Supplemental Information

A Manganese-Porphyrin Complex Decomposes H_2O_2, Inhibits Apoptosis, and Acts as a Radiation Mitigator in Vivo

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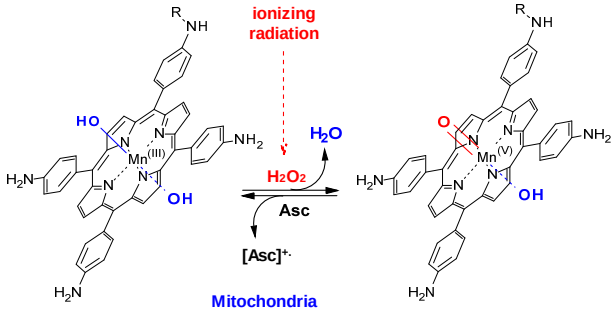
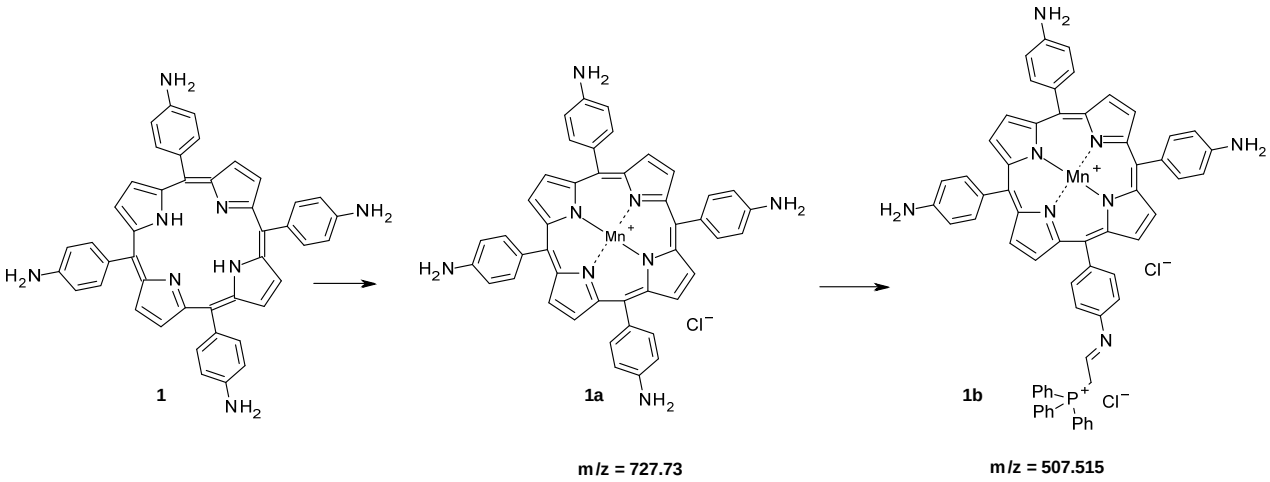


List of abbreviations: MEC, mouse embryonic cells; **1**, 4-[(5Z,10Z,14Z,19Z)-10,15,20-tris(4-aminophenyl)-21,23-dihydroporphyrin-5-yl]aniline; **1a**, 4-[(5Z,10Z,14Z,19Z)-10,15,20-tris(4-aminophenyl)-21,23-dihydroporphyrin-5-yl]aniline-$Mn^{(III)}$; **1b**, triphenyl-[(2E)-2-[4-[(1Z,4Z,9Z,15Z)-10,15,20-tris(4-aminophenyl)-21,23-dihydroporphyrin-5-yl]phenyl]iminoethyl] phosphornium-$Mn^{(III)}$; IR, ionizing radiation; IRP, radioprotectors; IRM, radiomitigators; CL, cardiolipin; Asc, ascorbic acid; GSH, glutathione; PS, phosphatidylserine; SOD, superoxide dismutase

Synthesis



*Preparation of **1a**:* $(CH_3CO_2)_2Mn$ (0.2 g) was refluxed for 6.5 hours in 2-propanol (200 mL) containing 5,10,15,20-tetrakis(4-aminophenyl)-21H,23H-porphine (**1**; 0.114 g). Thereafter, 50 mL of H_2O were added and the alcohol was rotor-evaporated (80 mm Hg). The remaining aqueous phase was saturated with NaCl, **1a** was extracted with ethyl acetate (3 x 50 mL), the combined extracts were dried over anhydrous Na_2SO_4, and the organic solvent was rotor-evaporated (80 mm Hg) to afford 125 mg of pure **1a** (yield, 92%).

