Research article

In vitro evaluation of glutathione peroxidase (GPx)-like activity and antioxidant properties of some Ebselen analogues

Ange Mouithys-Mickalad Mareque[1], Juan Mareque Faez[2], Léon Chistiaens[2], Stephan Kohnen[1], Carol Deby[1], Maryse Hoebeke[1,3], Maurice Lamy[1,4], Ginette Deby-Dupont[1,4]

[1]Centre for Oxygen Research and Development (CORD) and [2]Laboratory of Organic Chemistry, Institute of Chemistry, University of Liège, Liège, Belgium
[3]Laboratory of Biomedical Spectroscopy, Institute of Physics, University of Liège, Liège, Belgium
[4]University Department of Anaesthesia and Intensive Care Medicine, University of Liège, Sart Tilman, Liège, Belgium

Four analogues of Ebselen were synthesized and their glutathione peroxidase activity and antioxidant property evaluated and compared to Ebselen. Among the studied compounds, only diselenide [**3**] exhibited both glutathione peroxidase activity and radical-scavenging capability. Compounds [**3**] and [**4**] showed a strong inhibitory effect (53% and 43%, respectively) on the lipid peroxidation of linoleic acid compared to Ebselen and selenide derivatives ([**1**] and [**2**]) which were less active (28%, 26% and 18% inhibition, respectively). A concentration-dependent inhibitory effect was also found in the model of the formation of $ABTS^{\bullet+}$ radical cation: 65% and 89% inhibition for compound [**3**] at 10^{-4} M and 5×10^{-5} M, respectively, and 68% and 90% for compound [**4**], compared to 14% and 52% inhibition for Ebselen and the diselenides [**1**] and [**2**] (29%, 46% and 45%, 68%, respectively). By EPR spin trapping technique, the following inhibitory profile of the Ebselen analogues was observed towards the formation of thiyl radicals: Ebselen = [**3**] > [**1**] > [**2**] > [**4**]. Studies with compound [**3**] are in progress on oxidative stress cell models.

Keywords: Glutathione peroxidase, diselenide and selenide derivatives, Ebselen, electron paramagnetic resonance, lipid peroxidation

INTRODUCTION

Glutathione peroxidase (GPx) is a mammalian selenoenzyme which functions as a catalytic antioxidant and protects various organisms from oxidative stress and cellular membrane damage.[1] GPx catalyses the reduction of harmful peroxides using glutathione (GSH) as the reducing substrate[2] according to the following equation:

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O \qquad \text{Eq. 1}$$

Received 21 October 2003
Revised 27 February 2004
Accepted 19 March 2004

Correspondence to: Ange Mouithys-Mickalad PhD, CORD, Institute of Chemistry, B6a, University of Liège, B-4000 Liège, Belgium
Tel: + 32 4 366 47 73; Fax: + 32 4 366 28 66;
E-mail: amouithys@ulg.ac.be

On the other hand, it is well known that the nucleophilic reactivity and radical scavenging properties of selenium contribute to the biological activity of selenium compounds. Consequently, the use of low molecular weight compounds (GPx mimics) able to circumvent the difficulty linked to the use of the enzyme itself is strongly encouraged. With this in mind, some simple seleno-organic compounds have been widely studied[3] and shown to have GPx mimicking activity *in vitro*.[4] Among them the most promising drug was Ebselen (PZ 51, 2-phenyl-1,2-benzoisoselenazol-3-(2*H*)-one), a heterocyclic compound that functions as an antioxidant.[5] Recently, much attention has been devoted to the design of new synthetic analogues of Ebselen able to mimic the GPx activity. One of the first studies addressing this problem in a series of diselenides was the study of Wilson *et al*.[6] The Iwaoka and Tomoda groups and others continued working on this issue, thus

DOI 10.1179/135100004225004788

demonstrating the important role of basic amino groups in the intramolecular interaction between nitrogen atom and selenium.[7] In the same way, Mugesh and co-workers developed novel analogues to increase the GPx mimicking activity. Various derivatives having in their structure either the diferrocenyl diselenide or ditelluride moieties were thus designed and synthesized.[8,9] In this series, diselenide derivatives bearing a primary amino group were not studied in the GPx mimic model.

