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ANTIOXIDANT ACTIVITY OF EBSELEN AND RELATED SELENOORGANIC COMPOUNDS IN MICROSOMAL LIPID PEROXIDATION

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Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)one, and its derivatives were compared for their ability to protect microsomal membranes against iron/ADP/ascorbate-induced lipid peroxidation, measured as low-level chemiluminescence and accumulation of thiobarbituric acid-reactive substances (TBARS). The concentrations of the compounds required to double the lag time of the control with no added antioxidants were 0.13 μM for ebselen, 0.5 μM for the N-pyridyl analog, 0.3–0.7 μM for the selenylsulfides, about 1.0 μM for the selenoxide derivative and 2.0 μM for the sulfur analog of ebselen. The open-chain seleno- and thioether derivatives, on the other hand, exhibited comparatively low abilities to protect the membrane, the lag doubling concentrations for these compounds being 100–1,000 fold higher than that for ebselen.

The rate of loss of α-tocopherol in the microsomal membrane during peroxidation was significantly diminished in the presence of 0.1–0.5 μM ebselen, while the glutathione adduct of ebselen was equally effective in protecting the loss of α-tocopherol. The sulphur analogue and, the benzylated and methylated derivatives of ebselen did not afford protection. Ebselen was without effect in microsomes from vitamin E-deficient rats up to 20 μM, indicative of the dependence of its protective ability upon α-tocopherol.

KEY WORDS: Ebselen, ebselen derivatives, microsomal lipid peroxidation, thiobarbituric acid reactive substance, antioxidants, chemiluminescence, α-tocopherol.

INTRODUCTION

Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)one, **1**, is a selenium-containing heterocycle with antioxidant and glutathione peroxidase activity.[1,2] The antioxidant activity has been studied in microsomal system using iron/ADP/ascorbate[1,3] or CCl_4^3 as prooxidants to induce lipid peroxidation. Recently, we have demonstrated the ability of ebselen and some of its derivatives to scavenge 1,2-dichloroethane radical cations and halogenated peroxyl radicals by pulse radiolysis;[4] the second-order rate constant for oxidation of ebselen by trichloromethylperoxyl radicals was of the order of $10^8\,M^{-1}s^{-1}$, comparable to that of α-tocopherol.[5]

Here, we compare the antioxidant activity of derivatives of ebselen, a few of which have been identified as its metabolites,[6] by studying their ability to prolong the lag phase preceding the onset of active peroxidation. Lipid peroxidation was induced non-enzymically in microsomal membranes by iron/ADP/ascorbate and was measured as low-level chemiluminescence and accumulation of thiobarbituric acid reactive substances (TBARS). In addition, the loss in the content of α-tocopherol in the

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microsomal membrane was followed. Previous reports have shown that ebselen inhibits microsomal NADPH-cytochrome P-450 reductase *in vitro*,[7,8] thereby eliminating the iron/ADP/NADPH system to be employed as model prooxidant.

MATERIALS AND METHODS

Ebselen and analogs were obtained from A. Nattermann Co.,/Rhone-Pôulenc (Cologne). Other chemicals used were obtained from Merck (Darmstadt) and Boehringer (Mannheim).

Incubation of Microsomes

Microsomes were prepared from male Wistar rats as described previously[9] and stored at − 18°C until use. Microsomal fractions (0.5 mg protein/ml) were incubated under oxygenation at 37°C in 6.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 16 μM ferrous sulfate and 2 mM ADP. Lipid peroxidation was initiated by addition of 0.5 mM ascorbate. Ebselen and the different derivatives dissolved in dimethylsulfoxide (DMSO) were added in 10 μl aliquots. Control assays had 10 μl DMSO alone.