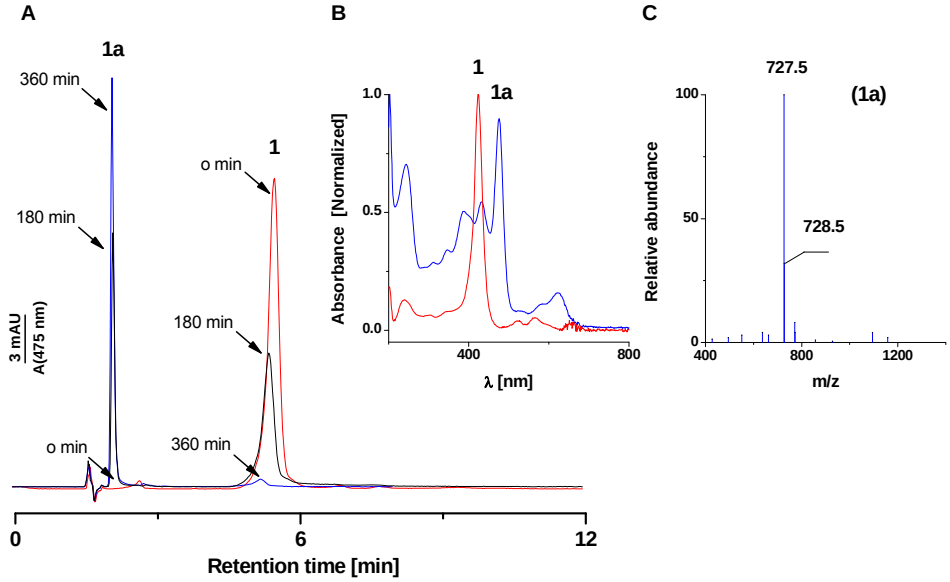


Figure 1SI. HPLC, UV and MS analysis of **1** (red lines, 0 min) and **1a (**blue lines, 360 min**)**. Isocratic chromatographic separations were carried out on a C18 column (Alltima 4.6 x 250 mm, 5u; Alltech Associates, Inc.; Deerfield, IL) with mobile phase consisting of 75 % methanol containing 0.02 M $LiClO_4$. Flow rate, 1 mL per min.

*Preparation of **1b**:* DMSO (0.3 mL) containing **1a** (11.4 mg) and 2-oxoethyl(triphenyl)phosphonium bromide ($[Ph_3P^+CH_2CHO]Br^-$; 5.2 mg) was heated at 70 ^oC for 1 hour. Then the reaction solution was subjected to a C18 semi-preparative HPLC separation (Figure 2SI; 5 mg of reaction product on the column). The fraction containing **1b** (Fig. 2SI*A*; 12 – 30 min; ***B*,** mass spectral analysis of the 12-30 min fraction) was collected and concentrated by rotor-evaporation. The remaining aqueous phase was saturated with NaCl and **1b**

extracted with ethyl acetate (3 x 100 mL). The unified extracts were dried over anhydrous Na_2SO_4 and then the organic solvent was rotor-evaporated to afford 2.7 mg of **1b**.

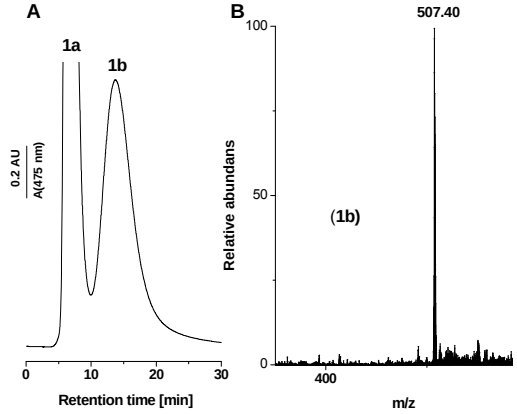


Figure 2SI. HPLC purification (*A*) and MS analysis (*B*) of **1b**. Column, octadecyl-functionalized silica gel (24 x 250 mm; 200-400 mesh; Sigma, MO); mobile phase, 75 % methanol containing 0.02 M $LiClO_4$; flow rate, 10 mL per min; injected sample, DMSO (1 mL) containing 5 mg of crude reaction product.

