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Design and Synthesis of a Mitochondria-Targeted Mimic of Glutathione Peroxidase, MitoEbselen-2, as a Radiation Mitigator

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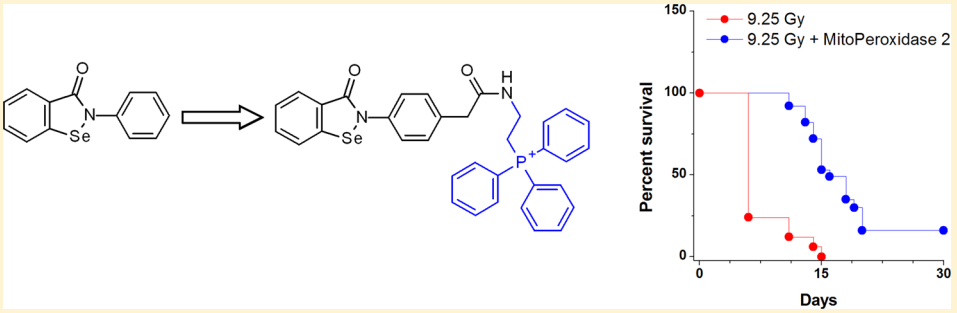
Supporting Information



ABSTRACT: Ionizing radiation (IR) triggers mitochondrial overproduction of H_2O_2 and accumulation of lipid hydroperoxides leading to the induction of apoptotic and necroptotic cell death pathways. Given the high catalytic efficiency of the seleno-enzyme glutathione peroxidase (Gpx) toward reduction of lipid hydroperoxides and H_2O_2, we tested the potential of mitochondria-targeted derivatives of ebselen to mitigate the deleterious effects of IR. We report that 2-[[2-[4-(3-oxo-1,2-benzoselenazol-2-yl)phenyl]acetyl]amino]ethyl-triphenyl-phosphonium chloride (MitoPeroxidase 2) was effective in reducing lipid hydroperoxides, preventing apoptotic cell death, and, when administered 24 h postirradiation, increased the survival of mice exposed to whole body γ-irradiation.

KEYWORDS: *Ebselen, mitochondria, H_2O_2, radiation, mitigators, apoptosis*

Exposure of eukaryotic cells to ionizing radiation (IR) results in a burst of species with high energy (e_{aq}^-; $H^\bullet$, $HO^\bullet$, and $O_2^{-\bullet}$) and indiscriminate reactivity with biomolecules, including DNA, on the millisecond time scale.[1] Studies by Patt, Bacq, and others have established that aminothiols such as cysteine and cysteamine protect mice from short-lived radiolytic intermediates.[2−4] Humans, however, do not tolerate the doses of cysteine and cysteamine, which would be required for analogous protection, and the early hope that these compounds might be useful as radiation protectors (RPs) has not been realized. These initial observations have been followed by a research program under the auspice of the Walter Reed Army Research Institute, which has led to the synthesis of 4400 aminothiols and their assessment as RPs.[5] Structural variables in the synthesis of RPs were the length and branching of the carbon chain that linked NH_2, SH, and OH functional groups. From this chemical library, only amifostine ($H_2O_3P-S-(CH_2)_2-NH-(CH_2)_3-NH2$) has found clinical applications.[6]

The immediate burst of radicals upon exposure to IR is followed by a dose-dependent and continuous mitochondrial overproduction of reactive oxygen species.[7,8] These primary lethal effects of IR, realized predominantly in rapidly proliferating hematopoietic cells and gut epithelial cells, initiate secondary effects whereby activated inflammatory cells massively generate reactive oxygen species (ROSs) thus producing "friendly fire" and inducing new waves of oxidation reactions culminating in apoptotic and nonapototic cell death.[9] Among these secondary oxidants, H_2O_2 and lipid hydroperoxides (LOOH) are most prominent in setting-up the "peroxide tone" and perpetuating enzymatic and nonenzymatic oxidations of critical biomolecules with signaling functions. In particular, peroxidase functions of mitochondrial intermembrane space hemoprotein, cytochrome c (cyt c), toward a mitochondria-specific phospholipid, cardiolipin (CL), has been associated with the accumulation of CL hydroperoxides required for the execution of mitochondrial apoptosis.[10] In addition, a group of cytosolic nonheme iron proteins, lipoxygenases (LOX), catalyze oxygenation reactions yielding hydroperoxides of free polyunsaturated fatty acids thus inducing necroptotic and ferroptotic cell death signals.[11]

