC and GSH did not cause any change in EPR signal at pH 6.3 on the timescale of the experiment (Supporting Figure S3). Dithioerithrol (DTT) and ascorbic acid react rapidly with cPTIO as indicated by the disappearance of the band at 560 nm, which might contribute to the limitations on the use of cPTIO *in vivo* as reported elsewhere.

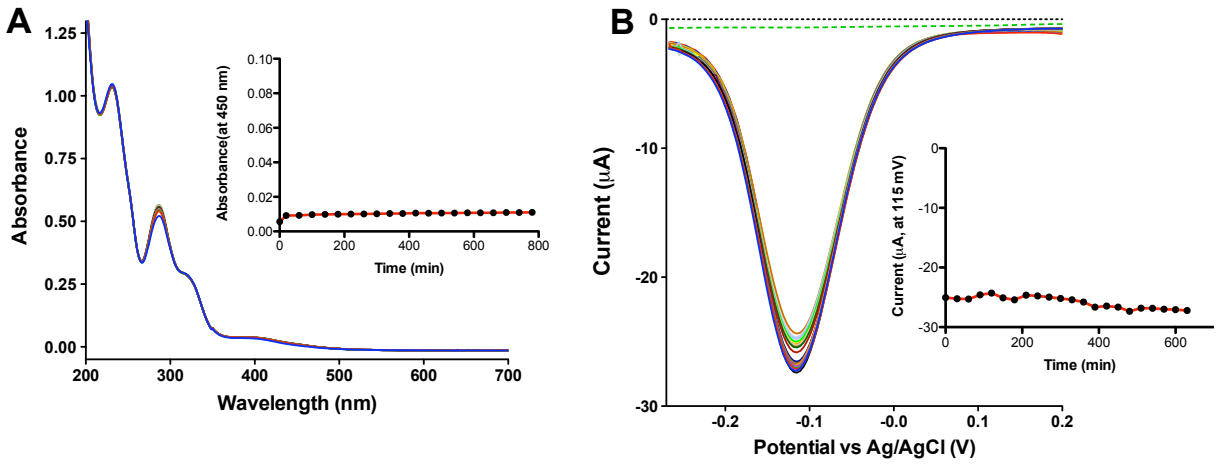

Figure S1. Ruthenium complex stability in 5 mM phosphate buffer pH 7.4, 100 mM NaCl at 37 ^{o}C, followed by (a) electronic spectroscopy (inset: kinetic trace for absorbance change at 450 nm) and (b) pulse differential voltammetry (inset: kinetic trace for current change at -115 mV).