Hydrogen peroxide detection using mitochondria targeted HyPer: Cells were transfected for 48 hours with pHyPer-dMito using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected cells were incubated with porphyrin complexes for 1 hour and then treated with H_2O_2. In irradiation experiments, cells were exposed to γ-rays at a dose of 10 Gy and then incubated with porphyrin complexes until harvest. Cells were analyzed using flow cytometry or an Olympus Fluoview 1000 confocal microscope (Malvern, NY, USA).

Biomarkers of Apoptosis: Phosphatidylserine externalization was determined by annexin V–FITC apoptosis detection kit (Biovision, Mountain View, CA) according to the manufacturer's instruction. Caspase–3/7 activity was measured using a luminescence Caspase–Glo^{TM} 3/7 assay kit (Promega, Madison, WI).

Western blot analysis: Samples were separated on 15% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with antibodies against cyt c (clone 7H8.2C12, BD Pharmingen) or actin (Novus; loading control), coupled with horseradish peroxidase detection.

*Isolation of mitochondria***:** Mitochondrial fractions were isolated by differential centrifugation. Briefly, cells were suspended in mitochondria isolation buffer (pH 7.4; MIB) containing mannitol (210 mM), sucrose (70 mM), HEPES (10 mM), EDTA (1 mM) and a protease inhibitor cocktail. Cells were lysed by Dounce homogenization. Unbroken cells, nuclei and debris were removed by a 5 min-centrifugation at 600×*g* (4 ^{o}C). Mitochondria, obtained by centrifugation at 10,000×*g* (10 min), were washed once with MIB.

*Assessment of **1b** in MEC and subcellular fractions:* Mouse embryonic cells (MEC; 2×10^6) were seeded in 100 mm cell culture dishes and let to attach overnight. Thereafter, the cells were incubated with **1b** (10 μM) in PBS at 37 ^{o}C for 30 min, washed with PBS (2 x 5 mL), and collected by trypsinization. Mitochondria were isolated as described above. In mitochondria, **1b** was analyzed by HPLC after precipitation of proteins with CH_3CN (final concentration, 70 %; incubation time, 30 min (4 ^{o}C); centrifugation, 5 min at 10,000g). **1b** from cytosolic fractions was extracted by following Folch's protocol ($CHCl_3$:CH_3OH:H_2O_2 = 2:1:1). After evaporation of $CHCl_3$, the dry residues was re-dissolved in minimal volume of methanol and **1b** separated by isocratic HPLC as indicated in Fig. 1SI.

*Superoxide anion radical reduces cytochrome c but not **1b**:* Comparative experiments for reduction of cytochrome c and **1b** by superoxide anion radical were carried out with crown ether/KO_2 ([crown-$K^+O_2^{\cdot-}$] + H_2O → KOH + $O_2^{\cdot-}$; Valentine et al. 1984 *Methods Enzymol* ***105***, 71; Fig. 3SI]. [crown-$K^+O_2^{\cdot-}$] was prepared via stirring KO_2 (7.1 mg) and 18-crown-6 (80 mg) for 60 min at 25 ^{o}C in dry DMSO (1 mL) under nitrogen. The resulting solution was stored at -20 ^{o}C and used within 24 hours.

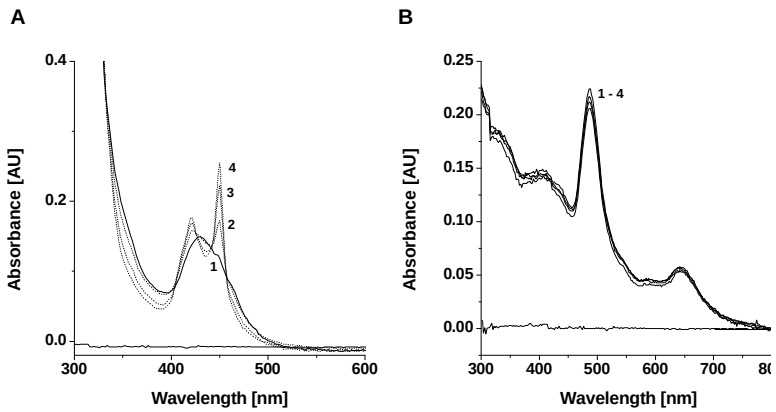


Figure 3SI. Absorption spectra of cytochrome c (50 μM; *A*) and **1b** (2 μM; *B*) in the absence (trace 1) and the presence of 50, 100 and 150 μM [crown-$K^+O_2^{\cdot-}$] (traces 2-4). Reactions were carried out at 25 ^oC in 0.1 M phosphate buffer (pH 7.4). Reduction of cytochrome c was monitored by the increases in absorbance at 450 nm.

