Supporting information for

Redox and Catalase-like Activities of Four Widely used Carbon Monoxide Releasing Molecules (CO-RMs)

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General information. All reagents and solvents were of reagent grade from commercial suppliers (e.g., Sigma Aldrich). A microplate reader (PerkinElmer Victor2, USA) was used for the H_2O_2 titration assay. Absorption spectra were measured on Varian Cary 100 Bio UV-Visible spectrophotometer. CO-RMs (CORM-2, CORM-3, CORM-A1 and CORM-401) were from Sigma-Aldrich and were used without purification. Catalase from bovine liver (2000-5000 units/mg) was from Sigma-Aldrich (C9322). Solutions of iCORM-2 and iCORM-3 were prepared according to literature procedures.[1, 2] The hydrogen peroxide fluorescent probe, PF-1, was synthesized according to literature procedures.[3]

Stock solution preparation. Stock solutions of CORM-2 (2 mM) were prepared either in methanol or DMSO. Stock solutions of CORM-3 (2 mM) and CORM-A1 (10 mM) were prepared in water. Stock solutions of CORM-401 (10 mM) and catalase were prepared in PBS buffer (pH=7.4, 0.01 M). Stock solutions of $Ru(DMSO)_4Cl_2$ (2 mM), Trolox (2 mM), BW-CO-111 (2 mM) and BW-CP-111 (2 mM) were prepared in DMSO. Stock solutions of DPPH (1 mM) were prepared in methanol. All stock solutions were freshly prepared before the experiments.

Hydrogen peroxide titration. The concentration of H_2O_2 was determined by a reported colorimetric assay with modifications.[4] The detection mechanism is based on the reaction among H_2O_2, 4-aminoantipyrine, phenol and horseradish peroxidase (HRP) to generate a pink quinone imine dye, which can be easily monitored by measuring the absorbance at 490 nm. Specifically, the stock solutions of phenol (500 mM), 4-aminoantipyrine (50 mM) and HRP (150 unit/ml) were prepared separately in water. The stock solutions of these compounds were further diluted by PBS buffer (pH=7.4, 0.01 M) to generate a detection mixture containing phenol (12 mM), 4-aminoantipyrine (1 mM) and HRP (1 unit/ml). To establish a standard curve, H_2O_2 solutions (final concentration: 25 to 440 μM) were added to the detection mixture. The resulting mixture was incubated for 30 min before measuring the absorbance at 490 nm by using a plate reader. For H_2O_2 titration, a suitable volume of the reaction solution was titrated into the detection mixture, and the final concentration of H_2O_2 should be in the detection range from the standard curve. The resulting mixture solutions were incubated for 30 min followed by measuring the absorbance at 490 nm.

Preparations of ABTS radical and DPPH solutions. The ABTS radical solution was prepared according to a literature procedure.[5, 6] A 7-mM solution of ABTS was prepared by dissolving ABTS in 20 mM acetate buffer (pH 4.5). A 2.45-mM potassium persulfate solution was prepared in water. The stock solution of ABTS radical was prepared by mixing the ABTS solution and potassium persulfate solution in a 1:1 ratio. The resulting mixture was incubated for 16 h in the dark to generate a dark green-blue solution, which was further diluted with ethanol for the reaction with CO-RMs and Trolox. The 0.2-mM solution of the DPPH radical was freshly prepared by dissolving DPPH in methanol.

ABTS and DPPH decolorization assays. 1 ml of ABTS solution or DPPH solution was incubated with various concentrations of CO-RMs under dark conditions for 6 min or 1 h, respectively. Then, the UV-Vis absorption spectra of the reaction solutions were measured.

GC method for semi-quantification of oxygen. In 6-ml headspace vials, various reaction solutions (group 1 to 6) were charged. The headspace vials were capped with an aluminum seal with PTFE/rubber liner during the 1 h incubation at room temperature. After incubation, for each group, 250 µL of gas sample was taken from the headspace by a gas-tight syringe (Hamilton®). The gas sample was analyzed by a GC system with TCD detection. Helium was used as the carrier gas with a flow rate of 30 mL/min. Gaseous components of the headspace were separated by passing through a packed column (60/80 Carboxen-1000 matrix support, L×O.D.×I.D. 15.0 ft (4.6 m)×1/8 in ×2.1 mm, Supelco). The column was heated to 35 °C for 5 min, and then the column temperature was increased to 225 °C at a rate of 20 °C/min while the detector temperature was held at 125 °C.

