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Horseradish peroxidase inhibition and antioxidant activity of ebselen and related organoselenium compounds

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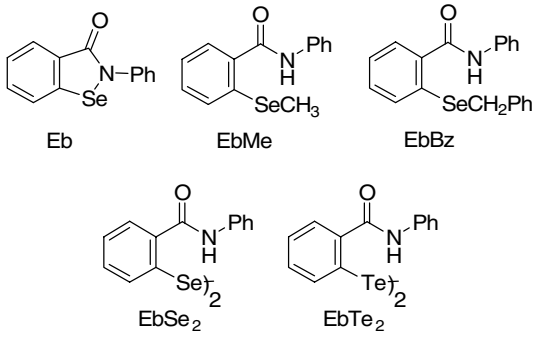
Abstract—Horseradish peroxidase (HRP) inhibition and glutathione peroxidase (GPx) activities of ebselen and some related derivatives are described. These studies show that ebselen and ebselen ditelluride ($EbTe_2$) with significant antioxidant activity, inhibit the HRP-catalyzed oxidation reactions. In addition, inhibition of lipid peroxidation and singlet oxygen quenching studies were carried out. Although the inhibition of HRP by ebselen is comparable with that of $EbTe_2$, the inhibitory effect on γ-radiation induced lipid peroxidation and the GPx activity of ebselen is found to be much higher than that of $EbTe_2$.

Ebselen (2-phenyl-1,2-benzisoselazol-3(2*H*)-one), an organoselenium compound, has been shown to protect tissue against oxidative damage by reducing hydrogen peroxide and other hydroperoxides.[1] The hydrogen peroxide reducing ability of ebselen, therefore, mimics the antioxidant selenoenzyme, glutathione peroxidase (GPx).[2] The catalytically active selenium in ebselen reduces hydroperoxides at the expense of thiol. Similarly to the GPx catalytic cycle, the selenol form of ebselen reacts with hydroperoxides to form a selenenic acid, which reacts with thiol to produce the corresponding selenenyl sulfide intermediate. This intermediate further reacts with an additional thiol to reproduce the selenol. Therefore, ebselen, when combined with suitable thiol compounds such as glutathione (GSH), dithioerythritol, *N*-acetyl cysteine, dihydrolipoate, etc.,[3] can reduce hydrogen peroxide to water and attenuate lipid peroxidation by reducing organic, cholestrol, cholesterol ester- and phospholipid peroxides.[4] In contrast to this reductive detoxification of hydroperoxides, ebselen is a poor free radical scavenger and consequently this is not regarded as a significant component of its pharmacodynamic activity.[5]

Although the biological activity of ebselen has been extensively studied,[6] the effect of ebselen and its derivatives on metalloproteins have not been studied in detail. However, a few reports have appeared in the literature, which show that some organoselenium compounds with reactive selenolate moiety can inhibit certain metalloproteins.[7] Recently, the role of metal coordination in the antioxidant activity of selenium has been reported.[8] Ebselen and other related compounds have been reported to be inhibitors of constitutive endothelial nitric oxide synthase (ceNOS),[9] lipoxygenases,[10] and c-Jun N-terminal kinase.[11] In this paper, we report the effect of ebselen on horseradish peroxidase (HRP) activity. In addition, we report the GPx-like antioxidant activity and the effect of ebselen and related derivatives on singlet oxygen and lipid peroxidation (see Scheme 1).



Scheme 1. Ebselen and related derivatives.

Keywords: Ebselen; Antioxidant; Selenium; Horseradish peroxidase; Lipid peroxidation.
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The inhibition of horseradish peroxidase (HRP) was studied by using spectrophotometric method.[12] The IC_{50} values for the inhibition of HRP by ebselen and other related compounds are summarized in Table 1. Interestingly, ebselen inhibited the HRP activity with an IC_{50} value of 16.9 ± 1.4 μM, indicating that ebselen may strongly inhibit peroxidase-catalyzed oxidation reactions. The Se-methylated and Se-benzylated derivatives of ebselen (EbMe and EbBz, respectively), on the other hand, do not show any significant inhibition. The ditelluride ($EbTe_2$) was found to be almost as potent as ebselen in the inhibition. These observations reveal that the oxidation of selenium or tellurium centres in ebselen and ditelluride ($EbTe_2$) may be responsible for the inhibition. At a concentration of 100 μM, the methyl and the benzyl derivatives inhibited only 25.5% and 33.1%, of the HRP activity, respectively. On the other hand, the diselenide ($EbSe_2$) exhibited a significant inhibition, although the inhibitory activity of this compound was found to be much lower than that of ebselen and the ditelluride (see Fig. 1).

