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In vitro glutathione peroxidase mimicry of ebselen is linked to its oxidation of critical thiols on key cerebral suphydryl proteins – A novel component of its GPx-mimic antioxidant mechanism emerging from its thiol-modulated toxicology and pharmacology

I.J. Kade [a,*], B.D. Balogun [a], J.B.T. Rocha [b]

[a] Department of Biochemistry, Federal University of Technology, Akure, Ondo State, Nigeria
[b] Programa Posgraduacao em Bioquimica Toxciologica, Centro de Ciencias Naturais e Exatas, Universidade Federal de Santa Maria, RS, Brazil

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ABSTRACT

The antioxidant mechanism of ebselen in rats brain is largely linked with its glutathione peroxidase (GPx) rather than its peroxiredoxin mimicry ability. However, the precise molecular dynamics between the GPx-mimicry of ebselen and thiol utilization is yet to be fully clarified and thus still open. Herein, we investigated the influence of dithiothreitol (DTT) on the antioxidant action of ebselen against oxidant-induced cerebral lipid peroxidation and deoxyribose degradation. Furthermore, the critical inhibitory concentrations of ebselen on the activities of sulphydryl enzymes such as cerebral sodium pump, δ-aminolevulinic acid dehydratase (δ-ALAD) and lactate dehydrogenase (LDH) were also investigated. We observe that ebselen (at ⩾42 μM) markedly inhibited lipid peroxidation in the presence and absence of DTT, whereas it inhibited deoxyribose degradation only in the presence of DTT. Furthermore, under *in vitro* conditions, ebselen inhibited the thiol containing enzymes; cerebral sodium pump (at ⩾40 μM), δ-ALAD (⩾10 μM) and LDH (⩾1 μM) which were either prevented or reversed by DTT. However, the inhibition of the activities of these sulphydryl proteins in diabetic animals was prevented by ebselen. Summarily, it is apparent that the effective *in vitro* inhibitory doses of ebselen on the activity of the sulphydryl proteins are far less than its antioxidant doses. In addition, the presence of DTT is evidently a critical requirement for ebselen to effect its antioxidant action against deoxyribose degeradation and not lipid peroxidation. Consequently, we conclude that ebselen possibly utilizes available thiols on sulphydryl proteins to effect its GPx mimicry antioxidant action against lipid peroxidation in rat brain homogenate.

1. Introduction

In the last few decades, concerted efforts have centered on the use of selenium based compounds in the prevention and management of oxidative stress-linked degenerative diseases. In this regard, synthetic organoseleniums have been of prime interest due to their promising pharmacological potentials. Ebselen (2-phenyl-1,2-benzioselenazol-3(2H)-one; Scheme 1), is a synthetic selenium-containing heterocycle that has been used experimentally and clinically with success in a variety of situations where free radicals are involved and is generally regarded as non-toxic [1,2]. Basically, antioxidant property of ebselen has been linked mainly to its glutathione peroxidase (GPx)- or thiol-peroxidase-like activity, suggesting that these compounds can decompose peroxides using either reduced glutathione or other thiols as electron donors ([3–6]; Scheme 2). In addition, literature data have also indicated that ebselen compound can be a substrate of mammalian thioredoxin reductase (TrxR) [6,7], which can be an efficient route for selenol formation and, consequently, peroxide degradation ([8,9]; Scheme 3). However, the exact contribution of each pathway to the antioxidant properties of ebselen is still unknown and thus still open [9]. Hypothetically, the operation of the two routes can be considered advantageous to the living cells, because the actual concentration of selenol is expected to be higher when the two pathways are active.

In the present study, our prime interest is specifically on the GPx mimicry of ebselen which is depicted in Scheme 2. This scheme is described as a catalytic cycle in which, the Se–N bond of ebselen is readily cleaved by thiols to produce the corresponding selenenyl sulfides, which upon reduction by excess thiols produces selenol. Ultimately, the selenol reacts with organic hydroperoxide to form ebselen via ebselen–selenic acid [10]. Hence, the main cytoprotective role of ebselen is related to its GPx-like activity that

* Corresponding author. Mobile: +234 703304 8800.
E-mail address: ijkade@yahoo.com (I.J. Kade).

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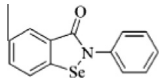
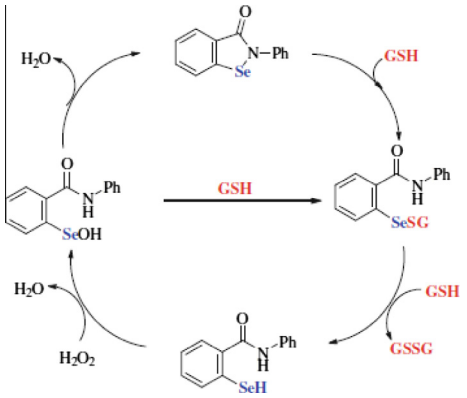
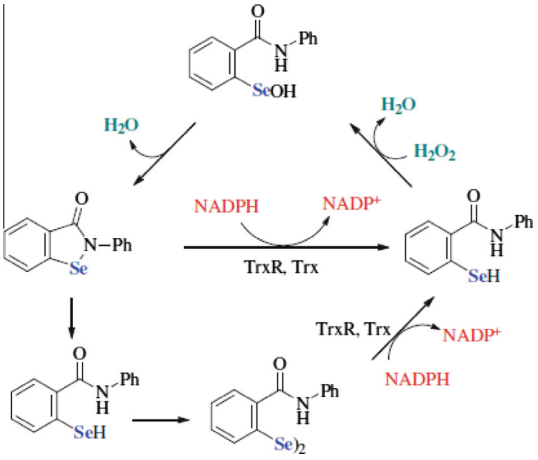


Scheme 1. Structure of ebselen.



Scheme 2. The mechanism of the catalytic reduction of hydroperoxides by ebselen as illustrated in Nogueira and Rocha [5].

inhibits the effects of membrane hydroperoxides, thus suggesting that the presence of thiols is a critical condition for the antioxidant action of this organoselenium [11–13]. Expectedly, this antioxidant mechanism of ebselen should hold sway under *in vitro* and *in vivo* systems involving its pharmacological testing. While the above speculation seems logical and have been variedly confirmed to be true under *in vitro* conditions [2,3,5,14,15], however experimental data from our laboratory and elsewhere have consistently shown that when ebselen was administered into animals either naïve or diseased models, the pharmacological action of ebselen was accompanied by an increase in the level of endogenous thiols in these animals [16,17]. Thus the dynamics between endogenous thiols and the pharmacological effects of ebselen is rather puzzling and may suggest that *in vitro* and *in vivo* reaction mechanisms of ebselen may differ considerably. Consequently, the antioxidant mechanism(s) of ebselen is rather a complex phenomenon and an enigmatic puzzle that is far from being completely understood. Unraveling this complex molecular drama has been the driving force in our laboratory especially in the understanding of the precise antioxidant chemistry of ebselen and other organoseleniums.