On the other hand, beside its thiol peroxidase-like activity, Ebselen has been found to have radical scavenging properties.[10] In animal model studies, Ebselen was shown to reduce oxidative stress in ischemia-reperfusion in heart[11] and to have neuroprotective effects in brain.[12,13] Nevertheless, some studies suggested that the unique mechanism of the antioxidant activity of Ebselen seemed specific to its capability to reduce the harmful peroxides rather than to its radical scavenging properties.[14] In the light of these findings and taking into account the increasing role played by this kind of compounds, we were interested in investigating not only the GPx mimic effect but also the antioxidant properties of new synthetic analogues of Ebselen. Here we report data obtained for four selenide and diselenide derivatives compared to Ebselen in *in vitro* experimental models: (i) glutathione peroxidase activity using kinetic assays and peroxidase-catalysed oxidation monitored by EPR spin trapping technique; and (ii) the antioxidant profile by using a free radical generating system (ABTS/H_2O_2/MetMb) and lipid peroxidation of an emulsion of linoleic acid followed by UV–visible spectrophotometry analysis.

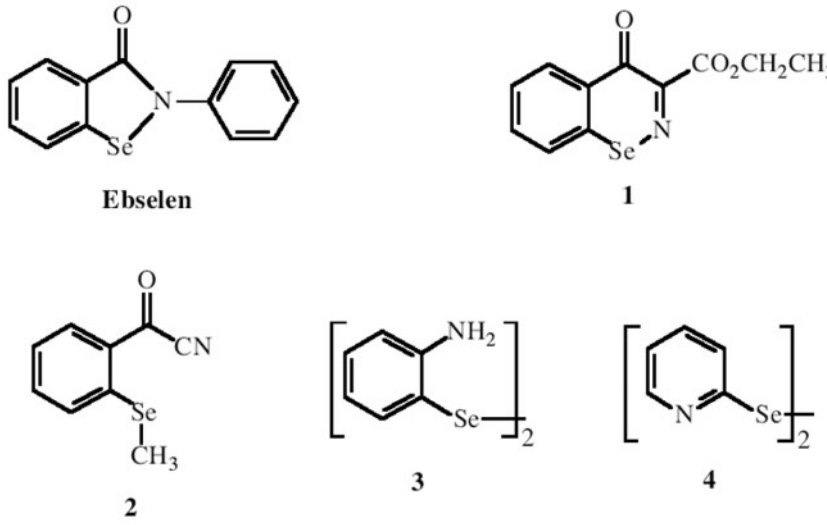
Materials and Methods

Chemicals

All synthetic compounds – (2-phenyl-1,2-benzoisoselenazol-3(2H)-one (Ebselen); ethyl-benzo-1,2-selenazine-4-one-3-carboxylate [**1**]; 2-methylselenobenzoylcyanide [**2**]; 2,2′-diamino-diphenyldiselenide [**3**]; and 2,2′-diselenopyridin [**4**] – were provided by the Laboratory of Organic Chemistry (University of Liège, Belgium) (Scheme 1). Benzenethiol (PhSH) was purchased from Janssen-Chemica (Belgium). Methanol was from UCB-Pharma (Belgium). 2-Thiobarbituric acid (TBA), trichloroacetic acid (TCA), phosphate salts ($NaH_2PO_4.2H_2O$ and Na_2HPO_4) and hydrogen peroxide were obtained from Merck (Belgium). CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) and diethylenetetramine pentaacetic acid (DTPA) were obtained from Sigma (Belgium). Horseradish peroxidase (HRP) was purchased from Boehringer Mannheim. The precise concentration of hydrogen peroxide (Merck, 30% H_2O_2) was determined by titration with potassium permanganate. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was obtained from Sigma-Aldrich (Belgium) with further purification with charcoal as previously described by Green and Hill.[15] For EPR and lipid peroxidation experiments, compounds were dissolved daily in DMSO or methanol (analytical grade, Merck). 2,2-Azinobis (3-ethylbenzothiazoline-6-sulfonate; ABTS) was purchased from Aldrich (Belgium). All other reagents were of analytical grade.

Kinetic analysis

The GPx-like catalytic activity of the tested compounds was initiated by the reaction of 37.5 mM H_2O_2 with a methanolic solution of 10 mM PhSH used as glutathione alternative.[7] The reaction was monitored at 25°C by UV spectroscopy at 305 nm by following the absorbance increase due to the formation of diphenyldisulfide (PhSSPh). The molar extinction coefficient of PhSSPh ($\varepsilon = 1.24 \times 10^3\ M^{-1}cm^{-1}$) at this wavelength was at least



Scheme 1. Chemical structure of Ebselen and the related analogues, selenides [1,2] and diselenides [3,4] used in this study.