Group 1: 4 ml of PBS buffer (pH=7.4, 0.01 M)

Group 2: 4 ml of PBS buffer (pH=7.4, 0.01 M), spiked with 230 µL (~0.01 mmol) O_2

Group 3: 4 ml of PBS buffer (pH=7.4, 0.01 M), spiked with 450 µL (~0.02 mmol) O_2

Group 4: 4 ml of 10 mM H_2O_2 in PBS buffer (pH=7.4, 0.01 M)

Group 5: 4 ml of 5 mM H_2O_2 in PBS buffer (pH=7.4, 0.01 M) containing 0.02 mM CORM-2

Group 6: 4 ml of 10 mM H_2O_2 in PBS buffer (pH=7.4, 0.01 M) containing 0.02 mM CORM-2

Table S1. Detection of oxygen generation from H_2O_2 disproportionation by GC.

Group	Oxygen peak (area)	
	1st	2nd
1	12665	12794
2	13939	14236
3	15524	15492
4	12601	12602
5	14530	14191
6	15950	15833

Detection of hydrogen peroxide in cell culture medium after treatment with CO-RMs. A fluorescence probe (PF-1) was used to measure the concentration of H_2O_2 in cell culture medium.[3] To generate a standard curve, 10 µM of PF-1 was incubated with 10 µM to 120 µM of H_2O_2 in FluoroBrite™ DMEM (A1896701, Thermo Fisher Scientific) for 1 h at 37 °C. After incubation, 100 µL of the solution from each reaction mixture was added to a single well in a 96

well plate. The fluorescence (excitation wavelength: 490 nm emission wavelength: 525 nm) was measured by using a plate reader. For CO-RM-treated groups, 100 μM of H_2O_2 solutions were first incubated with various concentrations of CORM-2, CORM-3, iCORM-2 or iCORM-3 in DMEM or DMEM (with 10% FBS, v:v) for 1 h at 37 °C. After that, 10 μM of PF-1 was added to each reaction. The resulting mixture was incubated for an additional 1 h at 37 °C followed by measurement of fluorescence intensity.

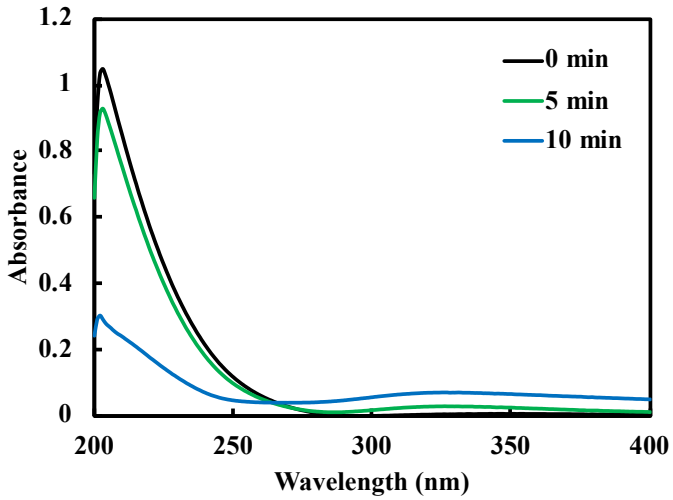

Figure S1. UV-vis spectra of 10 mM H_2O_2 upon treatment with CORM-2 (20 μM) in PBS buffer (pH = 7.4, 10 mM) at room temperature.

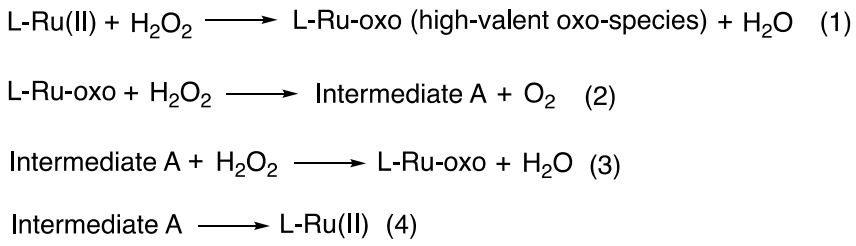

Figure S2. A proposed mechanism for Ru (II)-mediated H_2O_2 disproportionation.[7]