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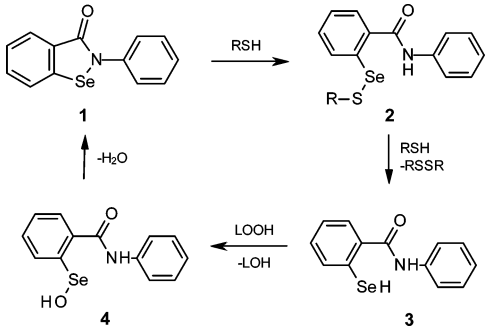
Hence, regulation of the levels of ROSs is important for the control of the major radiation-induced cell death pathways.[12]

The central regulator of LOOH is a seleno-enzyme, glutathione peroxidase 4 (GPx4), whose deficiency leads to cell death.[12] GPx has been shown to impede mitochondrial apoptosis via clearance of hydroperoxides of CL,[13,14] while overexpression of mitochondrial catalase has been found to increase radioresistance in vitro and in vivo.[15]

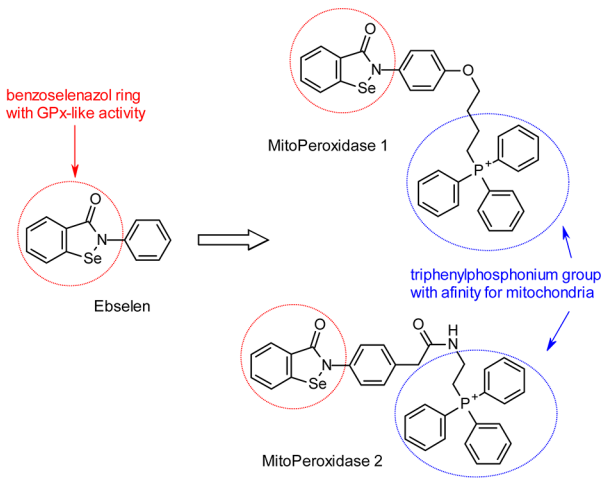
Recently, Tak and Park have reported that 2-phenyl-1,2-benzoselenazol-3-one (ebselen; Scheme 1, **1**), when administered for 14 days prior to radiation, provides substantial protection against killing and oxidative damage to mice exposed to whole-body irradiation (WBI).[16] Ebselen is a multifunctional drug that accumulates in the endoplasmic reticulum (ER),[17] reacts with cellular thiols, and mimics the activity of glutathione peroxidase (GPx) by clearing H_2O_2 and LOOH (Scheme 1).[18] Following recent clinical trials for the prevention and treatment of cardiovascular diseases, arthritis, stroke, and atherosclerosis,[18] ebselen has been included in the National Institutes of Health Clinical Collection,[19] a chemical library of bioavailable drugs considered clinically safe.

We have assessed the potential of the derivatives of ebselen 4-[4-(3-oxo-1,2-benzoselenazol-2-yl)phenoxy]butyl-triphenyl-phospho-nium iodide (MitoPeroxidase 1; synthesized as reported in ref 20; Scheme 2) and 2-[[2-[4-(3-oxo-1,2-benzoselenazol-2-yl)phenyl]acetyl]amino]ethyl-triphenyl-phosphonium chloride (MitoPeroxidase 2; Scheme 3, 10) to act as radiation mitigators (RMs). Because under conditions of oxidative stress sufficient concentrations of antioxidants at the sites of generation of reactive metabolites are critical to protection from oxidative damage, we have targeted the synthesis of ebselen derivatives that selectively compartmentalize to mitochondria (Scheme 2).