Scheme 3. The peroxiredoxin mimic activity of ebselen as exemplified by Nogueira and Rocha [2,5].

Recently, we attempted to protect deoxyribose sugar from Fenton derived ·OH assaults using ebselen. We observed that this organoselenium did not offer protection against ·OH-induced degradation of the pentose sugar but effectively inhibited lipid peroxidation induced by various neurotoxic and hepatotoxic agents. The latter observation is only in part confirmatory of earlier reported data [2,3,5,15]. More interestingly though is the fact that there is no available data resolving this intriguing, yet contrasting and puzzling observation in the literature. In any case, an elegant scientific explanation for the rationale behind the observed differential sensitivity of ebselen in its protection of lipids and deoxyribose sugar may provide an insight into the seemingly complex dynamics between the antioxidant action of ebselen and its influence on thiol redox systems. However, it is noteworthy that scientific approach(es) aimed at understanding the antioxidant chemistry of ebselen should as a necessity take into consideration its already established GPx mimicry and the ability of this organoselenium to serve as a substrate for TrxR. In this regard, taking into account our recent observation indicating that ebselen is not a substrate for the mammalian cerebral TrxR [9] thus showing that its main antioxidant mechanism in the brain may be its GPx mimicry and the fact that it inhibits cerebral lipid peroxidation but not deoxyribose degradation, we designed several experimental models with a view to gaining more insight into the molecular puzzles surrounding the pharmacological chemistry of ebselen vis-a-vis its strict dependence on thiols under *in vitro* conditions.

2. Materials and methods

2.1. Chemicals

Ebselen, dithiothreitol (DTT), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), and thiobarbituric acid (TBA), adenosine triphosphate (ATP), δ-aminolevulinic acid (δ-ALA), nicotinamide adenine dinucleotide (oxidized) (NAD^+) were obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade and were obtained from standard commercial suppliers.

2.2. Animals

Male adult Wistar rats (200–250 g) from our own breeding colony were used. Animals were kept in separate animal cages, on a 12-h light: 12-h dark cycle, at a room temperature of 22–24 °C, and with free access to food and water. The animals were used according to standard guidelines of the Committee on Care and Use of Experimental Animal Resources.

2.3. Preparation of tissue homogenate

Rats were decapitated under mild ether anesthesia and the brain was rapidly removed, placed on ice and weighed. Tissues were immediately homogenized in cold 50 mM Tris–HCl, pH 7.4 (1/10, w/v). The homogenate was centrifuged for 10 min at 4000*g* to yield a pellet that was discarded and a low-speed supernatant (S1). This was used for TBARS and enzyme assays. The amount of protein in S1 was carried out by the method of Lowry et al. [18].

2.4. Thiobarbituric acid reactive species (TBARS) assay

An aliquot of 100 μl of S1 was incubated at 37 °C in the presence of ebselen (1–167 μM) in the medium with and without DTT (2 mM) and/or Fe^{2+} (10 μM) or H_2O_2 (2 mM) for 30, 45 and 60 min. Productions of TBARS were determined as described by method of Ohkawa et al. [19] except that the acetate buffer for color

development had a pH of 3.4. The color reaction was developed by adding 300 μl 8.1% SDS to S1, followed by sequential addition of 500 μl acetic acid/HCl (pH 3.4) and 500 μl 0.8% of thiobarbituric acid (TBA). This mixture was incubated at 95 °C for 1 h. TBARS produced were measured at 532 nm and the absorbance was compared to that of a standard curve obtained using malondialdehyde (MDA).

2.5. Determination of thiol consumption in TBARS assay

The incubation system is essentially as described in Section 2.4 above. However, at the expiration of the incubation periods, 5% trichloroacetic acid was added and centrifuged to precipitate the proteins. The levels of thiols were estimated using Ellman's reagent following the method of Ellman [20].

2.6. Deoxyribose degradation

Deoxyribose degradation was determined as described in literature [21]. Deoxyribose is degraded by hydroxyl radicals with the release of thiobarbituric acid (TBA) reactive materials. Deoxyribose (3 mM) was incubated at 37 °C for 30 min with 50 mM potassium phosphate pH 7.4 plus ferrous sulphate (0.1 mM) and/or H_2O_2 (1 mM), ebselen (1–100 μM) and with or without DTT to induce deoxyribose degradation. After incubation, 400 μl of TBA 0.8% and 800 μl of TCA 2.8% were added, and the tubes were heated for 20 min at 100 °C and spectrophotometrically measured at 532 nm.

2.7. Determination of thiol consumption in deoxyribose degradation

The incubation system is essentially as described in Section 2.6. After incubation, the levels of thiols were estimated using Ellman's reagent following the method of Ellman [20].

2.8. Effect of ebselen on δ-ALA-D activity

δ-ALA-D activity was assayed according to the method of Sassa [22] by measuring the rate of product [porphobilinogen (PBG)] formation except that 100 mM potassium phosphate buffer and 2.5 mM δ-ALA were used. Incubation was carried out in the presence and absence of DTT. An aliquot of 100 μl of S1 was incubated for 1 h at 37 °C and the reaction product was determined using modified Erhlich's reagent at 555 nm.

2.9. Effect of ebselen on LDH activity

LDH activity was monitored spectrophotometrically by the rate of increase in absorbance at 340 nm at 35 °C resulting from formation of NADH. The assay medium contained 21 mM of glycine-KOH buffer (pH 10), 0.25 mM of NAD^+, ebselen (1–10 μM) and 15–20 μg of protein. The mixture was preincubated for 3 min, and the reaction was started by adding neutralized lactic acid (pH 6.8) to provide a final concentration of 50 μM. Incubation was carried out in the presence and absence of DTT. Values are expressed as change in absorbance/mg protein/min.

2.10. Effect of ebselen on Na^+/K^+-ATPase activity

The reaction mixture for Mg^{2+}-dependent-Na^+/K^+-ATPase activity assay contained 3 mM MgCl, 125 mM NaCl, 20 mM KCl and 50 mM Tris–HCl, pH 7.4 and 100–120 μg of protein, in a final volume of 500 μl. The reaction was initiated by addition of ATP to a final concentration of 3.0 mM. Controls were carried out under the same conditions with the addition of 0.1 mM ouabain. Na^+/K^+-ATPase activity was calculated by the difference between the two assays. Released inorganic phosphorous (Pi) was measured by the method of Fiske and Subbarow [23]. Incubation was carried out in the presence and absence of DTT. For all enzyme assays, incubation times and protein concentration were chosen to ensure the linearity of the reactions. All samples were run in duplicate. Controls with the addition of the enzyme preparation after mixing with trichloroacetic acid (TCA) were used to correct for nonenzymatic hydrolysis of substrates. Enzyme activity was expressed as nmol of phosphate (Pi) released min^{-1} mg $protein^{-1}$.