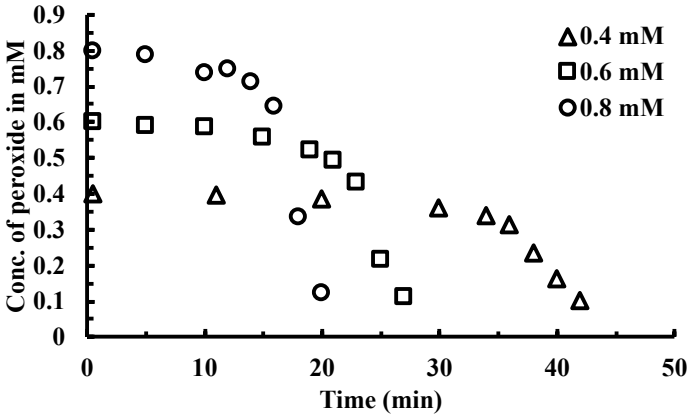

Figure S3. Time-dependent changes of H_2O_2 concentration upon incubations with CORM-2 (20 μM) in PBS (pH=7.4, 0.01 M, 1% DMSO) at 37 °C. The concentration of H_2O_2 was monitored by using a titration method.[4]

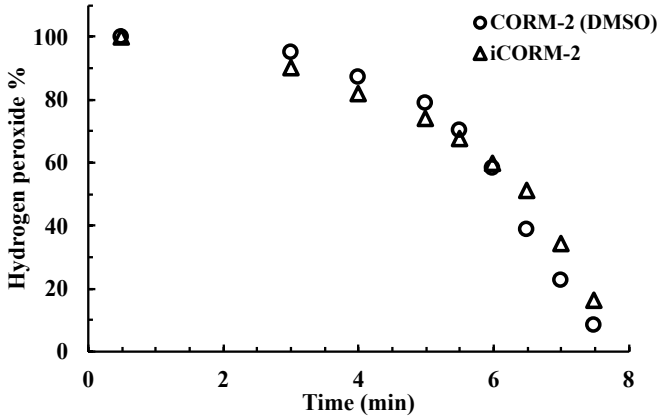

Figure S4. Time-dependent changes of H_2O_2 concentration. (expressed as a percentage of the initial concentration: 5 mM) over time upon incubations with CORM-2 (20 μM, diluted from DMSO stock solution) and iCORM-2 (20 μM) in PBS (pH=7.4, 0.01 M, 1% DMSO) at room temperature. The concentration of H_2O_2 was monitored by using a titration method.[4]

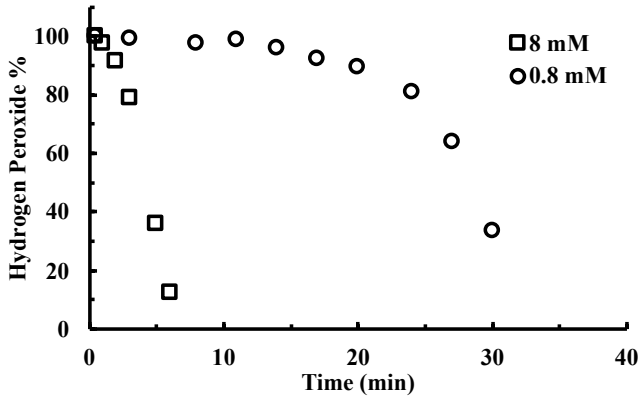

Figure S5. Time-dependent changes of H_2O_2 concentration.(expressed as a percentage of the initial concentration: 8 mM or 0.8 mM) over time upon incubations 20 μM CORM-3 in PBS (pH=7.4, 0.01 M) at 37 °C. The concentration of H_2O_2 was monitored by using a titration method.[4]

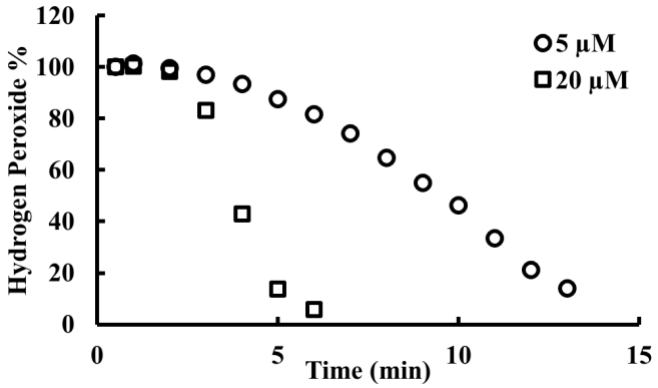

Figure S6. Time-dependent change in H_2O_2 concentration (expressed as a percentage of the initial concentration: 5 mM) upon incubations with 5 μM and 20 μM iCORM-3 in PBS (pH=7.4, 0.01 M) at 37 °C. The concentration of H_2O_2 was monitored by using a titration method.