Lipid Peroxidation Measurements

Low-level chemiluminescence was followed with a single-photon counting system equipped with a red-sensitive photomultiplier (EMI 9658 AM).[10] The photomultiplier was connected to an amplifier - discriminator (model 1121 A, Princeton Applied Research, Princeton, NJ, USA) and adjusted for single photon counting to a recorder. Chemiluminescence was assayed in a 35 mm × 5 mm × 56 mm cuvette maintained at 37°C, with constant stirring and oxygen bubbling during the course of the reaction. Peroxidation was also assessed by measuring the level of thiobarbituric acid-reactive substances formed as malondialdehyde equivalents.[11] The α-tocopherol content in the microsomal membrane during peroxidation was followed by HPLC[12] using an ESA model 5100A Coulochem electrochemical detector with the analytical cell potential set at + 0.3 V. β-Tocopherol (1 nmol) was used as an internal standard. Protein was measured by the method of Lowry *et al.*[13]

RESULTS AND DISCUSSIONS

Chemiluminescence Studies

The chemical structures and names of the different derivatives of ebselen used in this study are shown in Figure 1. A comparison of the antioxidant ability of the different derivatives is made in Figure 2. The lag time was measured by extending the slope of increasing chemiluminescence to intersect the baseline. The ratio of lag time in presence of antioxidant (τ) to the lag time in its absence (τ_o) is plotted versus the log of concentration of the compound used. Antioxidant capacity is expressed as the lag doubling concentration which is the concentration of compound required to double lag time of control. It is seen that ebselen, **1**, has the highest antioxidant capacity by affording protection at very low levels, the lag doubling concentration being 0.13 μM.

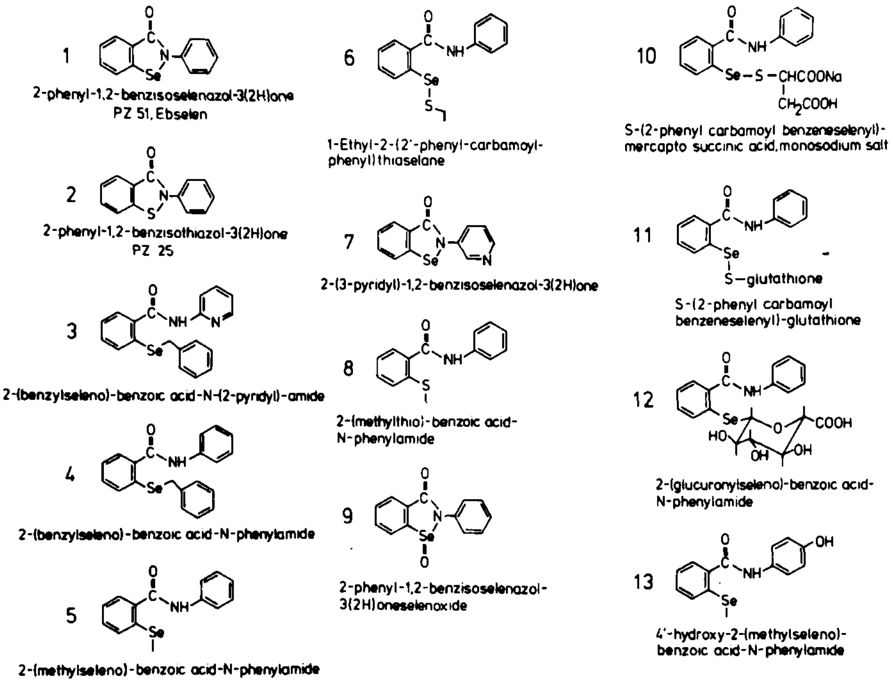

FIGURE 1 Chemical structures and names of ebselen and derivatives studied.