2.11. Thiol peroxidase activity

The catalytic effect of ebselen on the reduction of H_2O_2 was carried out in the presence of DTT. Free –SH groups were determined according to Ellman [20]. Ebselen at a concentration range of 50–150 μM were incubated in the medium containing DTT (2 mM) with and without H_2O_2 (2 mM) at 2.5 min intervals for 15 min. Aliquots of the reaction mixture (200 μl) were checked for the amount of DTT at the indicated time intervals.

2.12. Diabetes induction

Diabetes was induced by a single intravenous injection of STZ (50 mg/kg) previously diluted in 0.1 M citrate-buffer (pH 4.5) into adult male mice. Control rats received an equivalent amount of buffer. Diabetic state was checked 72 h after induction with STZ. Animals were considered diabetic when blood glucose level exceeded 250 mg/dL.

2.13. Treatment

The animals were divided randomly into the following groups: (group 1) control; (group 2) ebselen; (group 3) STZ; and (group 4) STZ + ebselen. Groups 2 and 4 were administered ebselen by gavage at a dose of 1 mg/kg (once/day) for 21 days after the administration of STZ. At the end of the experimental period, diabetic mice and the corresponding control animals were anesthetized with ether and euthanized by decapitation. Mice were fasted 12 h prior to euthanasia.

2.14. Ex vivo enzyme assay

Na^+/K^+-ATPase, δ-ALA-D and LDH activities were assayed as described above except that no preincubation was carried out.

2.15. Non-protein thiol assay

Non-protein thiols levels were estimated using Ellman's reagent after deproteinization of selected tissue with trichloroacetic acid (TCA) (5% in 1 mmol EDTA) by the method of Ellman [20].

2.16. Statistical analysis

All values obtained were expressed as mean ± SEM. The data were analyzed by appropriate ANOVA followed by Duncan's multiple range tests where appropriate and this is indicated in the text of results. The differences were considered significant when $p < 0.05$.

3. Results

3.1. General observation

We would like to state that for lipid peroxidation experiments, incubations were done at 30, 45 and 60 min and similar results were obtained. In fact, there were no statistical differences in the

results obtained for the different incubation times. In the results description below, we have simply described the data obtained for incubation time of 60 min.

3.2. Ebselen vs. Fe^{2+}-induced cerebral TBARS production vs. thiol utilization

Fig. 1 shows the antioxidant behavior of ebselen under basal and Fe^{2+}-induced cerebral lipid peroxidation in the absence and presence of the dithiol, DTT (panel a). In order to better explore the data obtained, separate statistical analysis were carried out. One-way ANOVA revealed that ebselen exerted marked inhibitory effect on the production of lipid peroxidation products from rats' brain irrespective of the incubation conditions. In fact, three way ANOVA [(with or without thiols) × (basal/Fe^{2+}-induced) × 5 concentrations of ebselen] yielded significant third order interactions ($p < 0.001$). The interaction is significant because ebselen exerted marked inhibitory effect on the lipid peroxidation process either in the presence or absence of the dithiol. Furthermore, it is of note that the antioxidant potency is independent of the different incubation times employed.

Analysis of the DTT utilization by ebselen showed that the organoselenium depleted the levels of DTT (Fig. 1 panel b) in a concentration dependent manner. This effect appears to be independent of the presence of Fe^{2+}. Three way ANOVA [(with or without thiols) × (basal/Fe^{2+}-induced) × 5 concentrations of ebselen] of the results obtained revealed a marked third order interaction ($p < 0.0005$). This interaction is significant because ebselen profoundly depleted the level of the thiols in a concentration dependent manner.

3.3. Ebselen vs. H_2O_2-induced cerebral TBARS production vs. thiol utilization

When H_2O_2 was used to induce lipid peroxidation, ebselen caused a concentration dependent inhibitory effect on the production of lipid peroxidation products in the rats' brain in a fashion similar to that observed when Fe^{2+} was used as prooxidant (Fig. 2 panel a). One-way and three-way ANOVA performed on the results obtained showed a strongly marked interaction because H_2O_2 markedly exacerbated the production of aldehydic products from cerebral lipids and ebselen markedly inhibited this effect. This inhibitory effect of ebselen is independent of the availability of the DTT whether in the basal or H_2O_2 induced lipid peroxidation. Consequently, we equally observed that the antioxidant effect of ebselen was accompanied by a concomitant reduction in the levels of DTT (Fig. 2 panel b).

3.4. Ebselen vs. free radical scavenging, Fe^{2+}-chelating and reducing properties

Fig. 3 shows the free radical scavenging property (panel a), Fe^{2+}-chelating (panel b) and reducing properties (panel c) of ebselen. As can be observed, ebselen neither exerted free radical scavenging ability nor Fe^{2+} chelating nor reducing properties.

3.5. Thiol peroxidase activity of ebselen

The oxidation of DTT by DPDS in the absence of peroxide was similar for control (no ebselen; data not shown for control); however, in the presence of hydrogen peroxide, the oxidation of DTT was significantly higher in the presence of 100 μM ebselen during all the sampling period (Fig. 4). Two-way ANOVA [(with and without H_2O_2) × (seven sampling times)] indicate that the rate of DTT oxidation oxidation in the absence of peroxide was similar for control (no ebselen. However, in the presence of hydrogen peroxide, the oxidation of GSH was significantly higher in the presence of ebselen during all the sampling periods.

3.6. Ebselen vs. Fe^{2+}/H_2O_2-induced deoxyribose degradation vs. thiol utilization

Fig. 5(a–d), shows the results obtained when deoxyribose sugar was assaulted by Fe^{2+} and/or H_2O_2 in the absence and presence of DTT. Apparently, ebselen alone did not exert any significant inhibitory effect on basal production of aldehyde products from degradation of deoxyribose sugar (Fig. 5 panel a). In the presence of DTT, however, ebselen showed a concentration dependent inhibition of TBARS production evoked by H_2O_2 (panel b), Fe^{2+} (panel c) and combination of H_2O_2/Fe^{2+} (panel d). In fact, separate three-way ANOVA [(with or without thiol) × (with or without Fe^{2+} or H_2O_2) × 5 concentrations of ebselen] yielded a significant third order interaction ($p < 0.0001$) because ebselen exhibited a marked concentration dependent inhibitory effect on the production of TBARS from deoxyribose degradation. In Fig. 6, the amount of DTT left during the deoxyribose assay was quantified for the various incubation conditions. The data shows that the levels of the thiols were markedly reduced and this effect is related to the ability of the ebselen to inhibit the Fenton process.