To understand the effect of H_2O_2 and $ABTS^{2-}$ on the HRP inhibition, we carried out some experiments in which the concentration of one of the substrates (peroxide or $ABTS^{2-}$) was varied while keeping other one constant (Figs. S1 and S2, supporting information). The Lineweaver–Burk plots obtained by increasing the H_2O_2 concentration reveal that the inhibition of HRP by ebselen is different from that of $EbSe_2$ and $EbTe_2$. While the H_2O_2 concentration does not have much effect on the inhibition of HRP by ebselen, the inhibition by $EbSe_2$ and $EbTe_2$ appears to be reversed by increasing the peroxide concentration.

Recently, it has been shown that Fe-containing peroxidases such as lactoperoxidase can be inhibited by organoselenium compounds that exhibit significant glutathione peroxidase-like antioxidant activities.[7] Therefore, ebselen may inhibit HRP by reducing hydrogen peroxide or by reacting with the oxidized enzyme, because ebselen is a well-known GPx mimic that reduces hydrogen peroxide and organic peroxides by using thiol co-substrates.[2,3] To this end, we have investigated the GPx activity[13] of ebselen and related derivatives (Fig. 2) and correlated the GPx activity of these compounds with their HRP inhibitory activities. While the HRP inhibition activities of ebselen and $EbTe_2$ correlate well with their GPx activities, the methyl (EbMe) and benzylic (EbBz) compounds, which show significant GPx activity, do not show any appreciable HRP inhibition. This indicates that the HRP inhibition by selenium or tellurium compounds cannot be directly correlated with their GPx-like behaviour, since the GPx activity of selenium/tellurium compounds also depend on the reactivity of these compounds with thiols in addition to their reactions with peroxide. It has been shown that the GPx activity of selenium compounds not only depends on the reactivity of the selenol intermediates towards hydrogen peroxide, but also depends on the reactivity of the selenenyl sulfide intermediates towards thiols.[3a] As there is no thiol present in the HRP inhibition experiments, only the reactivity of the selenium/tellurium compounds towards hydrogen peroxide may determine the HRP inhibition potency of the test compounds.

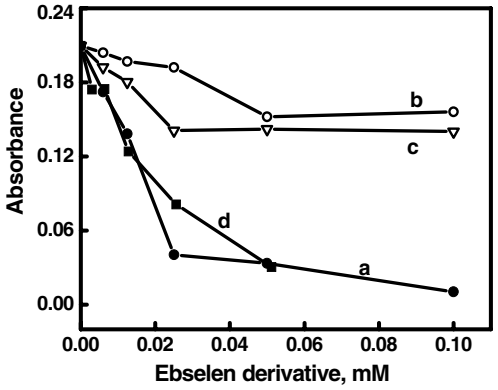

Figure 1. Absorbance at 645 nm ($ABTS^{\bullet-}$) in presence of different concentration of ebselen and its derivatives (a, Eb; b, EbMe; c, EbBz; d, $EbTe_2$).

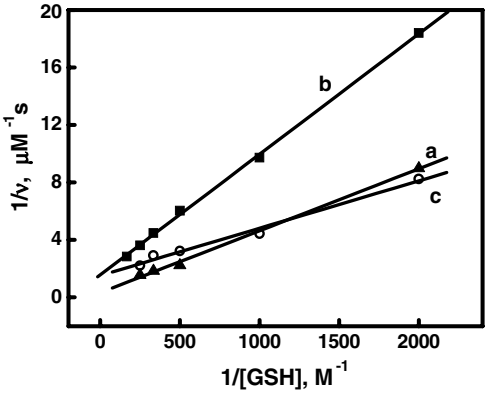

Figure 2. Lineweaver–Burk plots for the reduction of H_2O_2 by ebselen and its derivatives (a, Eb; b, EbMe; c, EbBz).

