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LETTER

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

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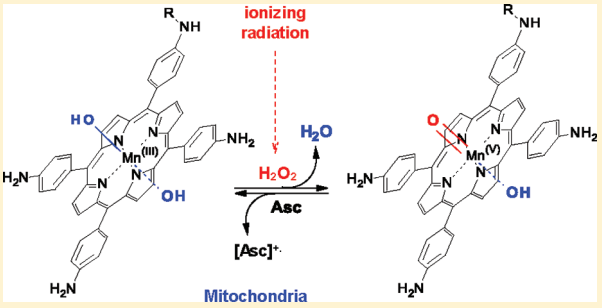
Supporting Information

ABSTRACT: Ionizing radiation triggers mitochondrial overproduction of H_2O_2 with concomitant induction of intrinsic apoptosis, whereby clearance of H_2O_2 upon overexpression of mitochondrial catalase increases radioresistance in vitro and in vivo. As an alternative to gene therapy, we tested the potential of $Mn^{(III)}$−porphyrin complexes to clear mitochondrial H_2O_2. We report that triphenyl-[(2*E*)-2-[4-[(1*Z*,4*Z*,9*Z*,15*Z*)-10,15,20-tris(4-aminophenyl)-21,23-dihydroporphyrin-5-yl]phenyl]iminoethyl]phosphonium-$Mn^{(III)}$ compartmentalizes preferentially into mitochondria of mouse embryonic cells, reacts with H_2O_2, impedes γ-ray-induced mitochondrial apoptosis, and increases the survival of mice exposed to whole body irradiation with γ-rays.



KEYWORDS: Manganese−porphyrin complex, H_2O_2, apoptosis, radiation mitigator

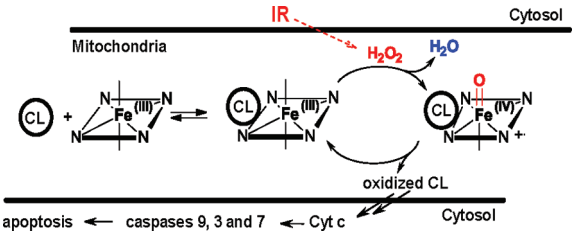
By radiolyzing water and damaging biomolecules via generation of O-, C-, S-, and N-centered radicals, ionizing radiation (IR) triggers cell death via three general mechanisms: mitochondria-mediated apoptosis, mitosis-linked death or loss of clonogenicity, and necrosis. The completion of these cell death pathways includes a cascade of reactions whose development may take from hours to days,[1] thus affording time for radiomitigation. In contrast, the initial radiolytic events happen instantaneously; therefore, protection of cells can be attained by radioprotectors (IRP) present at the time of radiation exposure. In search for IRP against IR, over 4000 scavengers of free radicals have been synthesized and tested in mice.[2] Currently, however, only 2-butylaminoethylsulfanylphosphonic acid (amifostine; WR-2721) has found clinical applications as an IRP.[3]

Apoptosis causes massive cell loss in radiosensitive tissues.[4] Recent studies suggest that inhibition of apoptosis and induction of regeneration-promoting cytokines are viable strategies in the discovery of mechanism-based radiomitigators (IRM).[4,5]

It has been shown that mitochondria exposed to IR overproduce H_2O_2.[6] Mitochondrial production of H_2O_2 can trigger inner-sphere oxidation of cardiolipin (CL) in its complex with cytochrome c ([CL-cyt c]; Scheme 1), which is an early apoptotic event.[7] Notably, clearance of H_2O_2 upon overexpression of mitochondrial catalase increases radioresistance in vitro and in vivo.[8]

As an alternative to gene therapy, we tested the potential of the porphyrin complexes **1a** and **1b** to act as IRM via clearance of mitochondrial H_2O_2 in cells exposed to IR (Scheme 2). The rationale for selection of **1a**,**b** was based both on the ability of amphiphilic organic cations to preferentially compartmentalize into mitochondria[9] and on the potential of $Mn^{(III)}$−porphyrin complexes to react with H_2O_2.[10,11]

Scheme 1



We speculated that reduction of the porphyrin−$Mn^{(V)}$=O complex **2** by ascorbic acid (Asc) and/or glutathione (GSH) would close a redox cycle that clears mitochondrial H_2O_2.