> 5 times larger than that of PhSH (ε = 9.0 $M^{-1}cm^{-1}$ under the same conditions). Kinetics of the reaction were compared with that of the reference compound Ebselen (Perkin-Elmer Lambda 15 UV:VIS spectrophotometer).

Lipid peroxidation experiments

Stock solutions of the drugs (10^{-2} M), prepared in DMSO, were diluted to the final concentration of 100 μM in 50 mM phosphate buffer pH 7.4 (total volume 2 ml). To a mixture containing 200 mg of CHAPS, 0.2 ml of linoleic acid (final concentration 6.4 mM) and 100 ml of the chelexed phosphate buffer were added 20 μl of 10^{-2} M drug. The mixture reaction was irradiated (from cesium-137 source at the total dose rate of 10,000 rads) to produce $^{\bullet}OH$ able to trigger lipid peroxidation. Then, 0.5 ml of the irradiated solution was added to 0.5 ml of TCA and 2 ml of TBA (26 mM) in 50 mM Tris-HCl buffer at pH 7.0 in order to detect the final amount of lipid peroxidation products.[16] After extraction with 2 ml of *n*-butanol, the absorbance of the lipid peroxidation product–TBA complex of each sample was measured by monitoring the absorbance at 540 nm, with *n*-butanol as internal reference (Perkin-Elmer Lambda 15 UV:VIS spectrophotometer). Each assay was done in triplicate and repeated at least three times.

Evaluation of the antioxidant activity of Ebselen analogues on the formation of the radical cation ($ABTS^{\bullet+}$)

The experiment was performed in 50 mM phosphate buffer (pH 7.4) with the peroxidase system metmyoglobin (MetMb)/H_2O_2 in the presence of the ABTS substrate (λ_{max}, 342 nm) with or without addition of the Ebselen analogue. The ferrylmyoglobin radical formed from MetMb and H_2O_2 produced the ABTS radical cation ($ABTS^{\bullet+}$) with maximal absorbance at 734 nm. The antioxidant capacity of the drug under investigation was followed by the absorbance value at 734 nm. The tested compounds were used at final concentrations ranging from 10^{-5}–10^{-4} M and added just after the reaction has been started by H_2O_2 addition. All measurements were carried out at room temperature on an UV-visible spectrophotometer (Perkin-Elmer, Lambda 15).

EPR spin trapping investigation on the peroxidase-catalysed thiyl radical generation

The experiments were performed in 50 mM phosphate buffer (pH 7.4) in the presence of 100 mM DMPO in a total volume of 1 ml. The reaction started after the addition of the drug (10^{-4} M, in 1% DMSO) to the following system: HRP (25 μg/ml), GSH (10 mmol/l), H_2O_2 (1 mM), and DTPA (0.5 mmol/l). The reaction mixture was immediately transferred into a quartz flat cell in the EPR cavity (Bruker spectrometer ESP 300 E; Bruker, Karlsruhe, Germany). The EPR signal, corresponding to the spin adduct of DMPO/thiyl ($GS^{\bullet}$) resulted from the reaction of DMPO with the $GS^{\bullet}$ radicals produced by the HRP enzymatic activity on GSH. The EPR signal obtained in the absence of the drug was taken as the control spectrum.

EPR spectra were monitored at room temperature with the following instrument settings: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; centre of field, 3480 G; and sweep width, 100 G. The other parameters are listed in the captions to the figures.

Results

Estimation of GPx-like activity

The results of the initial reduction rate of H_2O_2, obtained by monitoring the increase of the UV absorption at 305 nm due to the diphenyl disulfide (PhSSPh) formed, are listed in Table 1. In the absence of catalysts with potential GPx-like activity, an exceedingly slow oxidation of PhSH took place (k = 0.91 mM s^{-1}). Among the studied compounds, diselenide derivatives [**3**] and [**4**] strongly accelerated the reduction rate of H_2O_2 with k values of 6.79 and 12.23 mM s^{-1}, respectively. In contrast, the selenide derivatives [**1,2**] were weakly active, with an efficiency of 1.37 and 0.95 mM s^{-1} almost similar to that of the reference drug (Ebselen, k = 1.16 mM s^{-1}) which, in this model, was poorly active (Table 1).

Effect of Ebselen and analogues on the lipid peroxidation of linoleic acid

Radiation-induced lipid peroxidation was designed to follow the capability of Ebselen analogues to inhibit the for-

Table 1. Kinetics of the reduction of the hydrogen peroxide molecule catalysed by Ebselen analogues (10^{-4} M) in the presence of benzenethiol (PhSH) taken as glutathione alternative. The results represent the average of four separate assays.