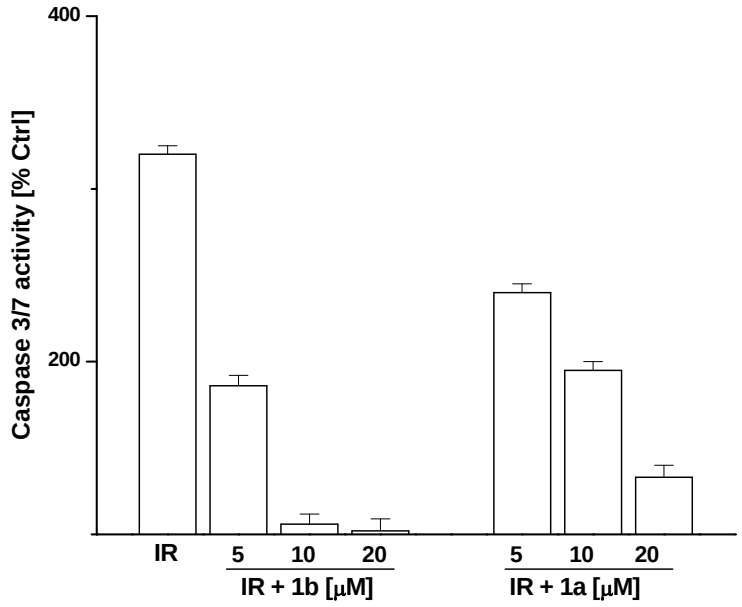


Figure 4SI. *Comparative analysis of the effects of **1a** and **1b** on caspase 3 activity in MEC exposed to γ-rays.* Cells (5×10^4) were seeded on 35 mm dishes and allowed to attach overnight. Cells were exposed to γ-rays (dose, 10 Gy) and, 15 min thereafter, treated with **1a** (or **1b**) for 30 min. Then, cells were washed, further incubated for 24 hours, and harvested for analysis of caspase 3 activity.