Table 1. Antioxidant profile of ebselen and its related derivatives

	$^1O_2$+substrate k ($\times 10^6$ M^{-1} s^{-1})	Lipid peroxidation IC_{50}, 280 Gy	GPx activity K_m ($\times 10^{-3}$)	GPx activity V_{max} (μM min^{-1})	HRP inhibition (IC_{50})[a] (μM)
Eb	4.16 ± 0.12	25 μM	13.15	182.9	16.9 ± 1.4
MeEb	14.7 ± 3.3	100 μM	5.25	37.5	>100
BzEb	6.65 ± 0.25	75 μM	2.2	39.5	>100
$EbSe_2$	Does not scavenge at 1 mM	19% at 200 μM	—	—	50.0 ± 1.0
$EbTe_2$	9.9 ± 0.41	175 μM	2.3	31.2	17.3 ± 1.7

[a] The IC_{50} value is the concentration at which lipid peroxidation is inhibited by 50%.

In order to find out the effect of ebselen and related derivatives on lipid peroxidation and singlet oxygen, we have carried out singlet oxygen quenching and lipid peroxidation experiments.[14,15] The extent of γ-radiation-induced lipid peroxidation in liposomes was monitored as thiobarbituric acid reactive substances (TBARS) in the absence and the presence of different concentration of ebselen and its derivatives. The peroxidation was found to be inhibited in the presence of ebselen and related compounds. At a constant γ-radiation dose of 280 Gy, inhibition of peroxidation was followed at different concentration (0.025–0.150 mM) of ebselen and its derivatives. The TBARS formation was found to decrease with increasing concentration of ebselen and its derivatives. As shown in Figure 3, ebselen is found to be the most potent antioxidant having IC_{50} value of 25 μM. In the series, ebselen diselenide ($EbSe_2$) was found to be the least effective. It showed only 19% protection to the liposomes at 200 μM. The IC_{50} values for EbMe, EbBz and $EbTe_2$ were found to be 100, 75, and 175 μM, respectively. When aqueous liposomal solutions were exposed to γ-radiation, the hydroxyl radicals produced during water radiolysis, induced lipid peroxidation in the liposomes.[16] Inhibition by ebselen derivatives may be due to the reaction with either hydroxyl radicals or peroxyl radicals produced during lipid peroxidation. Figure 3 shows the inhibition of TBARS formation by ebselen and its derivatives at a given concentration (50 μM).

The higher activity of the organotellurium compound ($EbTe_2$) as compared to the ebselen diselenide derivative ($EbSe_2$) indicates that peroxide-decomposing capacity as well as chain-breaking ability play role to inhibit γ-radiation induced lipid peroxidation in liposomes under the conditions used. These observations are further supported by the singlet oxygen quenching studies. Although replacement of sulfur by selenium is known to modify the biological activities considerably, a similar role of tellurium in biosystems has not been discovered probably due to their great sensitivity to light and air. However, in recent years, the biological importance of tellurium has attracted considerable attention, as evidenced by several model studies on the antioxidant and photochemotherapeutic properties of synthetic organotellurium compounds.[17]

The higher efficiency of ebselen as compared to that of its sulfur analogue in the prevention of lipid peroxidation initiated via radicals, on the thiol reduction of ferric cytochrome c and reduction of peroxynitrite are significant examples where, the redox properties of selenium plays an important role.[6a] However, basic structural modification in organoselenium compound can also alter their redox activity. Therefore, we carried out singlet oxygen quenching studies in the presence of different ebselen derivatives. The energy of the singlet oxygen is 22.5 kcal/mol above the ground state of the triplet oxygen.[18] Depending upon the substrates, singlet oxygen reacts either by electron transfer or by addition across the double bond to form endoperoxides.[18] The latter reaction is found to be crucial step in lipid peroxidation. Singlet oxygen in absence of any quencher has a lifetime of 66.0 ± 1.0 μs in dicholoroethane. However, in the presence of different concentration of ebselen and its derivatives, the lifetime of the singlet oxygen was found to decrease. The bimolecular rate constant for the reaction between singlet oxygen and ebselen derivatives (Eq. 2) was calculated from the Stern–Volmer plots (Fig. 4).