Compound	k (mM/s)
None	0.91
DMSO	1.24
Ebselen	1.16
[**1**]	1.37
[**2**]	0.95
[**3**]	6.79
[**4**]	12.23

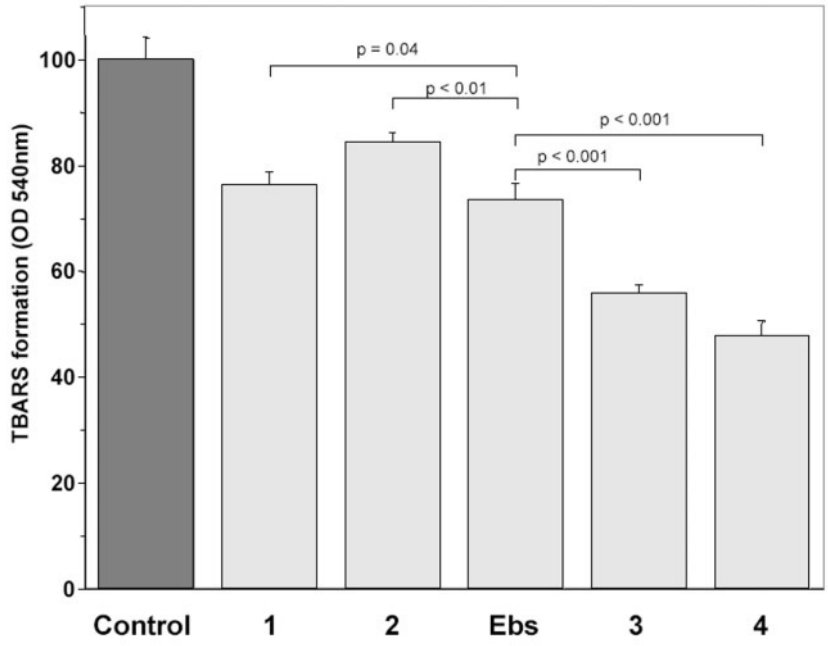


Fig. 1. Effect of Ebselen analogues [1–4] on *in vitro* lipid peroxidation (linoleic acid) induced by γ-radiation: comparison with Ebselen. Values are the mean ± SD of nine assays. $P < 0.001$ versus Ebselen was considered as extremely significant and $P < 0.05$ versus Ebselen was significant.

mation of peroxidized lipid derived from the reaction of hydroxyl radical with linoleic acid. The results indicated that the diselenide derivatives [**3**] and [**4**], at 10^{-4} M, significantly inhibited lipid peroxidation ($P < 0.001$ versus Ebselen; Fig. 1). Likewise, when Ebselen or selenide derivatives [**1**] and [**2**] were added to the reaction mixture, a decrease of the lipid peroxidation was also observed but to a lesser degree ($P < 0.05$ and $P < 0.01$ versus Ebselen, for compounds [**1**] and [**2**]), although still significant compared to control (Fig. 1). In the model of lipid peroxidation, the order of efficacy of the tested drugs was as follows: [**4**] > [**3**] > Ebselen > [**1**] > [**2**].

Evaluation of the antioxidant activity using the ABTS assay

The use of H_2O_2 and MetHb allowed the formation of ferrylmyoglobin (MbFe (IV)=O), with a subsequent electron transfer from the ABTS substrate, resulting in the formation of the ABTS radical cation, the production of which was followed spectrophotometrically (ABTS/MetMb/H_2O_2 system). This assay allowed the determination of the anti-radical activity of the selected compounds under hydrophilic conditions. In the absence of the Ebselen analogues, the absorbance due to the formation of the $ABTS^{\bullet+}$ radical cation was taken as the 100% reference value (Fig. 2). The diselenides [**3,4**] and the selenide [**2**] exhibited a dose-dependent inhibiting effect on the formation of the $ABTS^{\bullet+}$ radical cation ($P < 0.001$ versus Ebselen) at 5.10^{-5} and 10^{-4} M). Selenide [**1**] and Ebselen showed a less pronounced dose-dependent inhibitory effect ($P < 0.001$ versus control at 10^{-4} and 5.10^{-5} M). The order of efficacy of the compounds tested in this model was as follows: [**4**] > [**3**] > [**2**] > Ebselen = [**1**].