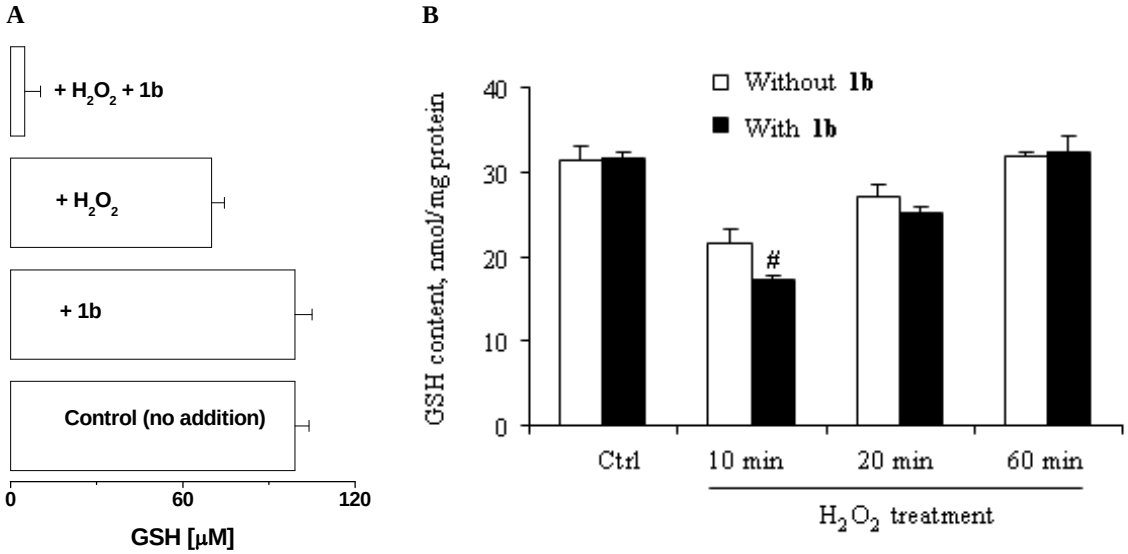


Figure 5SI. *Effects of H_2O_2 and **1b** on the oxidation of GSH in vitro: A*- Reactions were carried out for 5 min at 25 ^oC in 0.1 M phosphate buffer (pH 7.0) and then stopped by addition of catalase (400 units/mL). GSH was assessed with Elman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) by following changes in the absorbance of the reaction solution at 412 nm. GSH, **1b** and H_2O_2 were used at concentrations of 0.1 mM, 0.06 and 0.2 mM, respectively. The results represent the mean ± S.E (n = 3). *B*- MEC were incubated for up to 60 min with 0.1 mM H_2O_2 and levels of GSH were determined using a ThioGlo-1 kit (EMD, Gibbstown, NJ) following the manufacturer's protocol. The results represent the mean ± S.E (n = 3).

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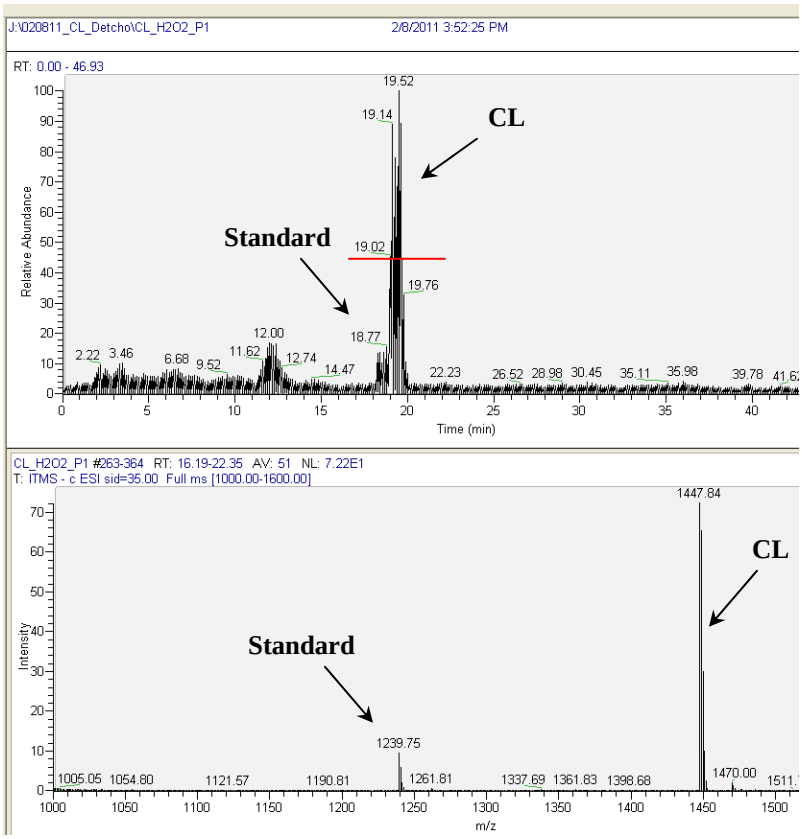

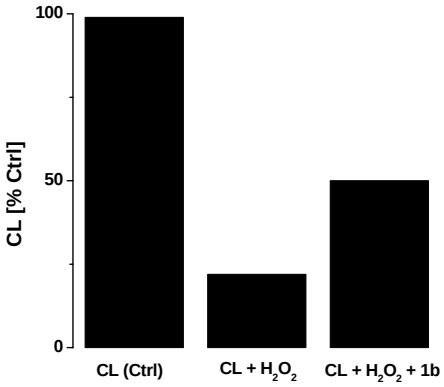

Figure 6SI. *Effects of* ***1b*** *on the oxidation of CL by cytochrome c and* H_2O_2. Reactions were carried out with CL-containing phosphatidylcholine (PC) liposomes for 30 min at 25 ^{o}C in 0.1 M Hepes (pH 7.4; 0.1 mM EDTA). Liposomes were obtained by sonication of phospholipids with ultra sound (10 x 5 sec; 30 seconds relaxation; ice bath), whereby PC, CL, cytochrome c and H_2O_2 were used at concentrations of 100, 25, 2 and 50 μM, respectively. After completion of the incubation, CL was extracted from the reaction suspension following Folch's protocol ($CHCl_3$: MeOH : H_2O = 2 : 1 : 1), the solvent was evaporated under nitrogen, the dry reside redissolved in methanol and subjected to HPLC-MS analysis. The results are representative of two independent experiments and did not differ more than 7%.