Figure 1A depicts the reaction of **1a** with H_2O_2 at pH 8.0, a value characteristic of intramitochondrial environment.[12] Consecutive additions of H_2O_2 to solution of **1a** resulted in a linear response of the H_2O_2-sensitive electrode, whose background readings were restored via decomposition of H_2O_2 with catalase (trace 1). The rates of the reactions of **1a** and **1b** with H_2O_2 markedly decreased at pH 7.0, which suggested the involvement of HOO^-, rather than H_2O_2 (Figure 1B,C); unexpectedly, at pH 7.0−7.4, H_2O_2 did not react with **1a** to any significant extent

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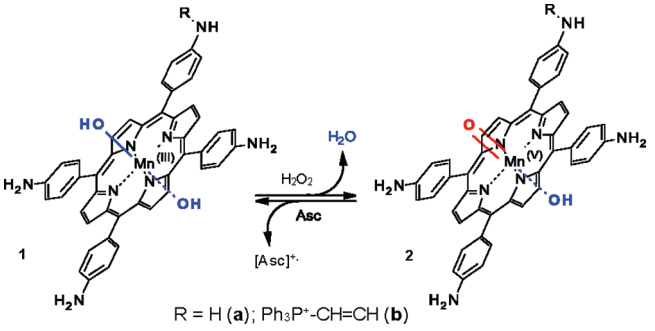
Scheme 2



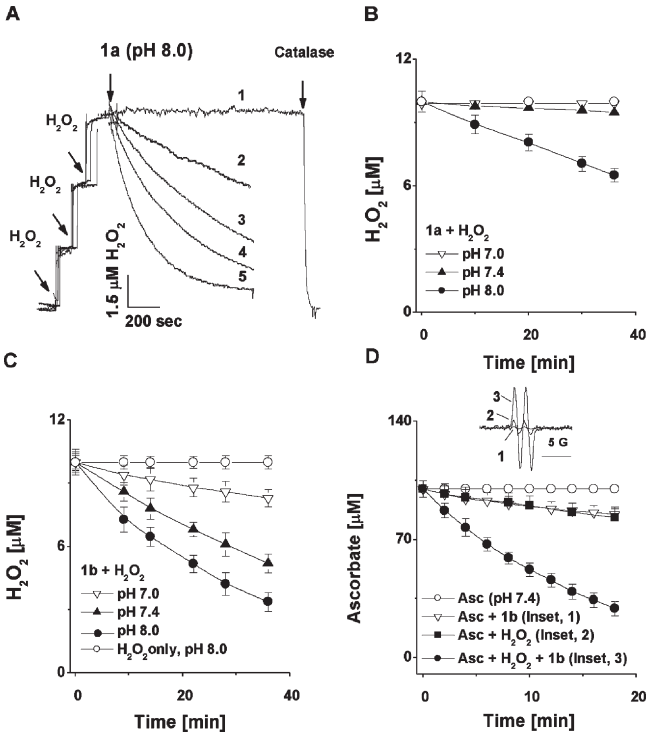


Figure 1. Complexes **1a** and **1b** react with H_2O_2. Experiments were carried out at 25 °C in 0.1 M phosphate buffer. Catalase, 400 units/mL (A); H_2O_2, 3.3 (A) and 200 μM (D); **1a**: 10, 20, 40, and 60 (A, traces 2−5) and 10 μM (B); **1b**: 10 (C) and 30 μM (D). The results in B−D represent the mean ± SE (n = 3).