EPR spin trapping investigation of the effect of Ebselen and its analogues on the thiyl radical generation

The peroxidase-catalysed oxidation system allowed investigation of the formation of the thiyl ($GS^{\bullet}$) radical intermediate triggered by the addition of H_2O_2 in the presence of a peroxidase (HRP). This system was designed to assess the capability of Ebselen or its analogues to protect glutathione from the oxidant attack by the H_2O_2/HRP couple.

Figure 3 shows the EPR spectrum characteristic of the thiyl radical produced by the enzymatic system HRP/GSH/H_2O_2 in the presence of 100 mM DMPO. This four-line EPR spectrum (with a^N [coupling constant of nitrogen] = 15.4 G and a^H [coupling constant of hydrogen] = 16.2 G), was totally abolished when 10^{-4} M Ebselen was added to the reaction mixture (Fig. 3F). A similar inhibition was obtained for compounds [**3**] and [**1**], the latter compound being slightly less active (Fig. 3G,H). The selenide derivative [**2**] only induced 55% inhibition while the diselenide [**4**] was inactive with the same signal as the control possibly with a slight increase (Fig. 3E). In the absence of HRP, no EPR spectrum was

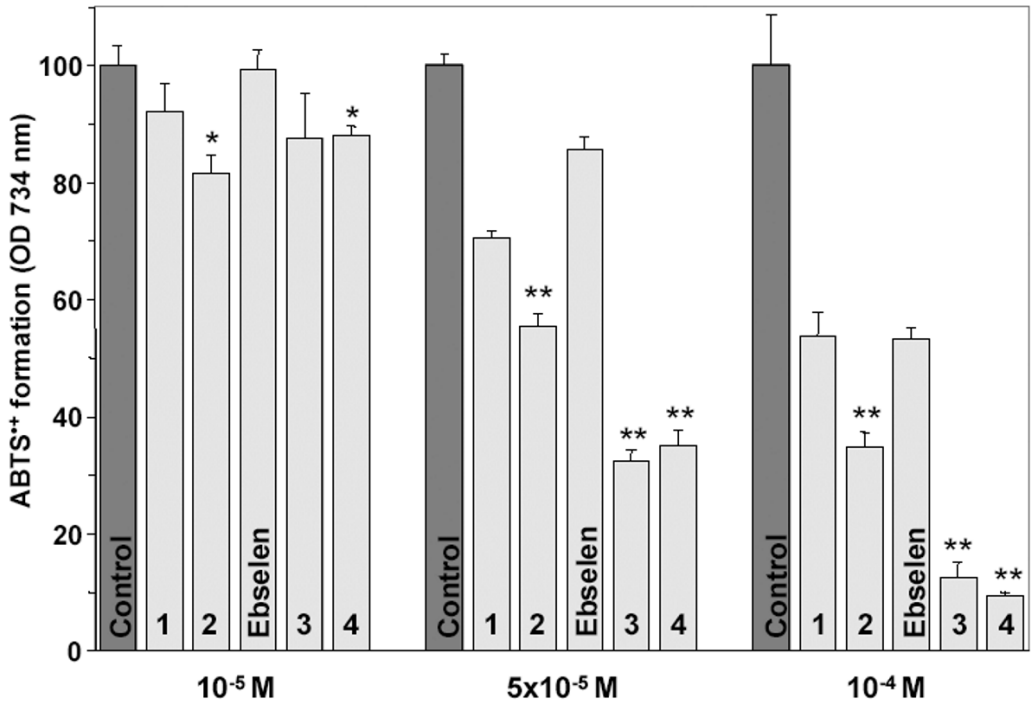


Fig. 2. $ABTS^{\bullet+}$ radical cation formation from the MetMb/H_2O_2/ABTS system in phosphate buffer at pH 7.4 and assessment of the antioxidant activity of selenides [1,2] and diselenides [3,4] compared to Ebselen. $ABTS^{\bullet+}$ radical cation formation was followed by the absorbance change at 734 nm. The results are expressed as mean ± SD with control value (no Ebselen or Ebselen analogue) taken as 100% (n = 4). *P < 0.01 versus Ebselen; **P < 0.001 versus Ebselen.

observed (Fig. 3C), and only a very weak EPR signal appeared in the absence of GSH (Fig. 3D). DMSO, used as a vehicle for the studied compounds, was without effect on the EPR spectrum (Fig. 3B). The efficacy of the compounds tested in this model can be summarized as follows: Ebselen = [**3**] > [**1**] > [**2**] > [**4**].