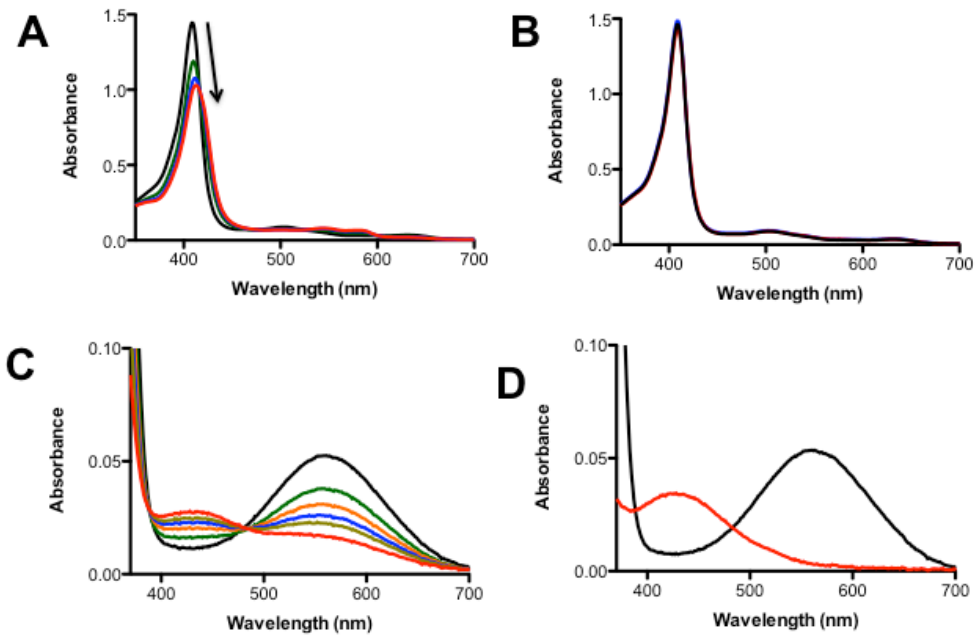

Figure S2. Detection assays for HNO in 0.1 M phosphate buffer pH 6.3, using (a) myoglobin (7.8 μM) and Angeli's salt (50 μM) (black 0 min last red 18 min); (b) myoglobin (7.8 μM) and DEA-NONOATE(100 μM)(black 0 min, last red 29 min; (c) carboxy-PTIO (45 μM) and Angeli's salt (45 μM) (black 0 min, last red 33 min); (d) carboxy-PTIO (45 μM) and DEA-NONOATE (45 μM) (black 0 min, red 2 min).

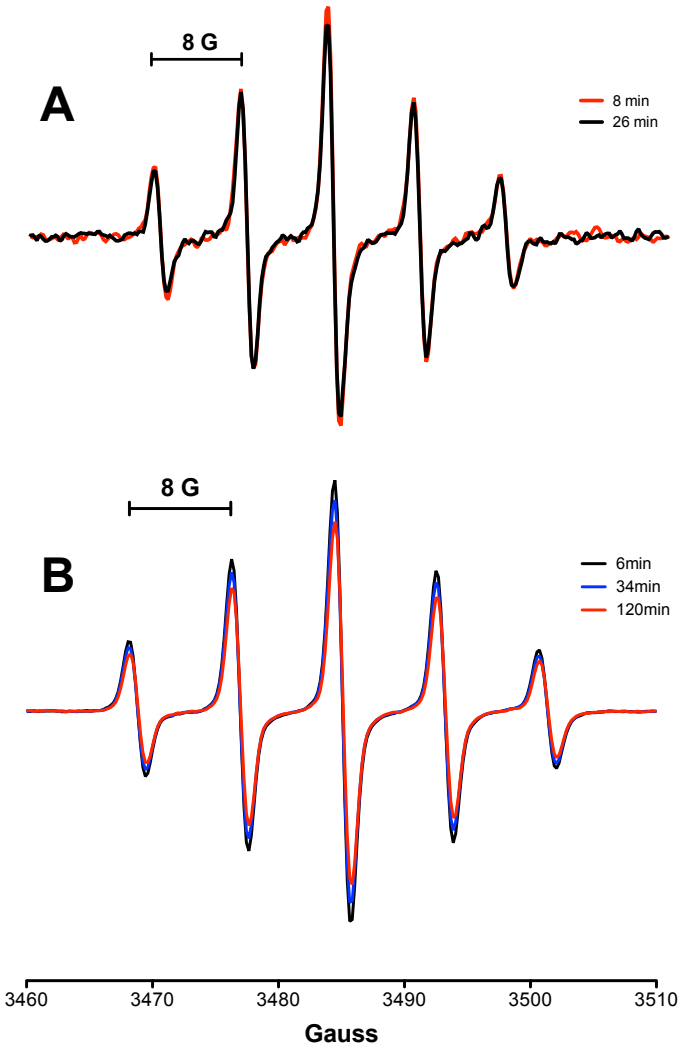

Figure S3. Effect of thiols on EPR signal of carboxy-PTIO (200 μM). (a) N-acetyl-L-cysteine (500 μM), (b) reduced glutathione (800 μM), in 0.1 M phosphate buffer (pH = 6.3).