(Figure 1B). Under these conditions, neither **1a** nor **1b** reacted with superoxide anion radical ($O_2^{-\bullet}$); Supporting Information, Figure 3SI). Both **1a** and **1b** (5−20 μM) were relatively stable in the presence of equimolar concentrations of H_2O_2. At concentrations exceeding 0.2 mM, H_2O_2 "bleached" the chromophores ($t_{1/2} \sim 60$ min), most likely due to oxidative release of $Mn^{(III)}$. In control experiments, we found that H_2O_2 (up to 0.2 mM) did not react with the porphyrin lacking $Mn^{(III)}$. Hence, the tentative mechanism presented in Scheme 1 presumes the addition of HOO^- to $Mn^{(III)}$ with concomitant heterolytic fission of the −O−O− bond ($HO-Mn^{(III)}-OOH \rightarrow HO-Mn^{(V)}=O + HO^-$). Similar reaction mechanisms have been proposed for the breakdown of peroxynitrite and peroxyacides by $Mn^{(III)}$-TMPyP.[13,14] In a reaction system consisting of **1a,b** (20 μM), H_2O_2 (100 μM), and phenol (10 mM), we did not observe formation of benzene-1,2-diol and benzene-1,4-diol, which attests for the lack of homolytic fission of $HO-Mn^{(III)}-OOH$ to $HO^\bullet$ and $Mn^{(III)}-O^\bullet \leftrightarrow Mn^{(IV)}=O$ ($C_6H_5OH + HO^\bullet + O_2 \rightarrow HO_2^\bullet + C_6H_4(OH)_2$;[15] data not shown). Alternatively, release of H_2O from $HO-Mn^{(III)}-OOH$ following a "push−pull" mechanism would yield the short-leaved dioxo species $O=Mn^{(V)}=O$. This pathway has been reported by Jin et al., who have shown that the rates of formation of $O=Mn^{(V)}=O$ from $Mn^{(III)}$TMPyP in the presence of HSO_5^-, H_2O_2, and *t*-BuOOH follow bell-shaped curves with maxima at pH 10.[16]

The reaction of **1b** with H_2O_2 yielded **2** (or $O=Mn^{(V)}=O$), as suggested by the enhanced one-electron oxidation of Asc (Figure 1D) to semidehydroascorbyl radical (Scheme 2, $[Asc]^{+\bullet}$; Figure 1D, inset) and by the increased oxidation of GSH by H_2O_2 in the presence of **1b** in both buffered aqueous solutions and in mouse embryonic cells (MEC; Supporting Information, Figures 5SI and 6SI). "Bleaching" of **1a,b** by H_2O_2 was fully inhibited by ascorbate (1 mM) and GSH (1 mM; data not shown), suggesting that the intracellular oxidation of **1b** to secondary porphyrin radicals will be hindered. In model experiments with CL-containing liposomes, **1b** also impeded oxidative modification of CL by Cyt c with H_2O_2 as a source of oxidizing equivalents (Supporting Information, Figure 7SI).

Next, we assessed the compartmentalization and IRM properties of **1b** in MEC. Upon incubation with MEC for 30 min, **1b** accumulated exclusively into mitochondria. Assuming that the volume of a single MEC is ~2.75 μm^3 and its mitochondrial compartment occupies ~10% of the volume of the cytoplasm,[17] we estimated the mitochondrial and cytosolic concentrations of **1b** to be ~3.5 and 0.005 mM, respectively (Figure 2A). These data indicate a considerable accumulation of **1b** at the sites of H_2O_2 production and execution of mitochondrial stages of apoptosis in cells exposed to IR.

Treatment of MEC with **1b** led to decreased levels of mitochondrial H_2O_2 (Figure 2B−D). Experiments with MEC were performed after mitochondria-targeted expression of HyPer, a fluorescent protein sensor for H_2O_2.[18] HyPer has been engineered via insertion of yellow fluorescent protein (cpYFP) into the regulatory domain of the *E. coli* protein OxyR.[18,19] Notably, changes in the fluorescence spectrum of HyPer are observed at submicromolar concentrations of H_2O_2 without any significant interference from other cellular oxidants. Flow cytometric experiments indicate that **1b** decreased the fluorescence of HyPer in control MEC and cells exposed to either H_2O_2 or IR (Figure 2B,C, respectively), suggesting that it competed with Hyper for mitochondrial H_2O_2. The effects of irradiation, H_2O_2, and **1b** on the fluorescence of HyPer were visualized by confocal microscopy (Figure 2D). Altogether, these data supported the hypothesis that **1b** can act as a radiation mitigator via clearance of mitochondrial H_2O_2. To further verify this hypothesis, we explored whether **1b** protects MEC against IR-induced apoptosis. MEC were irradiated at a dose of 10 Gy; 15 min thereafter, the cells were incubated with **1b**. Several end points of apoptotic cell death were investigated 48 h post-IR. Compound **1b** inhibited in a dose-dependent manner the activities of caspases 3/7 (Figure 2E), the externalization of phosphatidylserine (Anexin V positive cells; Figure 2F), and the release of cyt c from mitochondria (Figure 2F, inset). Similar protection was found with **1a**, although at higher concentrations (Supporting Information, Figure 4SI).