Discussion

Using four experimental models, we demonstrated that four Ebselen derivatives exhibited an antioxidant activity, but with variable efficiencies in relation to the experimental model. Two models were designed to investigate the GPx mimicking activity and antioxidant capability – the kinetic method of H_2O_2 reduction and the EPR study of thiyl radical formation. The kinetic method showed that the rate of the catalytic reduction of H_2O_2 was slightly accelerated by Ebselen (taken as control drug) or the selenide derivatives [**1,2**]. Interestingly, we found that selenide derivative [**1**] increased the rate of the catalytic reduction of H_2O_2 more than Ebselen. From the results obtained with this model, it can be stated that the enhancement of the catalytic reduction was linked to the presence of a selenium atom in the structure of the compound, but that the presence of selenium atom could not explain the variable effects of the tested compounds. According to the kinetic studies of Mugesh *et al*.,[8] the diselenides with quite strong Se–N intramolecular interactions did not show noticeable activity, whereas the diselenides with an in-built co-ordinating basic amino group but no Se–N interaction showed excellent activity. These authors also reported that the diselenides, in which the selenium atom was directly bonded to a redox-active group such as ferrocenyl, showed a dramatic increase in peroxidase activity. The presence of the tertiary amine function in the structure of the diselenide compounds would also play a pivotal role, because it activated the Se–Se bond and stabilized the resulting selenic acid intermediate against further oxidation.[6,7] We found that the compounds [**4**] and [**1**], which do not bear the tertiary amine function, behaved as good catalysts for the reduction of hydrogen peroxide and that compound [**3**] bearing a basic amino group with weak Se–N interaction and compound [**2**] without Se–N interaction also showed a good GPx activity compared to Ebselen. These results led us to use another model for studying the oxidation phenomenon of Ebselen and its analogues, based on a peroxidase-catalyzed oxidation system and using the EPR spin trapping methodology as previously described.[17] The use of H_2O_2 in this system allowed the formation of ferryl species from peroxidase.