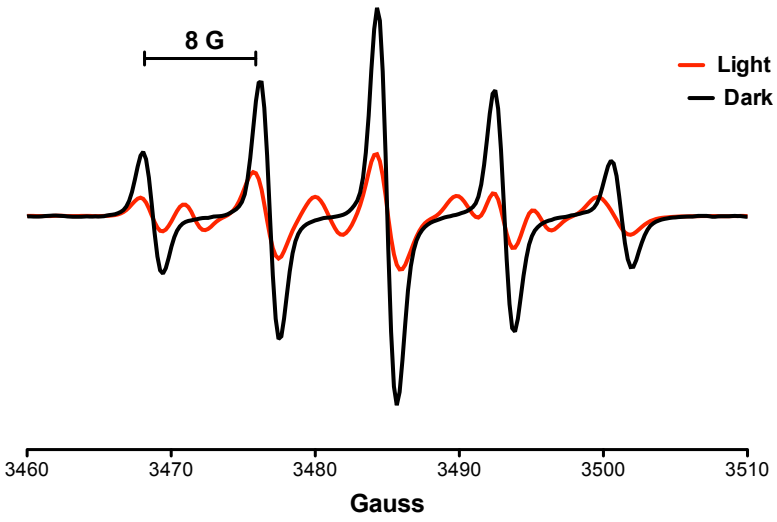

Figure S4. EPR spectrum of carboxy-PTIO in the presence of $[Ru(bpy)_2(SO_3)(NO)]^+$ in the dark or when irradiated with a blue LED (463 nm).

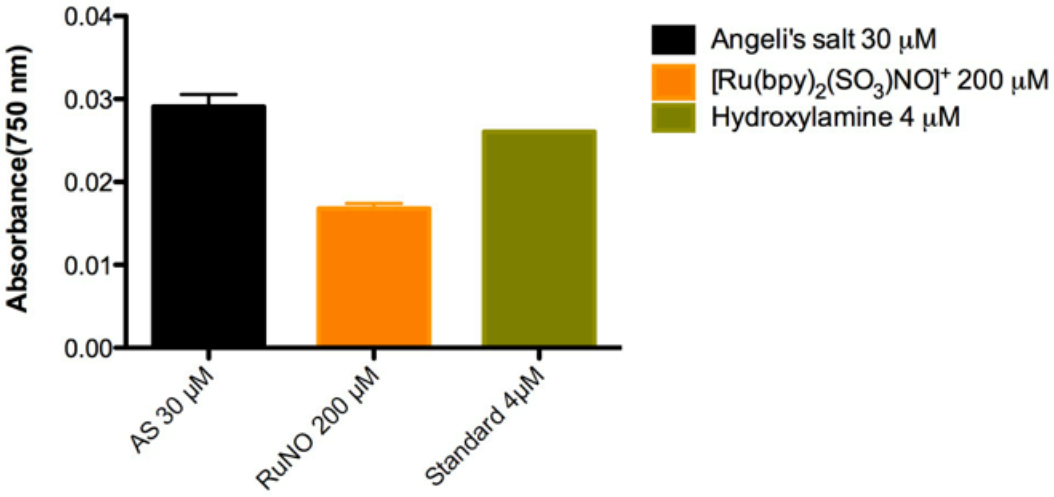

Figure S5. UV-vis detection of hydroxylamine, in a reaction of $[Ru(bpy)_2(SO_3)(NO)]^+$ (200 μM) with glutathione (2 mM), Angeli's salt (30 μM) with glutathione(2mM), and hydroxylamine (4 μM) with glutathione (2 mM).