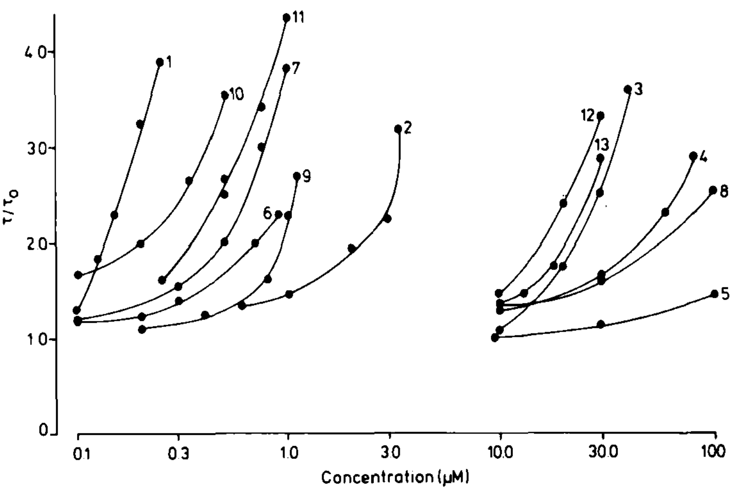

FIGURE 2 Comparative evaluation of antioxidant capacity of ebselen and analogs: dependence of lag time of chemiluminescence on concentration of the antioxidants. Incubation mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 16 μM ferrous sulfate, 2 mM ADP and microsomes (0.5 mg protein/ml) at 37°C with constant oxygenation. Lipid peroxidation was initiated by addition of 0.5 mM ascorbate. Ebselen and the other compounds were added in 10 μl DMSO. Control experiments had 10 μl DMSO. Ratio of lag phase in prescence of antioxidant to the lag phase in control (τ/τ_0) plotted vs concentration of antioxidant. Numbers correspond to the names in Figure 1. The curves are representative of three independent experiments.

The derivatives exhibited protection to varying extents in a concentration-dependent manner. The derivatives S-(2-phenyl-carbamoylbenzeneselenyl)mercaptosuccinate, **10**, the GSH adduct of ebselen, **11**, 2-(3-pyridyl)-1,2-benzisoselenazol-3(2H)one, **7**, 1-ethyl-2-(2'phenyl-carbamoylphenyl)thiaselane, **6**, and 2-phenyl-1,2-benzisoselenaz ol-3(2H)oneselenoxide, **9**, have potencies comparable to that of ebselen, with lag doubling concentrations of 0.2, 0.35, 0.5, 0.7 and 0.9 μM, respectively. Replacing Se in the benzisoselenazol ring of ebselen by sulfur diminished the antioxidant potency by 15-fold, the lag doubling concentration for **2** being 2.2 μM.

The two-electron oxidized product, **9**, and the glutathione adduct of ebselen, **11**, have been proposed[14,15] as probable intermediates during the catalytic reduction of hydroperoxides by ebselen. It has been suggested[14] that the selenoxide is formed in presence of high concentrations of hydroperoxides as in conditions where there is a preponderance of oxidative reactions, while the glutathione adduct is formed in presence of high thiol concentrations. In presence of thiols, the selenylsulfides have been shown to be formed[16] by attack of the thiolate anion on the Se atom of benzisoselenazol leading to ring-opening and adduct formation. Reaction of the selenoxide of ebselen with thiols also ultimately leads to the selenylsulfide.[17] The selenylsulfide has been proposed to constitute the storage and transport form of ebselen.[14] In the physiological system, the obvious choice for conjugation with ebselen would be glutathione, though the intermediate ebselen-SG was not detected in perfusion studies in rat liver, either due to its lability or low steady-state concentration.[6]

The Se-benzylated form of the N-pyridyl analog of ebselen, 2-(benzylseleno)-benzoic acid-N-(2-pyridyl)-amide, **3**, doubled the lag time of control at about 23 μM, while the corresponding derivative of ebselen, 2-(benzylseleno)-benzoic acid-N-phenylamide, **4**, was required at twice as high concentration to produce a similar delay in the lag phase.

The Se-methylated derivative of ebselen, 2-(methylseleno)-benzoic acid-N-phenylamide, **5**, and the corresponding sulfur analog, **8**, were practically inactive in preventing lipid peroxidation. However, para-hydroxylation of derivative **5**, to give 4'-hydroxy-2-(methylseleno)-benzoic acid-N-phenylamide, **13**, enhanced the antioxidant property. While the latter had a lag doubling concentration of 20 μM, more than 10-fold concentration of the former was required to produce similar prolongation of lag phase (data not shown). Compound **12**, 2-(glucuronylseleno)-benzoic acid-N-phenylamide was also found to have substantial antioxidant activity. The Se-methylated derivatives were identified as metabolites in the effluent perfusate during perfusion of isolated rat liver with ebselen,[6] while the Se-glucuronide and an O-glucuronide of **13** appeared in the bile; the syntheses and spectral characteristics of these metabolites have been described.[18]