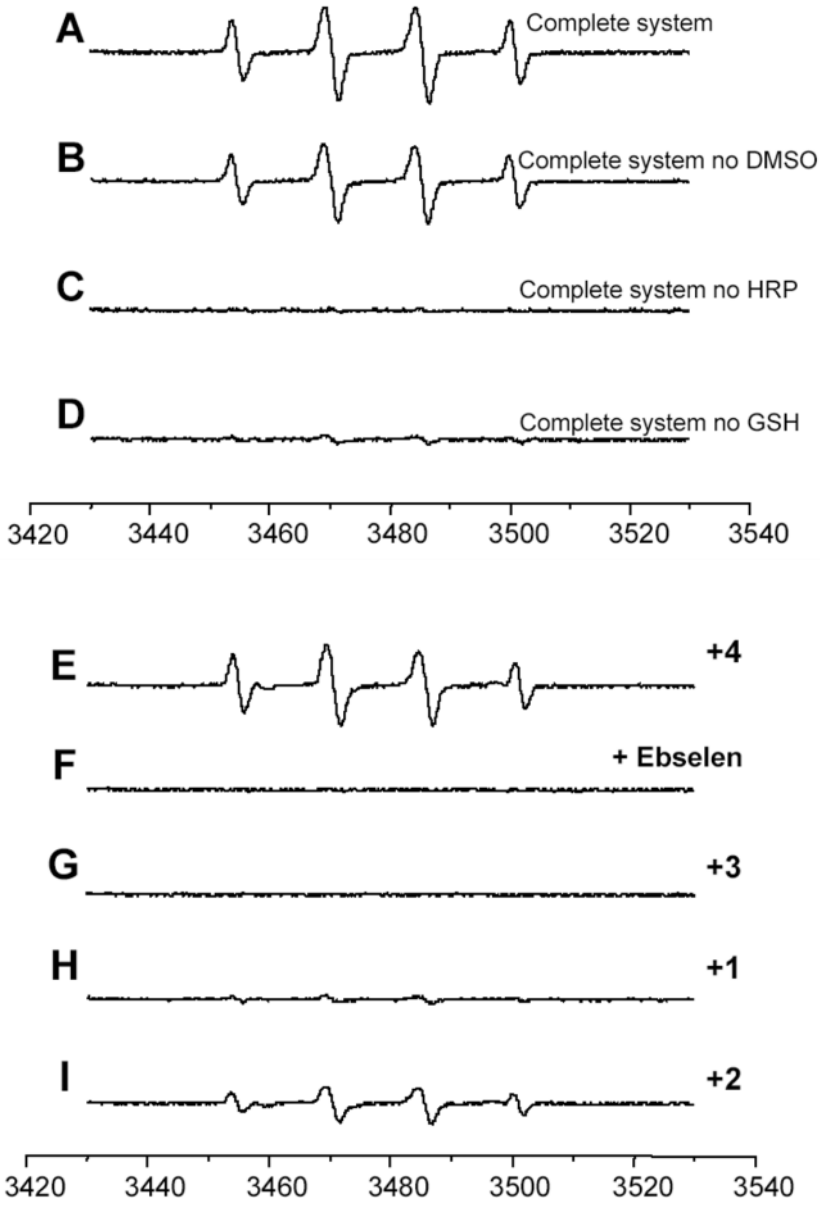


Fig. 3. Thiyl (GS$^{\bullet}$) radical produced by the enzymatic system (HRP/GSH/H_2O_2) and effects of synthetic analogues of Ebselen. (A) Complete system HRP/GSH/H_2O_2 + DMPO; (B–D) complete system without DMSO, HRP or GSH, respectively; E–I) same as (A) with compound [4], Ebselen, and compounds [3], [1] and [2], respectively. The results represent the average of three separate assays. Conversion time 40 ms, receiver gain 2×10^4 and number of scans 4.

This predictive model has been already extended to the study of oxidative phenomena occuring with antipsychotic drugs.[18,19] With this EPR method, we tested diselenide [**3,4**] and selenide [**1,2**] compounds and could monitor the ability of these compounds to prevent the formation of thiyl radical species (GS$^{\bullet}$) formed from the HRP/GSH/H_2O_2 system in the presence of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as spin trap. Only diselenide [**3**] and Ebselen prevented the generation of oxidized glutathione (GSSG) through its thiyl radical form. Both compounds completely abolished the formation of the GS$^{\bullet}$ radical, as illustrated by the disappearance of the signal area of the DMPO–SG adduct. Compounds [**1,2**] were less sensitive to the oxidation phenomenon. Compound [**4**] exerted no effect on thiyl radical formation because the intensity of the EPR spectrum remained identical to the control spectrum.

It is often evoked that the unique mechanism by which Ebselen exerts its antioxidant activity is linked to GPx-mimicking activity,[14] but other studies suggested that Ebselen may have radical scavenging properties. For instance, it was reported that Ebselen is a peroxynitrite

scavenger[20] and that this scavenging activity is linked to the capability of Ebselen to react with the peroxynitrite radical intermediates.[21] Our results on the lipid peroxidation model indicated that Ebselen had an antiradical effect less efficient than its analogues ([**3**] and [**4**]) and exerted its antioxidant capacity mainly by the GPx-mimicking activity because, by the kinetic assays and EPR, we found that Ebselen catalyzed the reduction of peroxide and inhibited the formation of oxidant species, decreasing so the attack of the substrate (*e.g.* thiol; Figs 2 and 3F,G). We also demonstrated that the Ebselen analogue [**3**] exhibited both profiles and should be used as a candidate for pharmacological investigation. Indeed, in the model of lipid peroxidation, the diselenide derivatives [**3,4**] showed the best inhibitory effect versus the reference drug Ebselen. To confirm the results obtained in the lipid peroxidation model, we evaluated the radical scavenging activity of Ebselen and that of its analogues, by monitoring the formation of the $ABTS^{\bullet+}$ radical cation by spectrophotometry,[22] in the presence or absence of the drug. The diselenides [**3,4**] inhibited, in a dose-dependent manner, the formation of the radical cation while the other compounds were less efficient, except compound [**2**] which displayed an inhibitory effect more pronounced than that of Ebselen and derivative [**1**]. These findings, taken together, indicated that Ebselen may exert its antioxidant activity not only by its GPx-mimicking activity, but also by the additional weak scavenging properties.

Conclusions

We tested four small and low-weight analogues of Ebselen and demonstrated that these compounds might have biological relevance, and that it should be interesting to use the diselenide derivative [**3**] as a pharmacological tool in cellular model studies. We also demonstrated that the main mechanism by which Ebselen exerted its antioxidant property is essentially as a GPx mimic, even if its anti-radical efficiency is weak.

Acknowledgements

This work was supported by the Fonds National de la Recherche Scientifique (FRSM grant No. 3.4542.00). We are grateful to Dr Messali Mouslim for providing us with the selenide derivatives.

