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THE PROTECTIVE EFFECTS OF SELENOORGANIC COMPOUNDS AGAINST PEROXYNITRITE-INDUCED CHANGES IN PLASMA PROTEINS AND LIPIDS

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Abstract: Many selenoorganic compounds play an important role in biochemical processes and act as antioxidants, enzyme inhibitors or drugs. The effects of a new selenocompound – bis(2-aminophenyl)-diselenide on oxidative/nitrative changes in human plasma proteins induced by peroxynitrite ($ONOO^-$) were studied *in vitro* and compared with the those of ebselen, a well-known antioxidant. We also studied the role of the tested selenocompounds in peroxynitrite-induced plasma lipid peroxidation. Exposure of the plasma to peroxynitrite (0.1 mM) resulted in an increase in the level of carbonyl groups and nitrotyrosine residues in plasma proteins (estimated using the ELISA method and Western blot analysis). In the presence of different concentrations (0.025-0.1 mM) of the tested selenocompounds, 0.1 mM peroxynitrite caused a distinct decrease in the level of carbonyl group formation and tyrosine nitration in plasma proteins. Moreover, these selenocompounds also inhibited plasma lipid peroxidation induced by $ONOO^-$ (0.1 mM). The obtained results indicate that *in vitro* bis(2-aminophenyl)-diselenide and ebselen have very similar protective effects against peroxynitrite-induced oxidative/nitrative damage to human plasma proteins and lipids.

Key words: Peroxynitrite, Tyrosine nitration, Carbonyl groups, Selenium, Ebselen

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Abbreviations used: DMSO – dimethylsufoxide; DNPH – dinitrophenylhydrazine; $ONOO^-$ - peroxynitrite, TBARS – thiobarbituric acid reactive substance.

INTRODUCTION

Selenium (Se), an essential trace element, is available in organic and inorganic (selenite, selenate) forms [1]. The selenoorganic compound selenocysteine is an integral component of several enzymes: glutathione peroxidases (GSH-Px), iodothyronine deiodinases (ID) and thioredoxin reductase (TrxR) [2-4]. Selenoorganic compounds are much less toxic than inorganic selenium compounds. The synthetic selenoorganic compounds are potential therapeutic and chemopreventive agents and may display a protective action against a broad spectrum of toxic substances, including different oxidants [5]. Selenocompounds, particularly in organic form, modulate platelet function and metabolism [6]. They may act as antiplatelet agents and play an important role in reducing the risk of many vascular diseases associated with blood platelet activation [7]. Among the selenoorganic compounds with anti-inflammatory properties, a well-known antioxidative agent called ebselen (2-phenyl-1,2-benzoisoselenazol-3(2H)-one) has been intensively studied over the last decade [5]. Ebselen is non-toxic organoselenium compound possessing therapeutic properties: it can be used effectively in the treatment of several diseases [5, 6]. Ebselen exerts an antiplatelet effect mainly due to its inhibition of platelet aggregation and the expression of P-selectin on platelet surface [7]. The pharmacological action of ebselen appears to be due to its unique antioxidative properties of scavenging organic hydroperoxides and peroxynitrite and mimicking the activities of glutathione peroxidase (an enzyme with selenium in form of selenocysteine as an integral component) [8]. Ebselen, like selenomethionine, is effective at protecting several model compounds against peroxynitrite-induced oxidation or nitration [9].

Peroxynitrite ($ONOO^-$) is a highly reactive species, but not a radical, produced *in vivo* by the reaction between the superoxide anion ($O_2^{\bullet -}$) and nitric oxide ($^{\bullet}NO$) [10, 11]. It is a physiologically relevant oxidant involved in the pathophysiology of various diseases. A variety of cells, including endothelial cells, neutrophils and activated macrophages, are the major sources of peroxynitrite. Several authors reported that $ONOO^-$ is capable of oxidizing a wide variety of biomolecules, including proteins, lipids and DNA [12, 13], and it has been implicated in several inflammatory disorders and atherosclerosis [14]. The exposure of proteins to $ONOO^-$ results in the oxidation of cysteine, methionine and tryptophane, the nitration of tyrosine, the formation of dityrosine, the fragmentation of molecules, and the formation of carbonyl groups [11, 15, 16]. Our earlier results showed that exposure of human plasma or blood platelets to peroxynitrite causes the oxidation and nitration of various proteins and the oxidation of lipids [15, 16], and that the resveratrol present in dietary sources and supplemented in the human diet, like other antioxidants (uric acid and deferoxamine), may suppress the toxic action of $ONOO^-$ on human plasma [16] or blood platelets [15].

The aim of this study was to determine the antioxidant activity of ebselen *in vitro* in terms of its ability to protect plasma proteins against peroxynitrite-promoted oxidation and nitration, and to compare this with the effects of bis(2-aminophenyl)-diselenide, a novel selenoorganic analogue of ebselen (Fig. 1). In our study, the formation of carbonyl groups was used as a marker of plasma protein oxidation. The nitration of plasma proteins induced by peroxynitrite was monitored via the measurment of the level of nitrotyrosine. The purpose of our study was also to assess the *in vitro* effects of selenocompounds on peroxynitrite-induced peroxidation of plasma lipids.