Accumulation of TBARS

The effect of ebselen and its derivatives on the time course of accumulation of TBARS was studied (Figure 3 and Table I) at the indicated concentration, which was close to their lag doubling concentration in chemiluminescence experiments. Derivatives **13** and **5** produced a prolongation of lag period by 1.5 to 2.5-fold with a reduction in the rate of peroxidation to 70% and 55%, respectively, of the control. On the other hand, the glutathione adduct, **11**, the benzylated derivative, **4**, and the selenoxide, **9** diminished the maximal level of TBARS to 43%, 22% and 13%, respectively, with considerable reduction in the rate of peroxidation and prolongation of the lag phase.

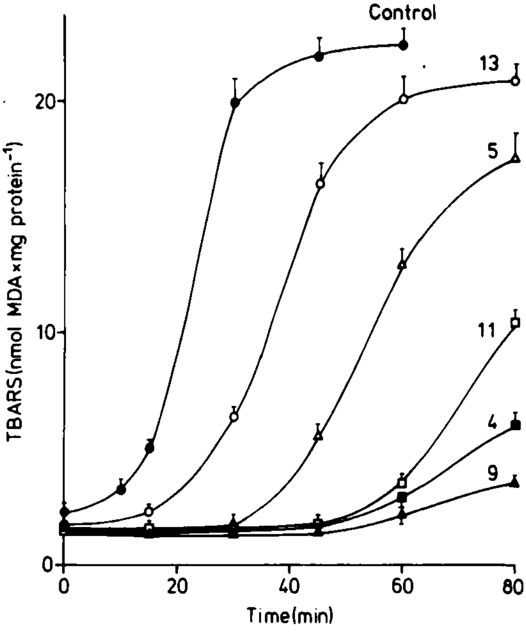

FIGURE 3 Effect of ebselen and derivatives on time course of accumulation of TBARS during microsomal lipid peroxidation. Control microsomes with no added antioxidants. (●); 2-(benzyl, seleno)-benzoic acid-N-phenylamide, **4**, at 30 μM, (■); 2-(methylseleno)-benzoic acid-N-phenylamide, **5**, at 100 μM, (△); 2-phenyl-1,2-benzisoselenazol-3(2H)one selenoxide, **9**, at 0.5 μM, (▲); glutathione adduct of ebselen, **11**, at 0.5 μM, (□); and, 4'hydroxy-2-(methylseleno)-benzoic acid-N-phenylamide, **13**, at 10 μM, (○). Incubation mixture as indicated in Figure 2. Control experiments had 10 μl DMSO alone. The level of TBARS measured as malondialdehyde equivalents at 535 nm. Curves are representative of 3 independent experiments.

TABLE I
Effect of ebselen and derivatives on accumulation of TBARS during microsomal lipid peroxidation.

Compound	Concentration of antioxidant (μM)	Rate of Accumulation (nmol MDA/mg protein/min)	% of maximum in control	(τ/τ_0)
Control	-	1.0	100	-
1[a]	1.6	0.33	35	4.4
2[a]	1.6	0.61	100	1.3
4	30	0.17	22	2.8
5	100	0.55	78	2.4
9	0.5	0.08	13	2.8
11	0.5	0.36	43	3.7
13	10	0.7	91	1.4

[a]Data from ref.[1], under similar conditions of assay, where maximal level of TBARS was measured 80 min after initiation of peroxidation. Incubation mixture and conditions of assay as mentioned in Figure 2. The level of TBARS measured as malondialdehyde equivalents at 535 nm and expressed as % of control, the maximal level of TBARS in control being 23 nmol MDA/mg protein. (τ/τ_0) represents the ratio of lag time in presence of antioxidants to the lag time in control. The lag period in control was 16 $\pm$ 1 min. Values are representative of 3 independent experiments.