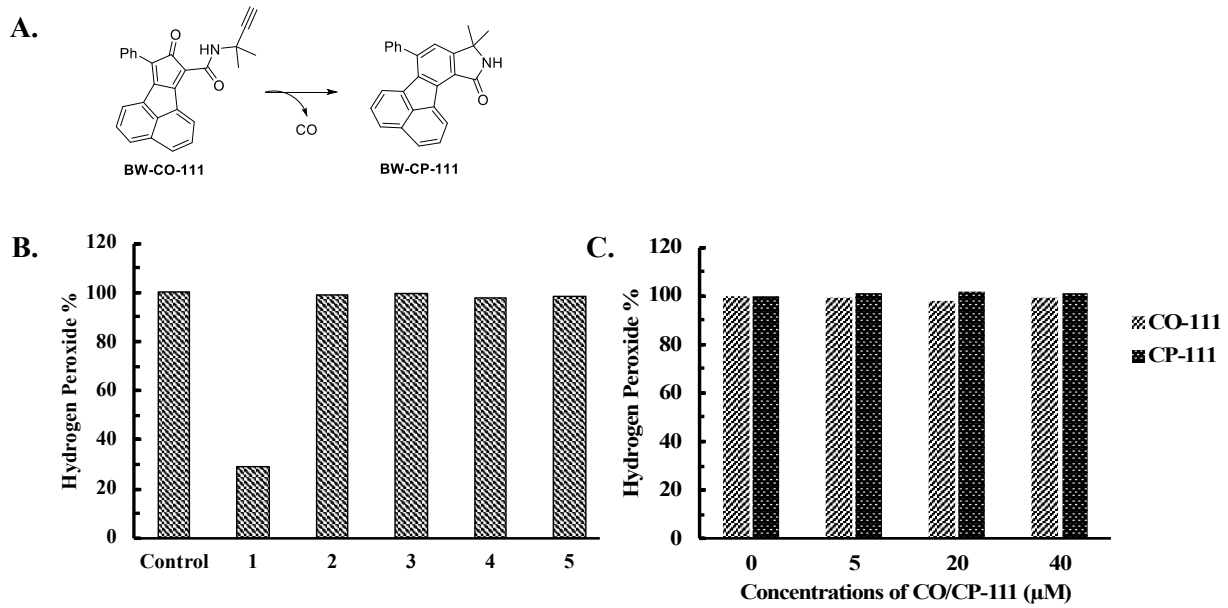

Figure S7. A) CO release from **CO-111**; B) Changes of H_2O_2 concentration (expressed as a percentage of the initial concentration: 5 mM) upon incubations with blank control; (1) 5 μM CORM-2; (2) 5 μM CO-111; (3) 20 μM CO-111; (4) 5 μM CP-111; and (5) 20 μM CP-111 for 15 min in PBS (pH=7.4, 0.01 M) at room temperature. C) Changes of H_2O_2 concentration (expressed in the percentage of the initial concentration: 5 mM) upon prolonged incubations with CO-111 and CP-111 for 1 h in PBS (pH=7.4, 0.01 M) at room temperature. The concentration of H_2O_2 was monitored by using a titration method.[4]

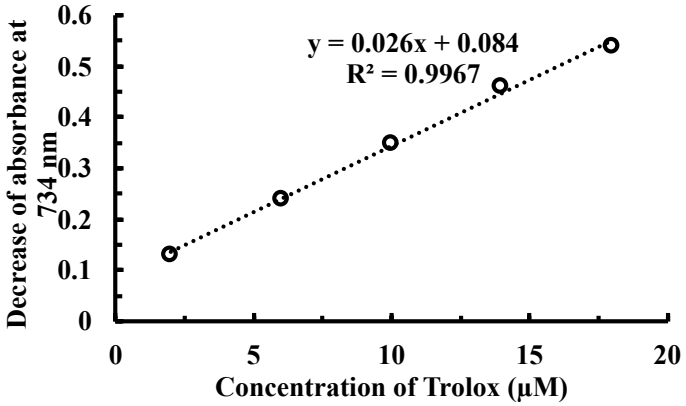

Figure S8. Decrease of UV absorbance (734 nm) of the ABTS radical upon treatment with various concentrations of Trolox.