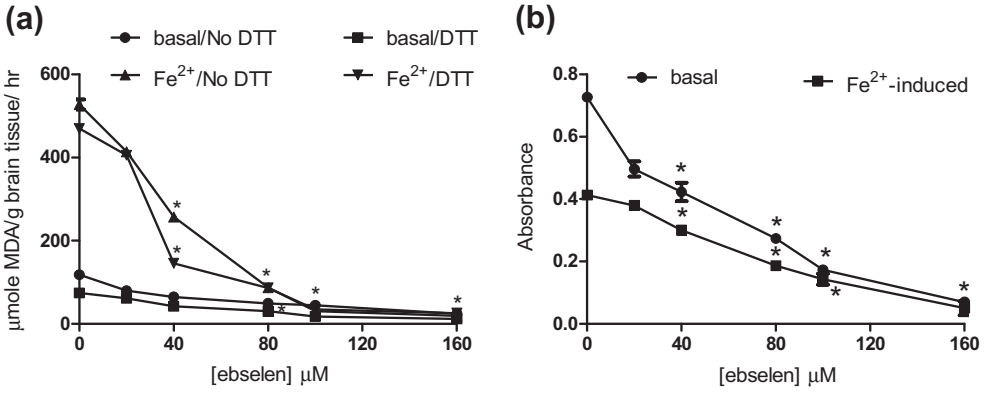


Fig. 1. (a) Effect of ebselen on lipid peroxidation in the presence and absence of Fe^{2+} with and without DTT. (b) Influence of ebselen on the oxidation of DTT in the presence and absence of Fe^{2+} in brain tissue homogenate. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

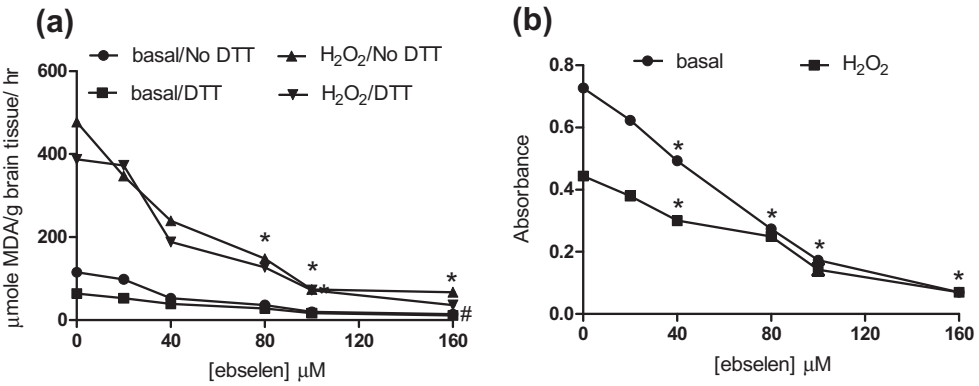


Fig. 2. (a) Effect of ebselen on lipid peroxidation in the presence and absence of H_2O_2 with and without DTT. (b) Effect of ebselen on the oxidation of DTT in the presence and absence of H_2O_2 in brain tissue homogenate. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

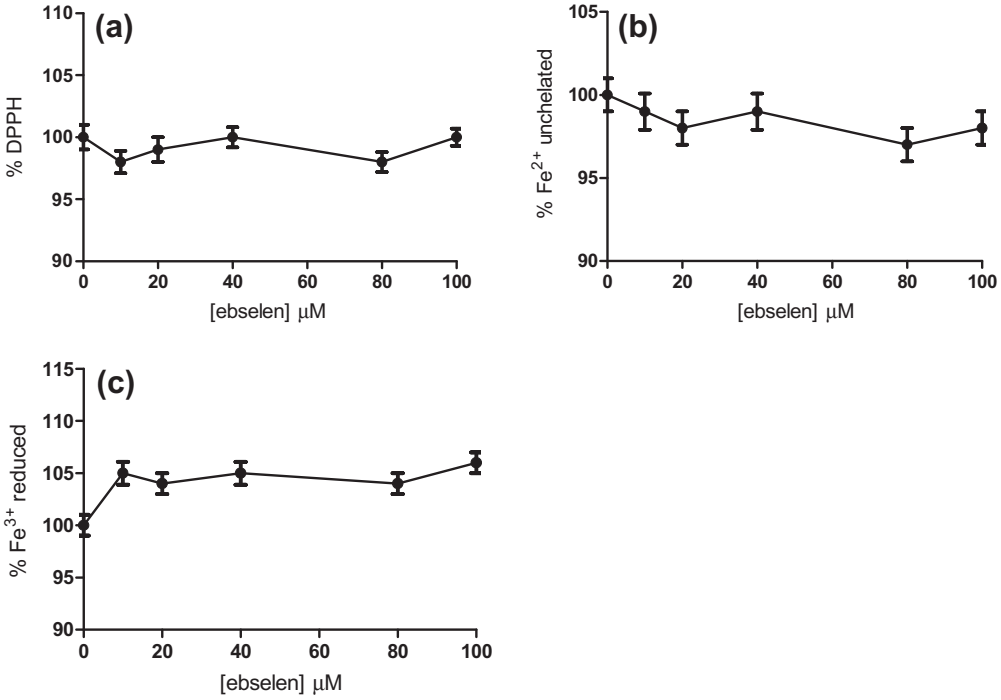


Fig. 3. (a) DPPH radical scavenging ability of ebselen. (b) Fe^{2+} chelating ability of ebselen. (c) Reducing property of ebselen. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test.

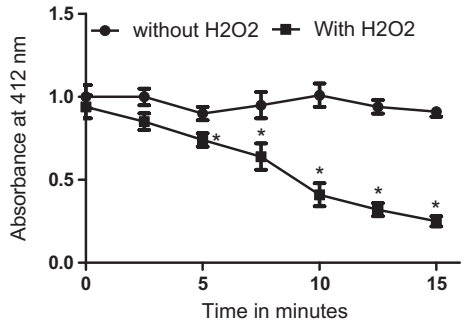


Fig. 4. GPx mimic of ebselen using DTT as the thiol source. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

3.7. Effect of ebselen on the activity of δ-ALAD

Fig. 7 shows that ebselen caused a concentration dependent inhibition on the activity of cerebral δ-ALAD. At 100 µM, the organoselenium completely inhibited the activity of the enzyme. Conversely, pre and post incubation of enzyme with DTT markedly abolished the inhibitory effect of ebselen on the activity of the enzyme ($p < 0.05$).

3.8. Effect of ebselen on the activity of LDH

Ebselen caused a significant concentration dependent inhibition on the activity of LDH which was significant at 1 µM (Fig. 8). DTT on the other hand prevented and reversed the inhibition caused by ebselen ($p < 0.05$).