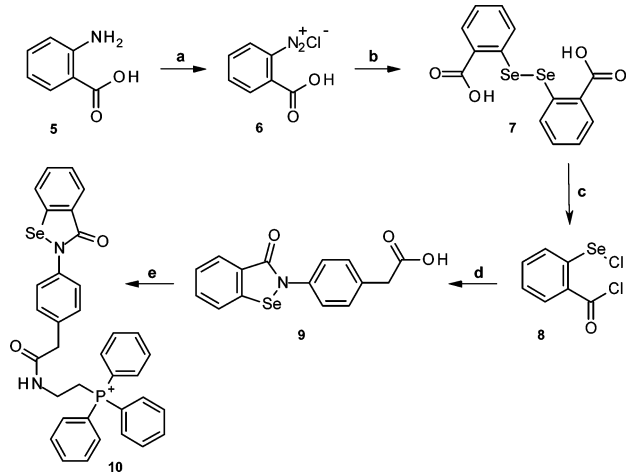
Scheme 1. Ebselen Exhibits GPx-Like Activity



Scheme 2. Design of Mitochondria-Specific Ebselen Derivatives



Scheme 3. Synthesis of MitoPeroxidase 2[a]



[a]Reagents and conditions: (a) aq. $NaNO_2$, HCl (18%), 0 °C; (b) Na_2Se_2, aq. NaOH, 40 °C (2 h), 55−63%; (c) $SOCl_2$, DMF, reflux (1 h), >95%; (d) 2-(4-aminophenyl)acetic acid, Et_3N, CH_3CN, 0 °C (1 h) to 25 °C (3 h), 72−76%; (e) $(Ph)_3P^+CH_2CH_2NH_2$, DCC, 0 °C (1 h) to 25 °C (4 h), 80−85%.

To date, several methods for delivery of drugs into mitochondria have been developed, including their derivatization with certain peptides or with a triphenylphosphonium group ($(Ph)_3P^+$).[21] Since mitochondria maintain a negative potential, the positive charge of the $(Ph)_3P^+$ group drives attached molecules inside the matrix and toward a diffusion equilibrium, thus affording up to a thousand-fold accumulation of the drug in mitochondria vs cytosol. The accumulation of organic cations in mitochondria may also be mediated by proteins such as the 2-oxoglutarate carrier.[22] Previous studies have shown that MitoPeroxidase 1 is taken up by isolated mitochondria, catalyzes the breakdown of H_2O_2 in the presence of thiols, and inhibits apoptosis induced by oxidants.[20] In agreement with the reactions presented in Scheme 1, incubation (5 min; t = 25 °C) of MitoPeroxidase 2 (15 μM; Figure 1A) and 6,8-bis(sulfanyl)octanoic acid (dihydrolipoic acid; DHLA; 15 μM) in He-deaerated methanol led to the formation of a selanyl-benzamide (Figure 1B), which readily reduced 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (10 μM; ROOH; Figure 1C) to the corresponding alcohol (ROH; $t_{incubation}$ = 5 min; 25 °C; Figure 1D; MS/MS spectra of both ROOH and ROH are included in Supporting Information). Similarly, the selanyl-benzamide reduced hydroperoxides of CL (Supporting Information).