$$^1O_2 \rightarrow {}^3O_2 + h\nu \ (1270\ \text{nm}) \qquad (1)$$

$$^1O_2 + \text{Ebselen-derivative} \xrightarrow{k_q} \text{Product} \qquad (2)$$

In these studies, methyl ebselen (EbMe) was found to be the most active compound with a bimolecular rate constant of $1.47 \pm 0.33 \times 10^7\ M^{-1}\ s^{-1}$ and ebselen diselenide was found to be the least active, which did not show any noticeable activity even at 1 mM concentration. Thus, all the derivatives except ebselen diselenide ($EbSe_2$) showed singlet oxygen quenching ability and the quenching rate constant was in the order of

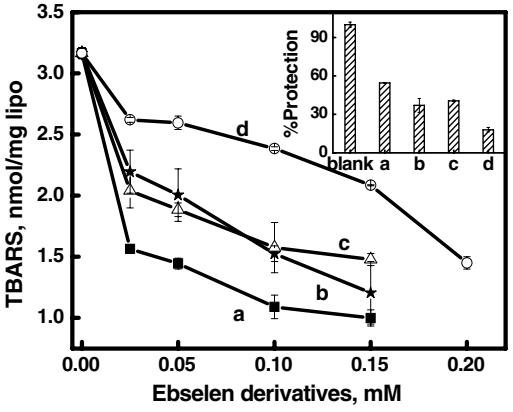

Figure 3. Effect of different concentration of ebselen and its derivative (0.025–0.15 mM) on the amount of TBARS formed on γ-irradiation-induced lipid peroxidation of liposomes at 280 Gy. Inset shows the relative protection of 50 μM of ebselen and its derivative on the γ-radiation-induced lipid peroxidation (a, Eb; b, EbMe; c, EbBz; d, $EbTe_2$).

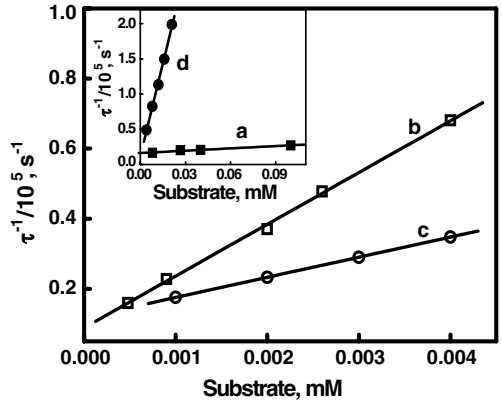

Figure 4. Stern–Volmer plot for quenching of singlet oxygen by ebselen and its derivatives (condition: Solvent: dichloroethane Sensitizer: Hypocrellin-A excitation wavelength: 532 nm). a, Eb; b, EbMe; c, EbBz; d, $EbTe_2$.

$\sim 10^7$–10^6 M^{-1} s^{-1}. It has been reported that singlet oxygen reacts with selenium compounds to form the corresponding selenoxide.[17] This observation is consistent with the involvement of a charge transfer complex in the deactivation of singlet oxygen.[19] The singlet oxygen reacts mainly with the selenium atom and the reactivity of ebselen and its derivative with singlet oxygen mainly depends upon the electron density on the chalcogen atom. In the case of methyl derivative, due to the electron-donating effect of methyl group, the density on the selenium atom increases, while in case of the benzyl derivative the electron-withdrawing effect on the benzyl group decreases the electron density on the selenium atom. The rate constant also depends upon the one electron reduction potential of the radical. In the series, methyl ebselen has the lowest reduction potential (E = 1.43 V vs NHE)[20] and has high reactivity with the singlet oxygen, whereas ebselen diselenide has high reduction potential and has least reactivity. Although ebselen and its derivatives seem to react with free radicals or singlet oxygen at different rates, their contribution to the total antioxidant capacity should be proportional to their respective physiological concentrations. Thus from the rate constant it can be inferred that very high concentration ($\sim 10^{-3}$ M) of the compounds are required to scavenge the singlet oxygen efficiently inside the cells.

n summary, we have shown that ebselen and ebselen ditelluride ($EbTe_2$) inhibit the HRP activity and their inhibitory potency was found to be much higher than that of the corresponding methyl and benzyl derivatives. The oxidation of selenium or tellurium in these compounds may account for their efficient inhibition properties.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.07.085.