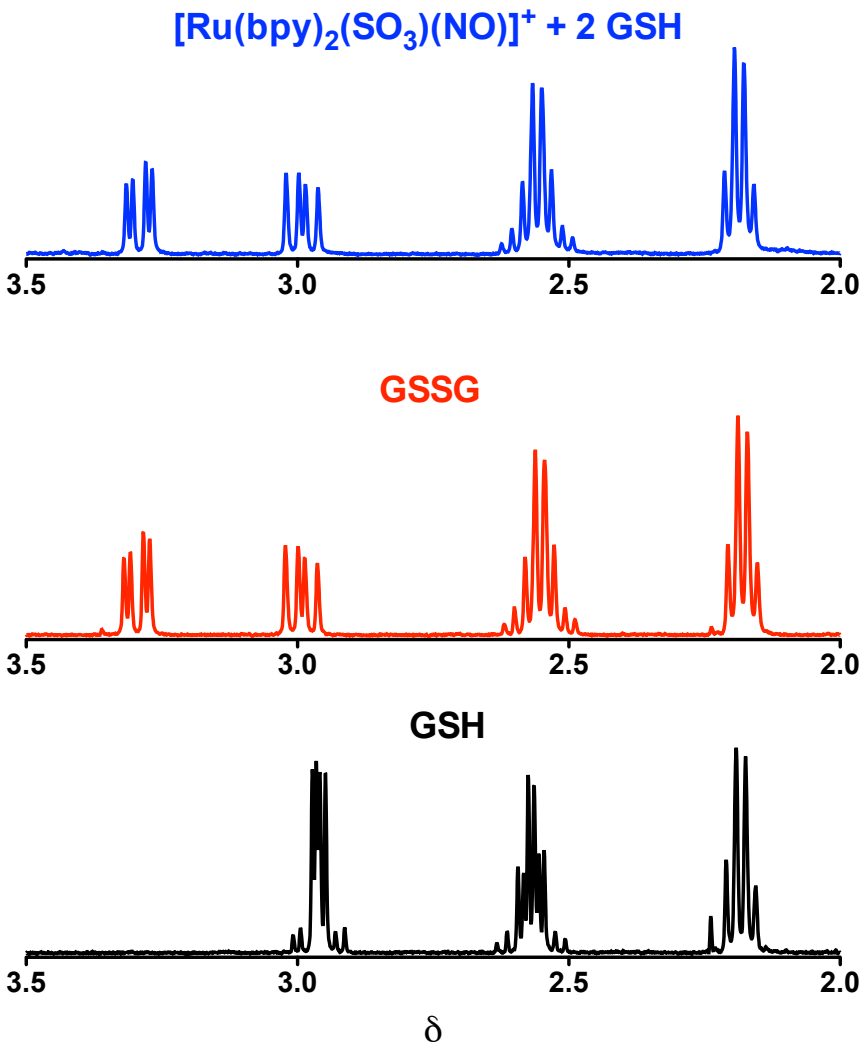

Figure S6. ^{1}H NMR spectra for in phosphate buffer solutions at pH 7.4 (D_2O) of the reaction of 6 mM of $[Ru(bpy)_2(SO_3)(NO)]^+$ with 12 mM of reduced glutathione (top); 12 mM of oxidized glutathione (middle), and 12 mM reduced glutathione (bottom).

[1] Antonini, E., and Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam.

[2] Zapata, A. L., Kumar, M. R., Pervitsky, D., and Farmer, P. J. (2013) A singular value decomposition approach for kinetic analysis of reactions of HNO with myoglobin, *Journal of inorganic biochemistry 118*, 171-178.

[3] Prodinger, W., and Svoboda, O. (1953) Nachweis und Bestimmung yon Hydroxylamin mit 8-0xy-chinolin, *Microchimica Acta 41*, 426-433.

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[5] Beccano-Kelly, D. A., Kuhlmann, N., Tatarnikov, I., Volta, M., Munsie, L. N., Chou, P., Cao, L. P., Han, H., Tapia, L., Farrer, M. J., and Milnerwood, A. J. (2014) Synaptic function is modulated by LRRK2 and glutamate release is increased in cortical neurons of G2019S LRRK2 knock-in mice, *Front Cell Neurosci 8*, 301-311.