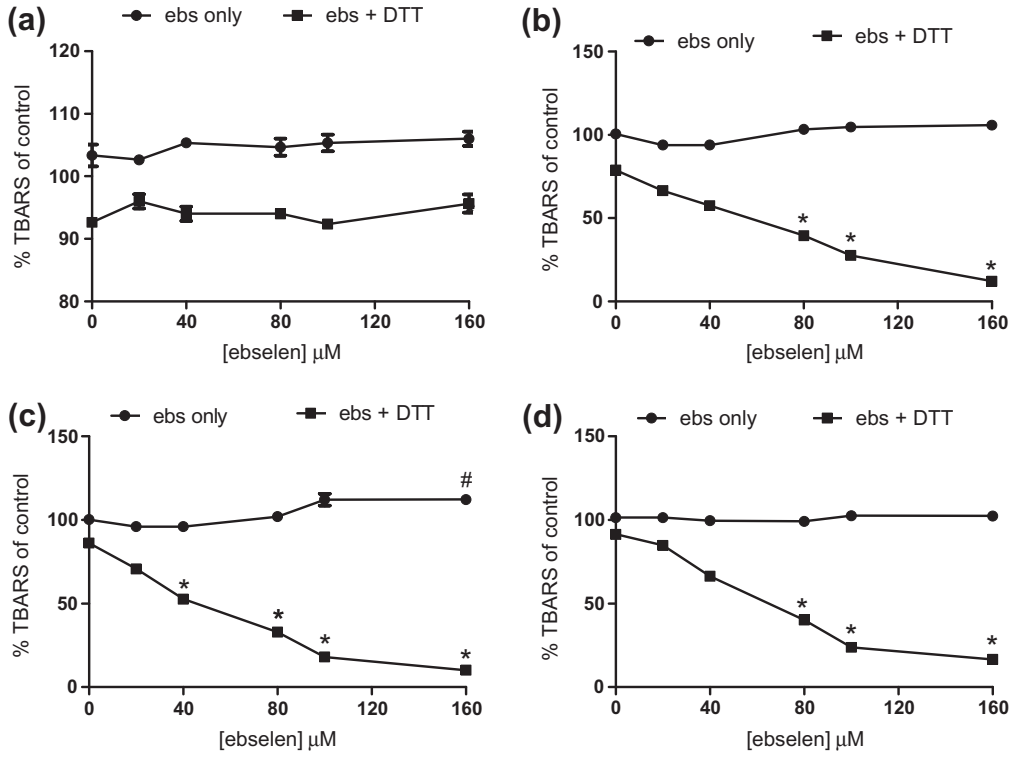


Fig. 5. Influence of the dithiol (DTT) on the inhibitory effect of ebselen on deoxyribose degradation: in the presence of ebselen alone (a); in the presence of Fe^{2+} alone (b); in the presence of H_2O_2 alone (c) and in the presence of both Fe^{2+} and H_2O_2. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

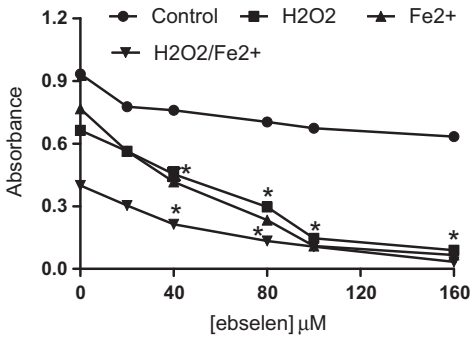


Fig. 6. Effect of ebselen on DTT oxidation in ·OH–induced deoxyribose degradation. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

3.9. Effect of ebselen on the activity of Na^+/K^+-ATPase

Fig. 9 shows that ebselen caused a marked inhibition on the activity of the cerebral sodium pump. On the other hand, pre and post treatment of enzyme with DTT markedly ($p < 0.05$) relieved the inhibition of the cerebral pump imposed by ebselen treatment.

3.10. Ex vivo effect of ebselen

Fig. 10 shows that in the brain of animals treated with ebselen oxidative stress caused a decrease in the level of thiol-containing enzymes tested, ebselen markedly restored the activity of these enzymes as demonstrated for cerebral Na^+/K^+-ATPase (panel a), LDH (panel b) and δ-ALA-D (panel c).

3.11. Effect of ebselen on non-protein thiols level in mice

The levels of non-protein thiols as expressed as GSH equivalent in both naïve and diabetic animals is presented in Fig. 11. The non-protein thiols levels in all the animals treated with ebselen were markedly higher ($p < 0.05$).

4. Discussion

One of the central antioxidant mechanisms of ebselen is centered on its GPx mimic (Scheme 2) and this has been attributed to its numerous pharmacological actions [2,3,5,14,15]. Thus the antioxidant action of ebselen whether *in vitro* or *in vivo* should be accompanied with oxidation of thiols. However, under *in vitro* assay conditions, exogenous thiols are often not components of the reacting systems involving ebselen antioxidant action, thus raising a complex puzzle. Herein, considering the inhibitory pattern of ebselen on Fe^{2+} and H_2O_2-induced cerebral lipid peroxidation, we observed that in the absence and presence of DTT, ebselen inhibited cerebral lipid peroxidation either in the basal or Fe^{2+} or H_2O_2-induced oxidative assaults with equal potency (Figs. 1a and 2a). As observed, ebselen inhibited lipid peroxidation in the absence of the dithiol, and thus we speculate that there is a possibility that the antioxidant action of ebselen is not strictly dependent on the availability of the dithiol. It may also imply that ebselen probably exhibits a GPx and non-GPx-mimic dual antioxidant mechanisms. The latter is further buttressed by our observations as presented in Figs. 1b and 2b where we observed that the utilization of DTT by ebselen under basal and prooxidant induced lipid peroxidation is similar suggesting a possible disconnect between the DTT oxidation and antioxidant action of ebselen. Conversely, the fact that ebselen neither exhibit radical scavenging