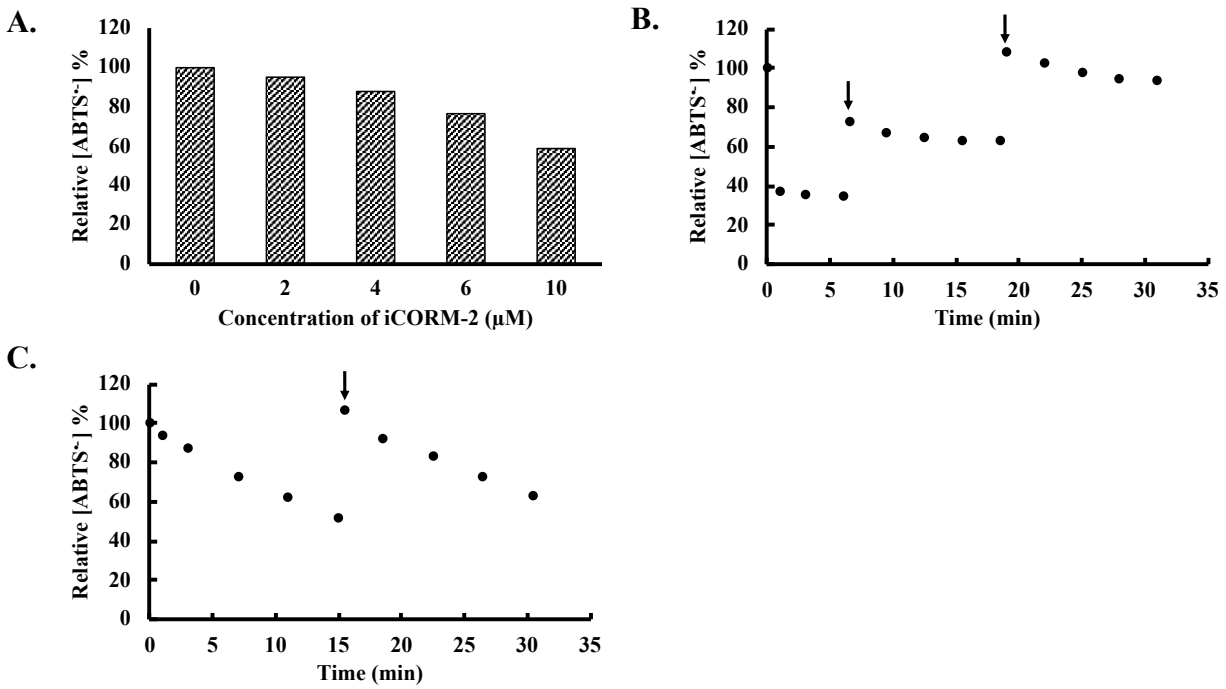

Figure S9. Decrease of ABTS radical concentration (expressed as a percentage of the initial concentration: 50 μM, determined using absorbance measured at 734 nm[5]) upon incubations with A) iCORM-2 for 6 min; B) Reinjection of ABTS radical during the Trolox (15 μM)-induced consumption of ABTS radical; C) Reinjection of ABTS radical during the iCORM-2 (5 μM)-induced consumption of ABTS radical in ethanol containing 20% acetate buffer (20 mM, pH 4.5, v:v).

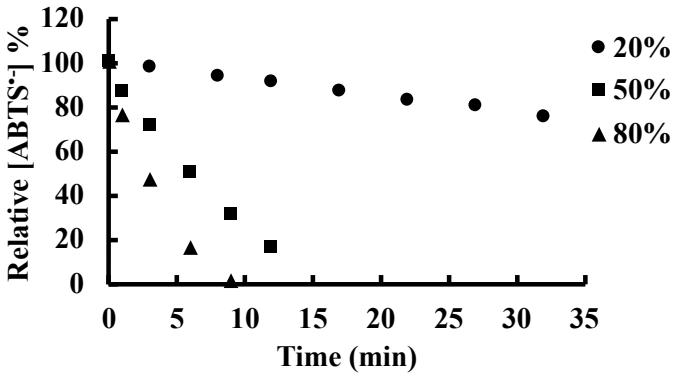

Figure S10. Time-dependent decrease of ABTS radical concentration (expressed as a percentage of the initial concentration: 50 μM, determined using absorbance measured at 734 nm[5]) upon incubations with CORM-2 (5 μM) in various ratios of ethanol solutions.