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12. HRP inhibition was measured by using $ABTS^{2-}$ as substrate. The initial rates were determined by following the decrease in absorbance at 645 nm (due to $ABTS^{\bullet-}$) in the presence and absence of different concentration of ebselen and its derivatives. Briefly, the solution contained HRP enzyme (70 nM) mixed with 25 μM $ABTS^{2-}$ in the absence and presence of different concentration of selenium compounds (0.06–0.1 mM). The reaction was initiated by addition of hydrogen peroxide (10 μM). The activity without test compound was set to 100%.
13. Glutathione peroxidase activity was determined by GSH–GSSG coupled assay by using glutathione reductase (0.3 U/ml) as ancillary enzyme in the presence of NADPH (0.25 mM). Different concentrations of glutathione (0.5–6 mM) in the presence of catalytic amount of ebselen derivative (0.025 mM) and hydrogen peroxide (1 mM) as substrate were used to measure the catalytic activity. In the assay, GSH is oxidized to GSSG, which is reduced back to GSH by glutathione reductase and NADPH. The rate of reaction is monitored by following decrease in the absorbance at 340 nm due to the decrease in the concentration of NADPH in presence of different concentration of thiols. The absorbance at 340 nm as a function of time is fitted to an exponential function, which gives the observed first-order rate constant. From this rate constant the initial rate (v) was calculated by using 6220 M^{-1} cm^{-1} as the extinction coefficient for NADPH. The kinetic parameters such as K_m and V_{max} were calculated from the double reciprocal plots (Lineweaver–Burk plots) of initial rate as a function of substrate concentration. For the GPx activity, the rates were corrected for the background reaction between H_2O_2 and GSH. Due to poor solubility of the organoselenium compounds in water, the compounds were dissolved in methanol. In most of the experiments, 2% of methanol was added to the buffer solution to obtain a homogeneous system.
14. Kinetics of singlet oxygen reactions with ebselen and its derivatives was studied using transient luminescence spectrometer (TL900, Edinburgh Instruments, UK). Singlet oxygen was generated by photoexcitation of hypocrellin-A (absorbance at 532 nm ~0.5) in dichloroethane by using second harmonic (532 nm) of Nd-YAG laser. Hypocrellin-A is a well-known dye, which generates singlet oxygen in high yield in dichloroethane. After laser

excitation, the decay profile of singlet oxygen emission was monitored at 1270 nm with the help of liquid nitrogen cooled germanium detector. Change in the lifetime of singlet oxygen was monitored in the presence of different concentration of (0.1–4 mM) test compound to determine the singlet oxygen-quenching rate constant as per the Stern–Volmer equation

$$\tau^{-1} = \tau_0^{-1} + k_q[\text{test compound}],$$

where τ and τ_0 is the lifetime of the singlet oxygen in presence and absence of quencher. k_q is the bimolecular rate constant for the quenching of singlet oxygen and the ebselen derivative. In this study, it is important to maintain the concentration of hypocrellin-A constant.

15. Lipid peroxidation studies were carried out in phosphatidyl choline liposomes. Lamellar phosphatidyl choline liposomes of 150 nm size, determined by light scattering studies, were suspended in pH 7.4 phosphate buffer and subjected to γ-radiation. γ-radiolysis was carried out using ^{60}Co γ-source with a dose rate of 48 Gy/min as measured by standard Fricke dosimetry. At a total absorbed dose of 280 Gy in N_2O/O_2-purged liposomal solution in absence and presence of the complex at pH 7.4 (phosphate buffer). The extent of lipid peroxidation was estimated in terms of thiobarbituric acid reactive substances (TBARS) using 15% w/v trichloroacetic acid, 0.375% w/v Thiobarbituric acid (TBA), 0.25 N hydrochloric acid, 0.05% w/v butylated hydroxyl toluene (BHT) as TBA reagent measuring the absorbance at 532 nm ($\varepsilon_{532} = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$).
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