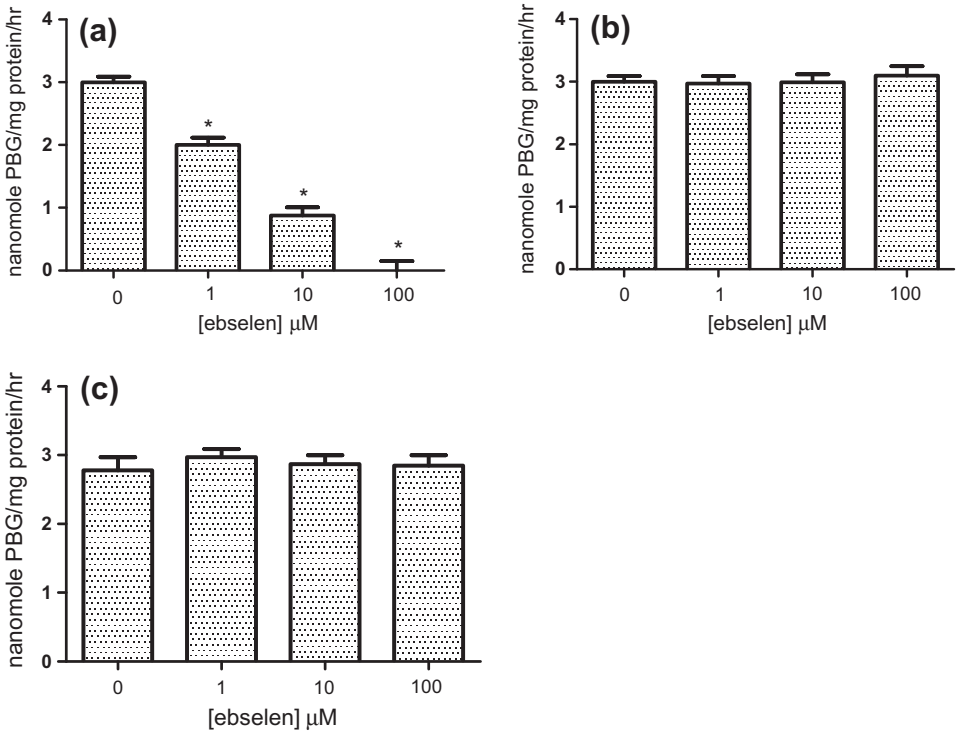


Fig. 7. Effect of ebselen on the activity of δ-aminolevulinic acid dehydratase. (a) Reaction was started by addition of δ-ALA after 10 min of pre-incubation in the presence ebselen. (b) Reaction was started by addition of δ-ALA after 10 min of preincubation in the presence of ebselen and DTT. (c) Reaction was started by addition of δ-ALA and DTT after 10 min of preincubation in the presence of ebselen. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

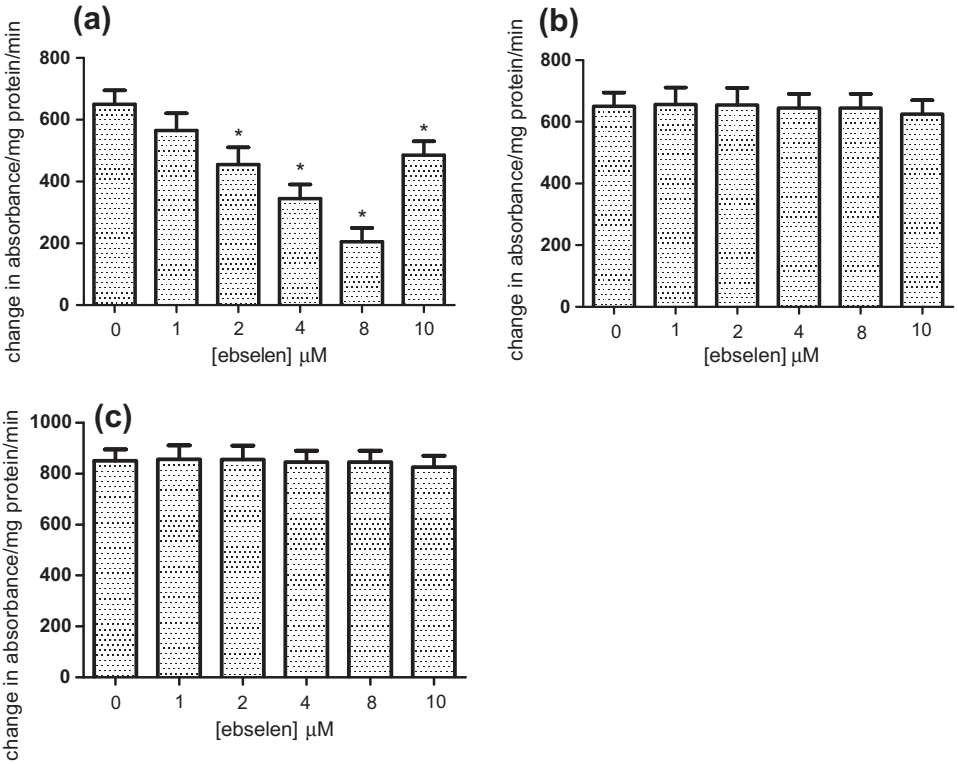


Fig. 8. Effect of ebselen on the activity of lactate dehydrogenase. (a) Reaction was started by addition of NAD^+ after 10 min of pre-incubation in the presence ebselen. (b) Reaction was started by addition of NAD^+ after 10 min of preincubation in the presence of ebselen and DTT. (c) Reaction was started by addition of NAD^+ and DTT after 10 min of preincubation in the presence of ebselen. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

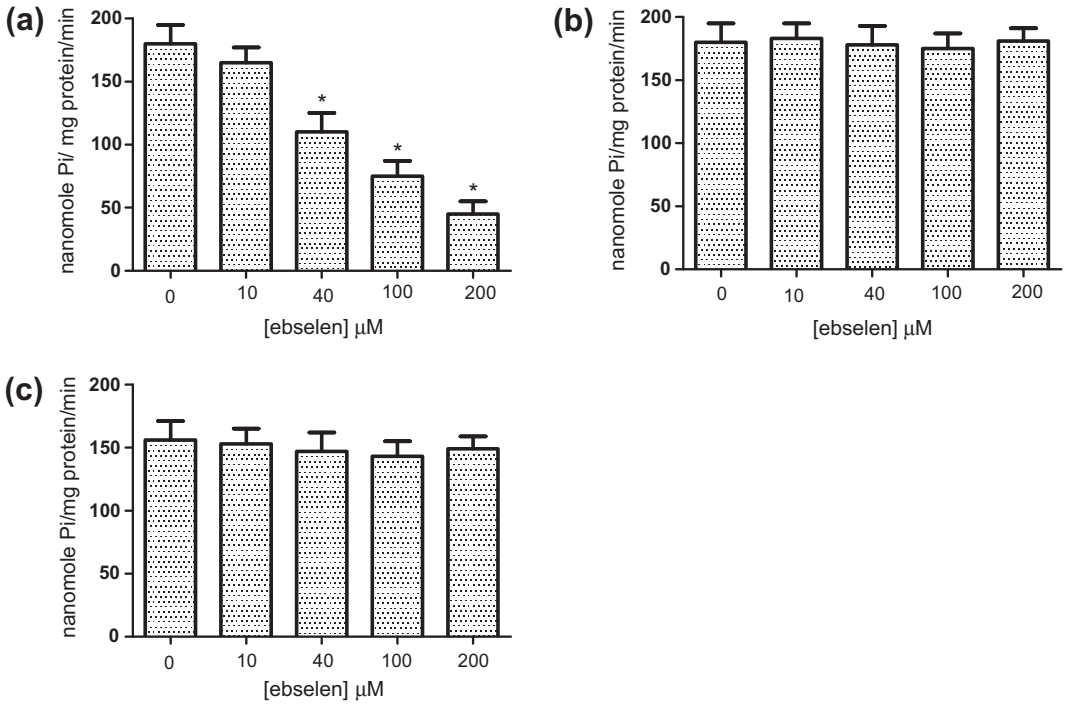


Fig. 9. Effect of ebselen on the activity of cerebral sodium pump. (a) Reaction was started by addition of ATP after 10 min of pre-incubation in the presence ebselen. (b) Reaction was started by addition of ATP after 10 min of preincubation in the presence of ebselen and DTT. (c) Reaction was started by addition of ATP and DTT after 10 min of preincubation in the presence of ebselen. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