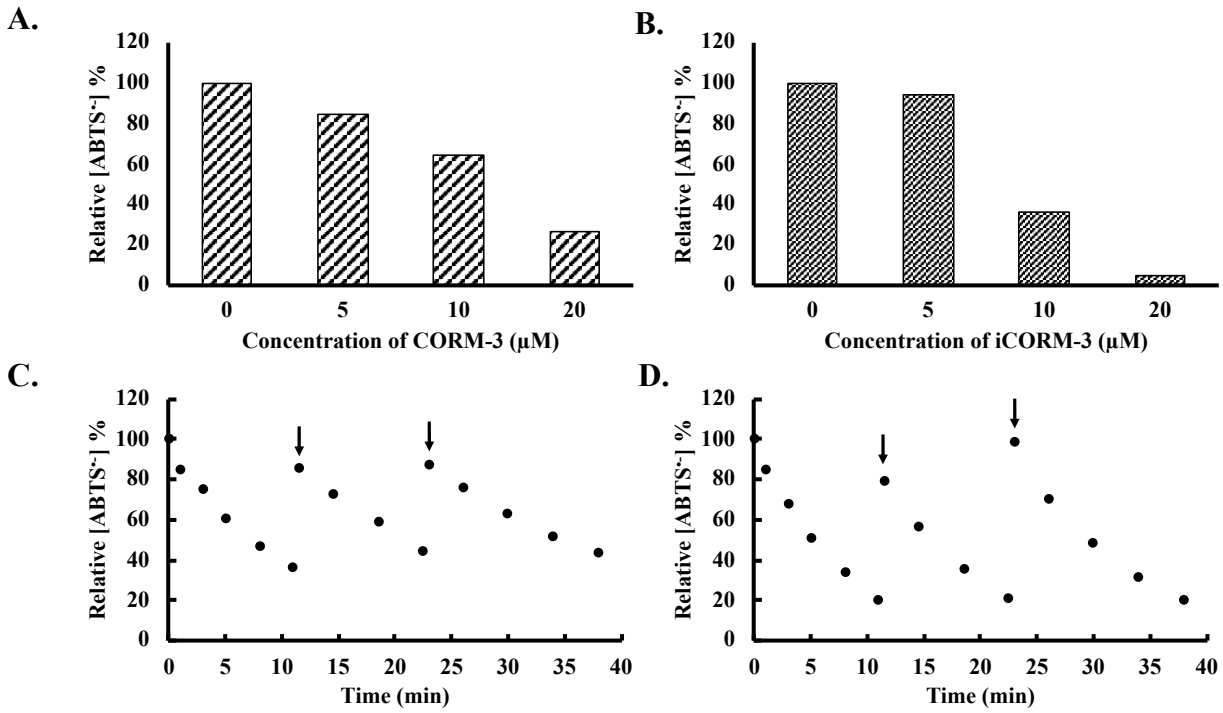

Figure S11. Decrease of ABTS radical concentration (expressed as a percentage of the initial concentration: 50 μM, determined using absorbance measured at 734 nm) upon incubations with A) CORM-3 and B) iCORM-3 for 6 min; C) Reinjection of ABTS radical during the CORM-3 (5 μM)-induced consumption of ABTS radical; D) Reinjection of ABTS radical during the iCORM-3 (5 μM)-induced consumption of ABTS radical in ethanol containing 20% acetate buffer (20 mM, pH 4.5, v:v).

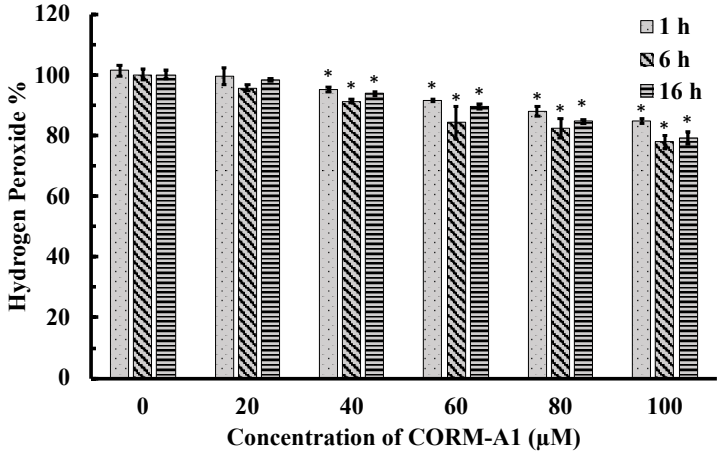

Figure S12. Time-dependent changes of H_2O_2 concentration (expressed as a percentage of the initial concentration: 300 μM) over time upon incubations with CORM-A1 in PBS (pH=7.4, 0.01 M) at 37 °C. The concentration of H_2O_2 was monitored by using the titration method. Values are

means ± SD. n = 3. Data with "*" indicates statistically significant differences between CORM-A1 samples and the negative control as determined by the t-test. *P<0.01 versus the control group.