Though the benzoselenazol ring is a common pharmacophore for ebselen and MitoPeroxidases 1 and 2, several factors may differentiate the pharmacological effect of these compounds. In contrast to ebselen, which accumulates in the ER, MitoPeroxidases 1 and 2 are expected to compartmentalize to mitochondria to reach millimolar concentrations.[23] In addition, the conjugated aromatic system of MitoPeroxidase 1 is extended to the phenolic oxygen, and thus, its Se−N bond is more polarized. This is likely to facilitate the conversion of

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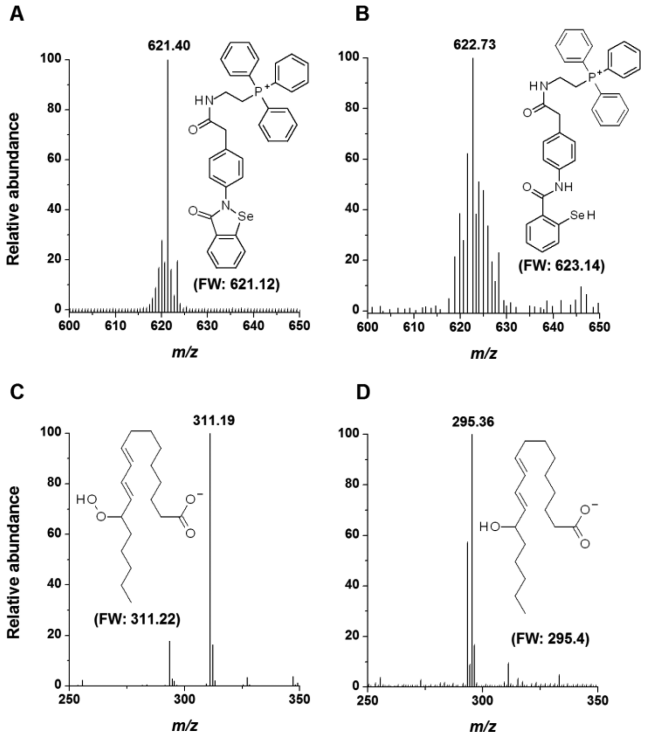

Figure 1. Mass-spectral analysis of the thiol-dependent reduction of ROOH by MitoPeroxidase 2. DHLA reduced MitoPeroxidase 2 (A) to selanyl-benzamide (B), which reacted with ROOH (C) to afford ROH (D). The mass spectra in A and B exhibit isotopic distribution characteristics for the Se isotopes.[20]

MitoPeroxidase 1 to a selenenyl sulfide (reaction **1** → **2**), which may result in an increased toxicity due to enhanced oxidation of cellular thiols.[24] However, weakening of the Se···O═C< interaction in **3** by the electron-withdrawing effect of the phenolic oxygen may increase the GPx activity of the parent benzoselenazol.[25,26]

Figure 2A depicts the toxicity of ebselen and MitoPeroxidases 1 and 2 in mouse embryonic cells (MEC). In contrast to ebselen and MitoPeroxidase 2, the toxicity of MitoPeroxidase 1 sharply increased in the concentration range of 10 to 20 μM. Comparable toxicity with MitoPeroxidase 2 was observed at

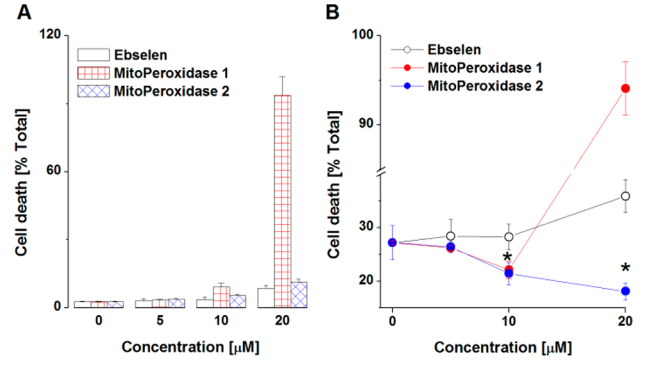

Figure 2. Toxicity (A) and radiomitigative properties (B) of ebselen and MitoPeroxidases 1 and 2. (A,B) Cell death was assessed flow-cytometrically by analysis of the externalization of phosphatidylserine. (B) Cells were exposed to IR and then treated with selenazols. The results represent the mean ± SD (n = 3; *p < 0.05).