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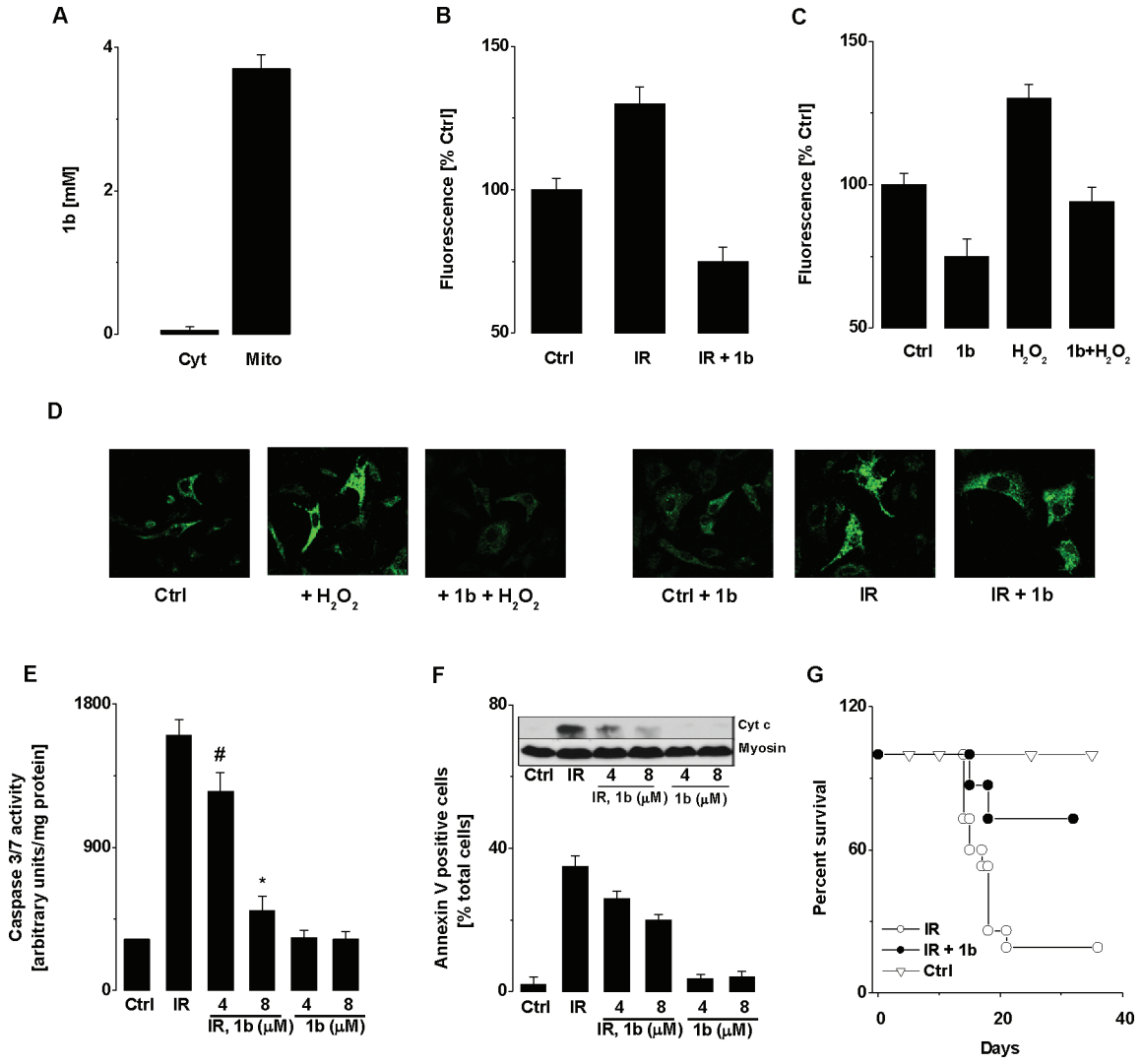