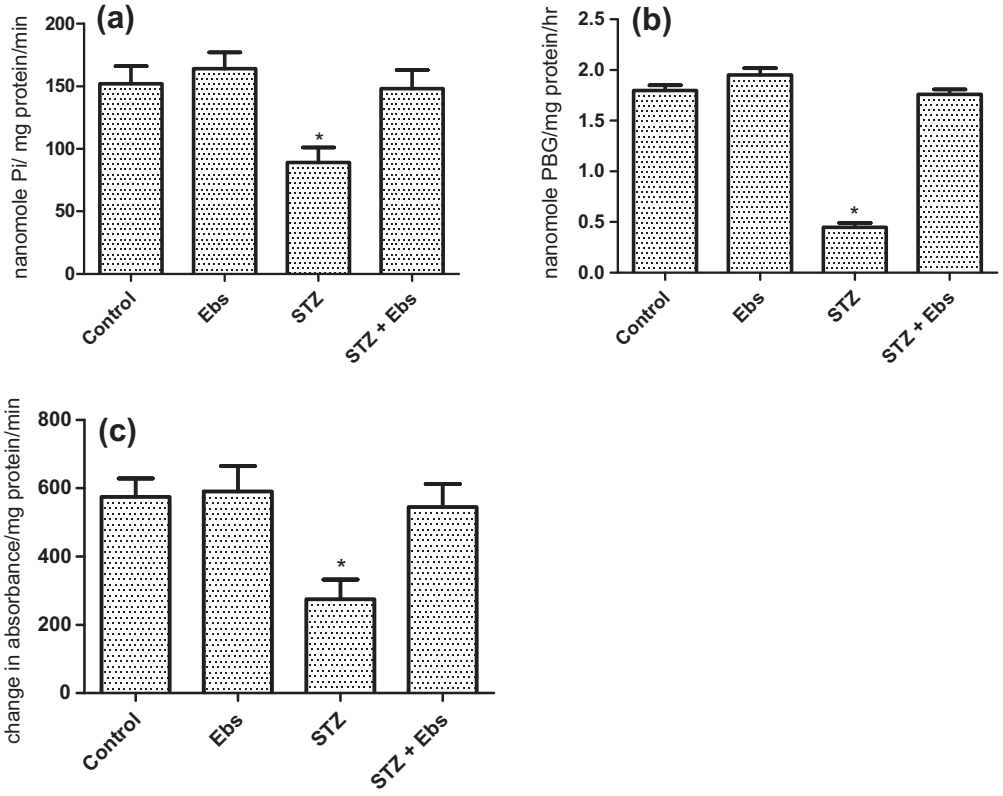


Fig. 10. Effect of ebselen on the activities of cerebral sodium pump (a); δ-aminolevulinic acid dehydratase (b); and lactate dehydrogenase (c) in streptozotocin-induced diabetic rats. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

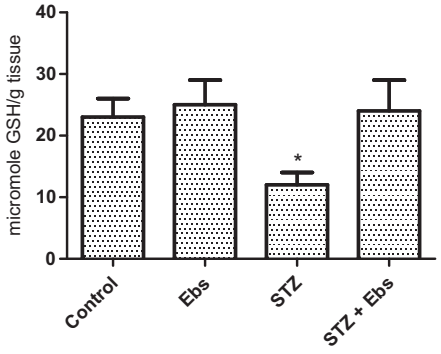


Fig. 11. GSH level in streptozotin-induced diabetic rats. Data show mean ± SEM values average from four independent experiments performed in quadruplicate in different days. Data are presented as mean ± SEM and post hoc comparisons were done by Duncan's multiple range test. *Indicate a significant difference in relation to the control ($p < 0.05$).

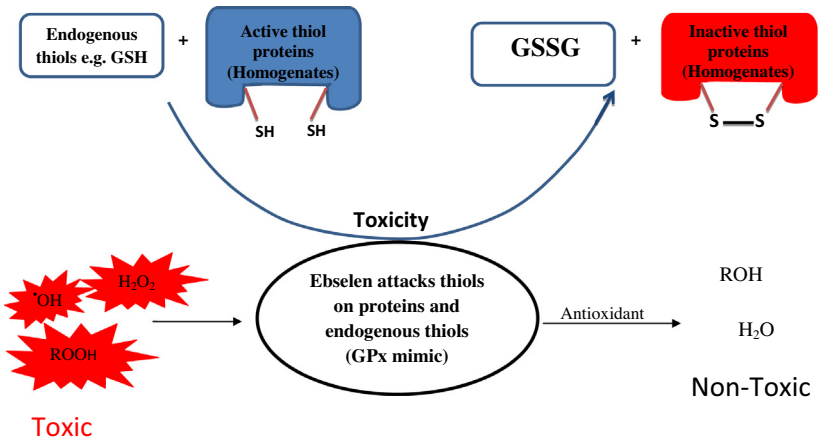
ability nor reducing nor Fe^{2+}-chelating properties (Fig. 3(a–c)) strongly suggest that the GPx mimic of ebselen (Fig. 4) is key to its antioxidant action. Aside lipid peroxidation, we also attempted to use ebselen to protect deoxyribose degradation mediated by hydroxyl radicals generated via Fenton reaction. Herein it is striking to observe that ebselen alone did not protect ·OH assault on deoxyribose sugar in the absence of DTT. On the other hand, it markedly inhibited ·OH assaults on the pentose sugar in the presence of the dithiol (Fig. 5c and d). The fact that this latter effect was accompanied by marked concomitant depletion of DTT in the reaction tubes (Fig. 6) indicate that the molecular events associated with the observed differential antioxidant sensitivity of ebselen in the presence and absence of DTT in models of Fenton reaction – derived ·OH assaults on cerebral lipids and deoxyribose sugar is rather an intriguing complex phenomenon. However, thus far and considering Figs. 5 and 6, we can reasonably and safely conclude that the antioxidant mechanism of ebselen is strictly based on its GPx mimic, since it appear that the inhibition of deoxyribose degradation elicited by ebselen is strictly dependent on the presence of DTT. However, the antioxidant action of ebselen even in the absence of DTT in lipid peroxidation (Figs. 1a and 2a) is in sharp contrast with Fig. 5, where deoxyribose sugar was not prostected in the absence of the thiol. With regards to this observation we speculate that free endogenous thiols derived from the tissue homogenates may play a role. In this case, considering the low level of these endogenous thiols [estimated value: 0.2 μmol GSH/in reaction tube; or 20 μmol GSH/g tissue], we speculate that the utilization of these thiols by ebselen must be associated with highly efficient thiol recycling system which is not supported by Scheme 2, thus making this possibility unlikely.