~40 μM concentration, while ebselen did not exhibit any significant toxicity in this concentration range.

We next assessed the radio-mitigative properties of the three benzoselenazols. Exposure of MEC to γ-irradiation (10 Gy) resulted in ~28% cell death (Figure 2B), whereas treatment with MitoPeroxidases 1 and 2 (but not ebselen), prior to or 30 min postirradiation, afforded radioprotection/mitigation. MitoPeroxidase 1 exerted ~25% radioprotection/mitigation, while at concentrations higher than 10 μM its toxicity prevailed the radioprotective effect. In contrast, 20 μM MitoPeroxidase 2 afforded ~50% radioprotection/mitigation. In this model system, MitoPeroxidase 2 acted as a potent inhibitor of radiation-induced activation of caspase 3 (Figure 3), an

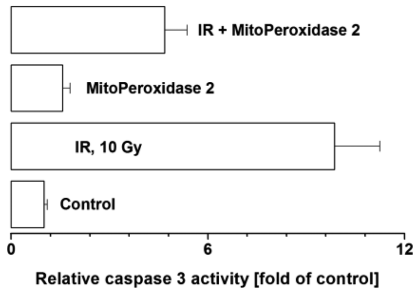

Figure 3. MitoPeroxidase 2 impedes the activity of caspase 3 in MEC. Cells were exposed to IR (10 Gy) and then treated with 20 μM MitoPeroxidase 2 as indicated in Figure 2. Caspase 3 activity was determined by EnzChek Caspase 3 Assay Kit (Z-DEVD-AMC substrate; Life Technologies, Grand Island, NY). The results represent the mean + SD (n = 3).

executioner caspase in apoptosis. Treatment of irradiated MEC with 3-hydroxypropyl(triphenyl)phosphonium chloride, which structurally mimics the triphenylphosphonium moieties of MitoPeroxidases 1 and 2, did not afford any radiomitigation (data not shown).

We further assessed whether the in vitro radiomitigative effect of MitoPeroxidase 2 was translated into an in vivo effect. Groups of 15 mice were treated with MitoPeroxidase 2 i.v. at 24 h after WBI. As shown in Figure 4, at two different radiation doses, administration of MitoPeroxidase 2 afforded an increase in survival.

In conclusion, the data presented herein indicate that MitoPeroxidase 2 acts as a potent radiation mitigator. Our data complement previous studies on the medicinal chemistry of ebselen within the context of radiomitigation upon late, 24 h

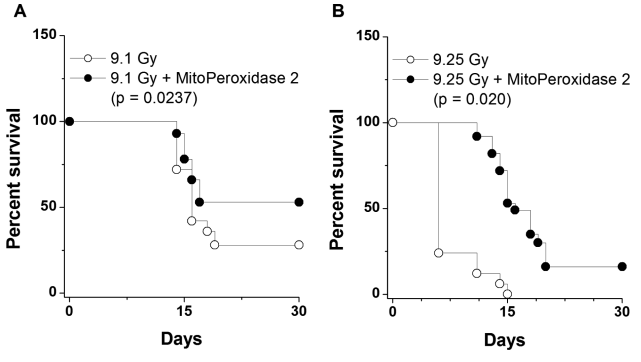

Figure 4. Radiomitigative properties of MitoPeroxidase 2 administered 24 h after exposure to γ-rays in mice (n = 15 mice per group).

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postirradiation treatment. This is an important pharmacological advantage, as IR damage to organisms is often the result of accidents, whereby immediate post-IR treatment may be impractical.

■ ASSOCIATED CONTENT

Supporting Information

Details of biological assays, chemical synthesis, and structural analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

GPx, glutathione peroxidase; ER, endoplasmic reticulum; IR, ionizing radiation; RMs, radiation mitigators; RPs, radiation protectors; WBI, whole-body irradiation; MEC, mouse embryonic cells

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