References

1. Loschen G, Azzi A, Richter C, Flohe L. Superoxide radicals as precursors of mitochondrial hydrogen peroxide. *FEBS Lett* 1974; **42**: 68–72.
2. Ursini F, Maiorino M, Brigelius-Flohe R *et al*. Diversity of glutathione peroxidases. *Methods Enzymol* 1995; **252**: 38–53.
3. Galet V, Bernier JL, Hénichart JP *et al*. Benzoselenazolinone derivatives designed to be glutathione peroxidase mimetics feature inhibition of cyclooxygenase/5-lipoxygenase pathways and anti-inflammatory activity. *J Med Chem* 1994; **37**: 2903–2911.
4. Muller A, Cadenas E, Graf P, Sies H. A novel biologically active seleno-organic compound-I. Glutathione peroxidase-like activity *in vitro* and antioxidant capacity of PZ 51 (Ebselen). *Biochem Pharmacol* 1984; **33**: 3235–3239.
5. Schewe T. Molecular actions of Ebselen – an antiinflammatory antioxidant. *Gen Pharmacol* 1995; **26**: 1153–1169.
6. Wilson SR, Zucker PA, Huang R-RC, Spector A. Development of synthetic compounds with glutathione peroxidase activity. *J Am Chem Soc* 1989; **111**: 5936–5939.
7. Iwaoka M, Tomoda S. A model study on the effect of an amino group on the antioxidant activity of glutathione peroxidase. *J Am Chem Soc* 1994; **116**: 2557–2561.
8. Mugesh G, Panda A, Singh HB, Punekar NS, Butcher RJ. Diferrocenyl diselenides: excellent thiol peroxidase-like antioxidants. *Chem Commun* 1998; 2227–2228.
9. Mugesh G, Panda H, Kumar S, Apte S, Singh HB, Butcher RJ. Intermolecularly coordinated diorganyl ditellurides: thiol peroxidase-like antioxidant. *Organometallics* 2002; **21**: 884–892.
10. Aruomo OI. Scavenging of hypochlorous acid by carvediol and Ebselen *in vitro*. *Gen Pharmacol* 1997; **28**: 269–272.
11. Maulik N, Yoshida T, Das DK. Oxidative stress developed during the reperfusion of ischemic myocardium induces apoptosis. *Free Radic Biol Med* 1998; **24**: 869–875.
12. Dawson DA, Masayasu H, Graham DI, Macrac IM. The neuroprotective efficacy of Ebselen (a glutathione peroxidase mimic) on brain damage induced by transient focal cerebral ischemia in the rat. *Neurosci Lett* 1995; **185**: 65–79.
13. Namura S, Nagata I, Takami S, Masayasu H, Kikuchi H. Ebselen reduces cytochrome c release from mitochondria and subsequent DNA fragmentation after transient focal cerebral ischemia in mice. *Stroke* 2001; **32**: 1906–1911.
14. Maiorini M, Roveri A, Ursini F. Antioxidant effect of Ebselen (PZ 51): peroxidase mimetic activity on phospholipid and cholesterol hydroperoxides vs free radical scavenger activity. *Arch Biochem Biophys* 1992; **295**: 404–409.
15. Green MJ, Hill AO. Chemistry of dioxygen. *Methods Enzymol* 1984; **105**: 3–22.
16. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978; **52**: 302–310.
17. Liégeois JF, Mouithys-Mickalad A, Bruhwyler J *et al*. JL 13, a potential successor to clozapine, is less sensitive to oxidative phenomena. *Biochem Biophys Res Commun* 1997; **238**: 252–255.
18. Mouithys-Mickalad A, Kauffmann JM, Petit C *et al*. Electrooxidation potential as a tool in the early screening for new safer clozapine-like analogues. *J Med Chem* 2001; **44**: 769–776.
19. Kohara T, Koyama T, Fujimura M *et al*. Y-931, a novel atypical antipsychotic drug, is less sensitive to oxidative phenomena. *Chem Pharm Bull (Tokyo)* 2002; **50**: 818–821.
20. Daiber A, Zou MH, Bachschmid M, Ullrich V. Ebselen as a peroxynitrite scavenger *in vitro* and *ex vivo*. *Biochem Pharmacol* 2000; **59**: 153–160.
21. Squadrito GL, Pryor WA. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. *Free Radic Biol Med* 1998; **25**: 392–403.
22. Cano A, Alcaraz O, Acosta M, Arnao MB. On-line antioxidant activity determination: comparison of hydrophilic and lipophilic antioxidant activity using the $ABTS^{\bullet+}$ assay. *Redox Report* 2002; **7**: 103–109.