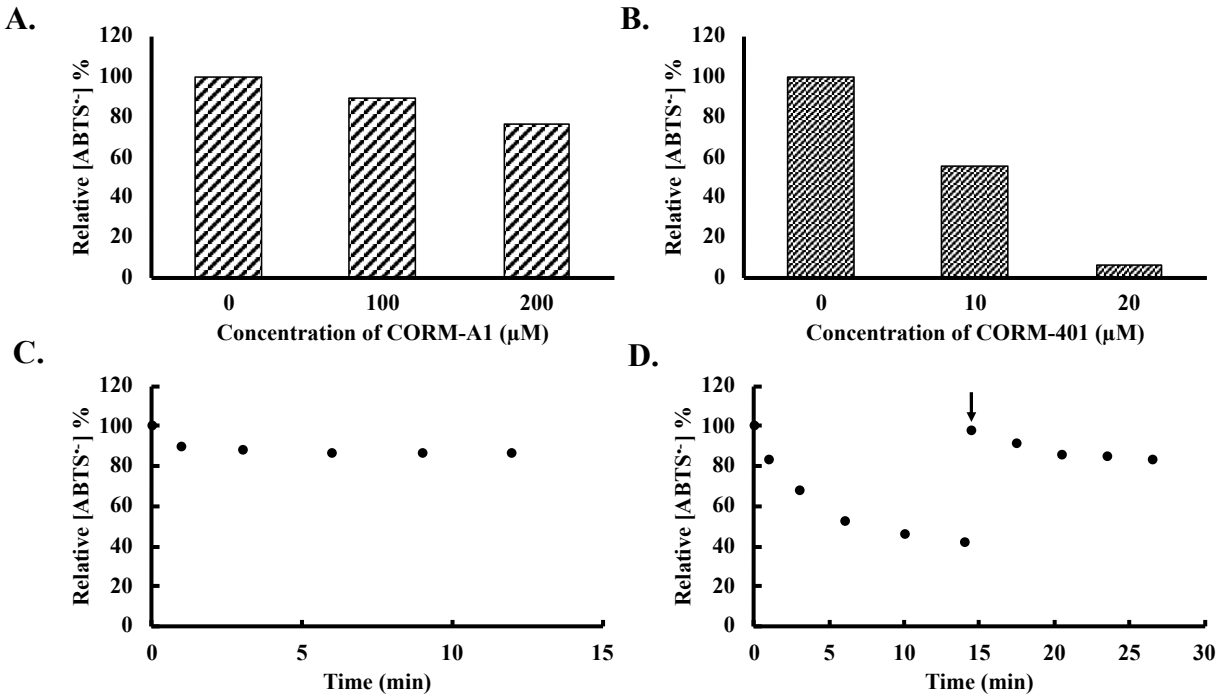

Figure S13. Decrease of ABTS radical concentration (expressed as a percentage of the initial concentration: 50 μM, determined using absorbance measured at 734 nm[5]) upon incubations with A) CORM-A1 and B) iCORM-401 for 6 min; C) Time-dependent decrease of ABTS radical upon incubation with CORM-A1 (100 μM); D) Reinjection of ABTS radical during the CORM-401 (10 μM)-induced consumption of ABTS radical in ethanol containing 20% acetate buffer (20 mM, pH 4.5, v:v).

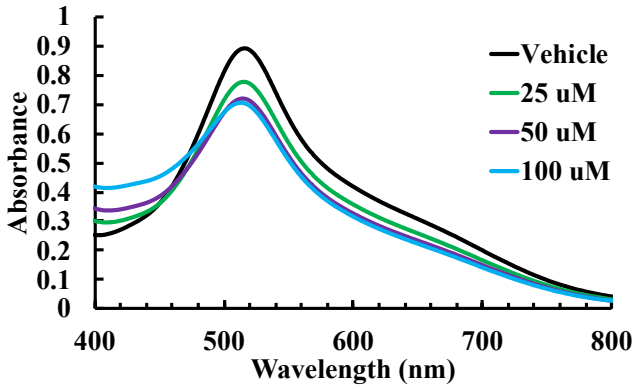

Figure S14. UV-vis spectra of DPPH upon treatment with various concentrations of CORM-A1 (25, 50 and 100 μM) in methanol for 1 h at room temperature.