It is noteworthy however, that our group and others have shown that aside its classical GPx mimicry, ebselen can also be metabolized by TrxR to form selenol intermediates which possibly mediates its diverse pharmacological mechanisms [5–7,9]. In fact, ebselen have been shown to be good substrates of rat liver, Escherichia coli, human placenta and calf thymus TrxR [6]. Consequently, it has been strongly postulated that the TrxR-like (that is the ability of TrxR to form the selenol intermediate of ebselen) is more important than the glutathione peroxidase-like activity of ebselen [9,24]. Plausible as these findings may have contributed to our understanding of the antioxidant mechanisms of ebselen, more recent observation in our group have shown that ebselen is not a substrate for the rat brain TrxR [9]. Hence, it is apparent that ebselen action in the rat brain is strictly based on the classical GPx mimic (Scheme 2). Thus in the present study, we speculate that in assay conditions in which exogenous dithiol were not added, then the other possible source of thiols must necessarily be the proteins derived from the tissue homogenates. Our earlier data [25–27] which is also confirmed herein show that ebselen inhibited thiol containing proteins such as δ-ALA-D (Fig. 7), LDH (Fig. 8) and Na^+/K^+-ATPase (Fig. 9) and this fact is further confirmed by the fact that DTT prevented this inhibition. Therefore, we speculate that in the absence of exogenous thiols and under basal or prooxidant induced cerebral lipid assaults (Figs. 1a and 2a), the observed antioxidant action of ebselen is likely at the expense of free varieties of endogenous thiols and available free thiols on proteins and these thiols are strongly essential to the normal functioning of these sulphydryl enzymes. In consonant with our argument, earlier report have suggested that unlike the native GPx, the redox cycle of ebselen is not strictly dependent on GSH and as such ebselen is not a high-fidelity mimic of GPx and can alternatively utilize a range of structurally diverse thiol containing molecules [28] and in this instance, we speculate its utilization of free available thiols on sulphydryl proteins to mediate its generally observed antioxidant action under *in vitro* conditions in the tissue homogenates. This phenomenon in the case of enzymes eventually leads to the loss of activity by the proteins and a reduction of hydroperoxides.

Hence in order to explain the observed antioxidant effect of ebselen in Figs. 1a and 2a, we will substantiate our arguments on two premises mainly centering on the proteins as the sources of thiol for GPx-mimicry of ebselen action under *in vitro* conditions. First, there is a strong concentration overlap between the effective pharmacology (antioxidant) (42 μM) and toxicity (for δ-ALA-D, ebselen is markedly toxic at 10 μM; for LDH at 1 μM and for Na^+/K^+-ATPase at 40 μM) of ebselen, suggesting that ebselen possibly mediates its antioxidant action via its toxicity, with the toxicological effect preceeding its antioxidant effect. Furthermore, as earlier mentioned, under basal conditions, the thiol consumption of ebselen is similar to that when Fe^{2+} and H_2O_2 are employed to induce lipid peroxidation (Figs. 1b and 2b). Based on the report of Borges et al. [27], and our present data which in part is confirmatory, we logically conclude that in the absence of supplied exogenous thiols, when ebselen was added to the basal tissue homogenates, it attacks thiols of importance on biological proteins thus expressing its toxicity, and the introduction of exogenous thiols only serve to relieve this inhibitory effect of ebselen on the proteins. Consequently, the observed depletion of DTT in the basal homogenate indicates that this dithiol was probably oxidized in an attempt to salvage sulphydryl biological proteins previously inactivated by ebselen (Figs. 7–9). Consequently, guided by our present data, we hypothesize that the celebrated *in vitro* antioxidant effect of ebselen in brain homogenate is a pseudo-effect and highly misleading and consequently as depicted in Scheme 4, we propose for the first time a likely link between thiol mediated *in vitro* toxicity and pharmacology of ebselen in brain homogenate.

The proposition of Scheme 4 may naturally pose a worry since ebselen is already undergoing clinical trials and has exhibited profound pharmacological promise in adverse neurological situations. However reports [16,17] and current data (Figs. 10 and 11) have shown that administration of ebselen into animals is always associated with an elevated amount of thiols strongly suggesting that the antioxidant mechanism of ebselen under *in vivo* conditions is probably not associated with its generally accepted classical GPx mimic. Consequently, we further speculate that there is an apparent disconnect between the observed antioxidant mechanisms of ebselen under *in vitro* and *in vivo* conditions. Apparently, the initial testing and eventual use of ebselen appear to have been a case of "good chance" since its observed *in vitro* action which was the likely premise for its further testing *in vivo* was erroneous and disingenuous.

Therefore, this report opens a challenge to experts in organoseleniums research to devise a holistic methodology for the evaluation of organoseleniums for their well observed *in vitro* antioxidant effects that is translatable *in vivo*. In our opinion, this would require



Scheme 4. Proposed *in vitro* ambivalent model for the antioxidant action of ebselen mediated by its toxicity.

some molecular approaches that will take into consideration the delicate balance between their toxicology and pharmacology. Another approach is to sensitize experts in the field of organoselenium synthesis to devise a critical approach in the rational designs of organoseleniums to incorporate conditions for their sensitivity towards dangerous biological oxidants and less sensitive to thiols. Again since there is possible discrepancies between the *in vitro* and *in vivo* data associated with ebselen's action, we hypothesize that under *in vivo* conditions, there is likely existence of intermediate metabolic product(s) of this compound that possess potent antioxidant property and with diverse antioxidant mechanisms which will definitely exclude the GPx mimic. Another possibility is that ebselen or its metabolic products possibly up-regulated the pathways of thiols biosynthesis under *in vivo* conditions. Consequently, identification of these metabolites may open a new exciting field in the rational design of organoselenium compounds that are potent candidates in the management of oxidative stress related diseases.

Conflict of interest statement

There are no conflicting interest.

Acknowledgements

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