Figure 2. Cellular compartmentalization and radiomitigative properties of **1b**. (A) Compound **1b** preferentially compartmentalizes into mitochondria. (B and D) Effects of γ-rays (10 Gy), H_2O_2 (100 μM), and **1b** (10 μM) on the fluorescence of HyPer. (E and F) Effects of **1b** on caspases 3/7 activities and PS externalization in control and irradiated MEC; (inset E) Western bolt analysis of cytosolic Cyt c in control and irradiated MEC. (F) Radiomitigative effects of **1b** on mice exposed to γ-rays (9.25 G). The results in G are from two independent experiments and in A−C and E and F represent the mean ± SE (n = 3).

We further assessed whether the in vitro radiomitigative effects of **1b** was translated into an in vivo effect in the mouse. Groups of 20 mice received **1b** (5 mg/kg body weight) 15 min after whole body irradiation (9.25 Gy). As shown in Figure 2G, ~ 80% of irradiated control mice were dead by day 21 (open circles), while nonirradiated mice receiving only ethanol as a vehicle (open triangles) survived the treatment. Irradiated mice post-treated with **1b** showed a significant increase in survival, with over 75% of mice alive at day 35 (closed circles).

In conclusion, the data presented herein indicate that **1b** acts as a potent radiation mitigator. It has been reported that the polycationic porphyrin complex $Mn^{(III)}$−$TMPyP^{5+}$, when pre-administered for 14 days, significantly increased the survival of mice exposed to whole-body irradiation.[20] The pharmacological action of $MnTMPyP^{5+}$ has been associated with its superoxide dismutase (SOD)-like activity. Cytotoxic, IR-induced mitochondrial production of ($O_2^{-\bullet}$) precedes that of H_2O_2 ($2O_2^- + 2H^+ \rightarrow H_2O_2$),[21] and it has been associated with inactivation of a number of mitochondrial enzymes.[22] While the reduction potentials of monocationic $Mn^{(III)}$−porphyrin complexes preclude reactions with ($O_2^{-\bullet}$), targeted synthesis of SOD mimetics has been achieved via conjugation of the porphyrin ring with charged groups (reviewed in ref 23). This structural modification leads to decreased electron density near $Mn^{(III)}$, thus increasing its ability to undergo one-electron reduction. However, the charge of these complexes reduces their ability to cross cell membranes (and thereby their bioavailability) and increases their toxicity due to intercalation into nucleic acids.[23] Our data complement previous studies on the medicinal chemistry of $Mn^{(III)}$−porphyrin complexes within the context of clearance of mitochondrial H_2O_2[11] rather than $O_2^{-\bullet}$. The radiomitigative properties of porphyrin−$Mn^{(III)}$ complexes may also reflect detoxification of peroxynitrite,[24] a possibility that has not been explored thus far. It is noteworthy that **1b** is a lipophilic compound whose bioavailability is not restricted by its molecular charge; we found millimolar mitochondrial concentrations of **1b** vs micromolar concentrations of $MnTMPyP^{5+}$, reported

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in ref 25. Furthermore, **1b** acted as a radiation mitigator when administered to mice after exposure to IR. This is an important pharmacological advantage, as IR damage to organisms is often the result of accidents, whereby post-IR treatment with free radical scavengers is not a viable option. It is possible that derivatives of **1b** containing functions with SOD-like activity may further enhance its pharmacological effectiveness and radiomitigative potential.

■ ASSOCIATED CONTENT

Supporting Information. Additional details for biological assays and the synthesis and characterization of **1a,b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

GSH, glutathione; Asc, ascorbate; CL, cardiolipin; MEC, mouse embryonic cells; IR, ionizing radiation; IRP, radioprotectors; IRM, radiomitigators

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