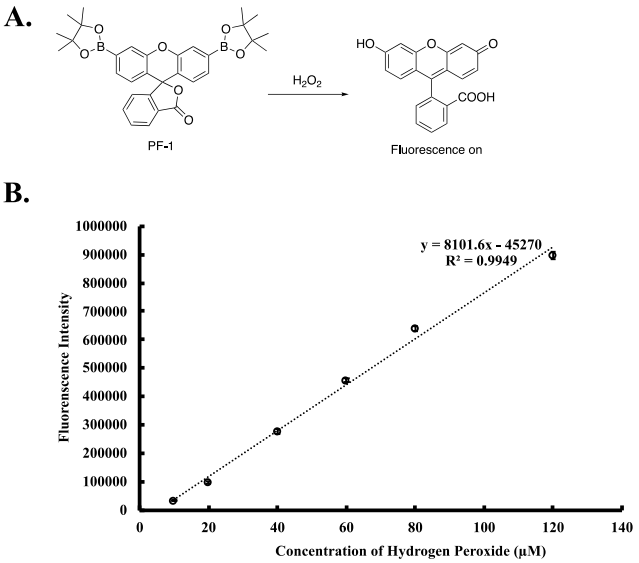

Figure S15. Fluorescence detection of H_2O_2 by PF-1. A) Detection mechanism of PF-1; B) Fluorescence response of 10 μM of PF-1 to various concentrations of H_2O_2 in DMEM (excitation wavelength: 490 nm; emission wavelength: 525 nm).

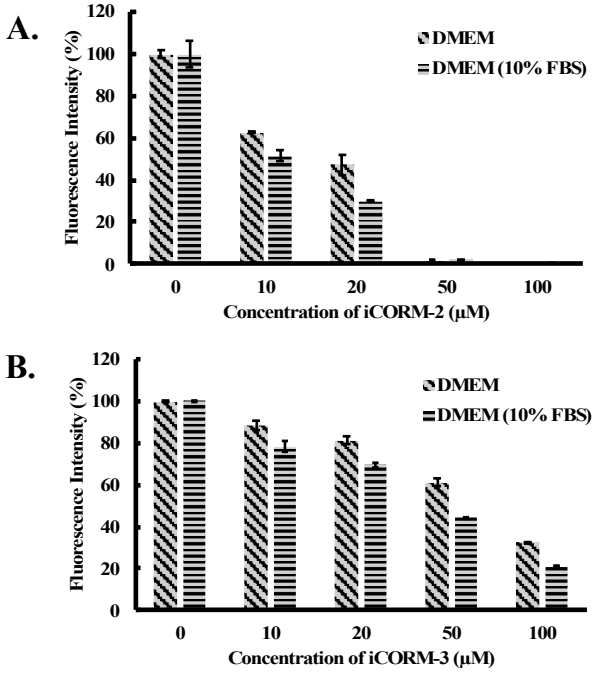

Figure S16. Fluorescence detection of H_2O_2 in cell culture medium by PF-1. 100 μM of H_2O_2 solutions were incubated with A) iCORM-2 and B) iCORM-3 in DMEM or DMEM (10% FBS) for 1 h at 37 °C. Values are means ± SD. n = 3.

Reference

1. E. Clark James, P. Naughton, S. Shurey, J. Green Colin, R. Johnson Tony, E. Mann Brian, R. Foresti and R. Motterlini, *Circ. Res.*, 2003, **93**, e2-e8.
2. D. Babu, G. Leclercq, R. Motterlini and R. Lefebvre, *Front. Pharmacol.*, 2017, **8**, 31.
3. M. C. Y. Chang, A. Pralle, E. Y. Isacoff and C. J. Chang, *J. Am. Chem. Soc.* , 2004, **126**, 15392-15393.
4. C. D. Fernando and P. Soysa, *MethodsX*, 2015, **2**, 283-291.
5. R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang and C. Rice-Evans, *Free Radic. Biol. Med.* , 1999, **26**, 1231-1237.
6. M. Ozgen, R. N. Reese, A. Z. Tulio, J. C. Scheerens and A. R. Miller, *J. Agric. Food Chem.* , 2006, **54**, 1151-1157.
7. S. Choua, P. Pacheco, C. Coquelet and E. Bienvenüe, *J. Inorg. Biochem.* , 1997, **65**, 79-85.