Relation to α-Tocopherol

Loss of α-tocopherol in the membrane after initiation of lipid peroxidation was monitored (Figure 4 and Table II). In control experiments with no added antioxidants, the content of α-tocopherol decreased at an initial slow rate of 2.0 pmol/mg

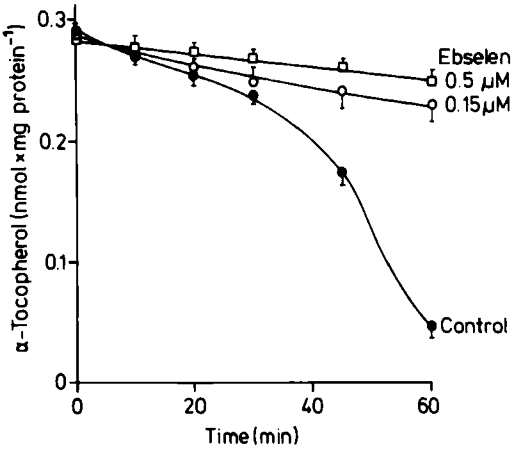

FIGURE 4 Loss of α-tocopherol in microsomal membrane during lipid peroxidation and effect of ebselen. Incubation mixture as indicated in Figure 2. β-Tocopherol was added as internal standard. Control with no added antioxidant, (●); 0.15 μM ebselen, (○) and 0.5 μM ebselen, (□). Values are mean ± SEM (n = 3).

TABLE II
Loss of α-tocopherol in microsomal membrane during lipid peroxidation and effect of ebselen and derivatives.

Compound	Concentration (μM)	Rate of Loss of α-Tocopherol (pmol/mg protein/min)	Level Remaining after 60 min (% of initial)
Control			
slow	–	2.0	–
rapid		9.0	17
Ebselen, **1**	0.15	1.1	81
	0.5	0.5	90
Ebselen-SG, **11**	0.2		
slow		1.1	
rapid		7.0	20
	0.5	0.8	83
2	2.0		
slow		3.1	
rapid		22.0	10
4	50		
slow		1.9	
rapid		5.8	17
5	50		
slow		1.8	
rapid		8.0	12

Data from experiments such as those shown in Figure 4. Values are average from three independent experiments. Ebselen at the concentrations studied and ebselen-SG at 0.5 μM concentration did not produce a biphasic pattern of loss of α-tocopherol.

protein/min up to 30 min followed by a rapid phase at 9.0 pmol/mg protein/min, with 17% of the initial content remaining after 60 min. In presence of ebselen the overall rate diminished significantly to 1.1 pmol/mg protein/min at a concentration of 0.15 μM with 81% of the initial α-tocopherol content remaining after 60 min. This effect was more pronounced at higher levels, with the rate of loss being 0.5 pmol/mg protein/min at 0.5 μM ebselen. The glutathione adduct was effective to a similar extent at 0.5 μM concentration with 83% of the initial α-tocopherol being present after 60 min. It is not known whether ebselen acts by 'sparing' α-tocopherol or by regenerating the reduced form of α-tocopherol, though it appears likely that the latter process is predominant as ebselen did not afford protection to microsomal membranes in the absence of vitamin E. This was evident in studies with microsomes isolated from rats maintained on vitamin E-deficient diet for about 15 weeks (data not shown). The lag time of control experiments for vitamin-E deficient microsomes was 7 ± 0.1 min, and ebselen did not prolong the lag phase up to 20 μM concentration. This clearly indicates the dependence of ebselen on vitamin-E for its protective antioxidant activity. The sulfur analog, **2**, the benzylated and the methylated derivatives, **4** and **5**, respectively, did not have any effect on the loss of α-tocopherol, which supports the results from chemiluminescence and TBARS studies.

Besides its role as a catalyst for reduction of hydroperoxides [2], ebselen was also shown by pulse radiolysis to be an efficient scavenger of free radicals.[4] A high rate constant for oxidation of ebselen by halogenated peroxyl radicals was observed, which was comparable to that of α-tocopherol[5], while the sulfur analog, PZ 25, exhibited significantly lower rate constants. Interestingly, the Se-methyl derivative, **5**, had rate constants comparable to that for ebselen, which would not be expected from the results of the present study where this compound was least effective as an antioxidant.

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