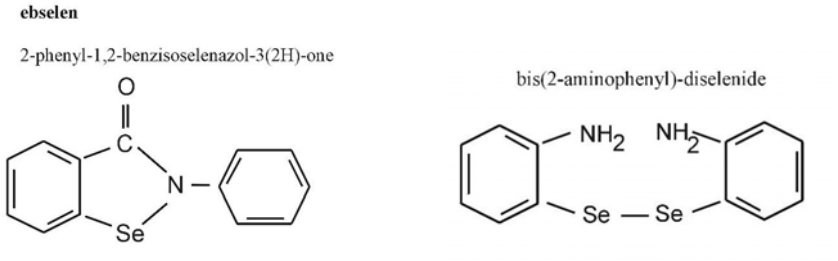

Fig. 1. The chemical structures of ebselen and bis(2-aminophenyl)-diselenide.

MATERIALS AND METHODS

Materials

Peroxynitrite was synthesized according to the method of Pryor and Squadrito [11]. Freeze fractionation (-70ºC) of the peroxynitrite solution formed a yellow top layer, which was retained for further assessment. The top layer typically contained 80-100 mM peroxynitrite as determined spectrophotometrically at 302 nm in 0.1 M NaOH (ε_{302nm} = 1679 M^{-1} cm^{-1}). Some experiments were also performed with decomposed $ONOO^-$, which was prepared by allowing the $ONOO^-$ to decompose at neutral pH (7.4) in 100 mM potassium phosphate buffer (15 min, room temperature). Ebselen, rabbit anti-DNP antibodies, anti-rabbit antibodies and anti-goat/sheep antibodies coupled with peroxidase were purchased from Sigma (St Louis, MO). Sheep anti-nitrotyrosine polyclonal antibodies were from Oxis (Portland, USA). Biotynylated anti-goat/mouse/rabbit antibody and streptavidin-biotynylated horseradish peroxidase were from DAKO (Glostrup, Denmark). Bis(2-aminophenyl)-diselenide was synthesized in the Department of Organic Chemistry, Biochemistry and Biotechnology, Wroclaw University of Technology (Wroclaw, Poland) and was donated by Prof. J. Mlochowski. Stock solutions of ebselen and bis(2-aminophenyl)-diselenide were made in 50% dimethylosuloxide (DMSO) at a concentration of 10 mM (the final concentration of DMSO in the samples was less than 0.5%) and kept frozen. All the other reagents were of analytical grade and were provided by commercial suppliers.

Incubation of plasma with peroxynitrite and antioxidants

Human blood from healthy volunteers was collected into sodium citrate (5 mmol/l final concentration) and immediately centrifuged (3000 × g, 15 min) to obtain plasma. Samples of human plasma were pre-incubated (2 min at room temperature) with ebselen or bis(2-aminophenyl)-diselenide at final concentrations of 0.025-0.1 mM and were then treated with $ONOO^-$ (at a final concentration of 0.1 mM). The reaction was performed by placing a small aliquot of peroxynitrite (5-6 μl) in the side of a tube containing the human plasma, immediately followed by vigorous vortexing. As DMSO has some antioxidant properties, the effect of the vehicle (solvent of the drug) was also assessed.

Determination of nitrotyrosine content in the human plasma proteins via the C-ELISA method

Detection of nitrotyrosine-containing proteins via a competition ELISA (C-ELISA) method in samples of plasma (treated with $ONOO^-$ alone or with selenium compounds and $ONOO^-$) was performed according to the procedure of Khan *et al.* [17] as described previously [15]. The nitro-fibrinogen (at a concentration of 0.5 μg/ml and 3-6 mol nitrotyrosine/mol protein) was prepared for use in the standard curve. The linearity of the C-ELISA method was confirmed by the construction of a standard curve ranging form 10 to 500 nM nitrotyrosine-fibrinogen equivalent. The concentrations of nitrated proteins that inhibit anti-nitrotyrosine antibody binding were estimated from the standard curve and are expressed as nitro-Fg equivalents. The amount of nitrotyrosine present in fibrinogen after treatment with peroxynitrite (at a final concentration of 1 mM) was determined spectrophotometrically (at pH 11.5, ε_{430nm} = 4400 M^{-1} cm^{-1}) [17].

Carbonyl group detection in human plasma proteins by the ELISA method

Detection of carbonyl groups via the ELISA method (using anti-DNP antiobodies) in plasma (treated with $ONOO^-$ alone or with selenium compounds and $ONOO^-$) was carried out according to a method described by Buss *et al.* [18]. Human plasma proteins reacted with dinitrotrophenylhydrazine (DNPH), and then proteins were non-specifically adsorbed onto an ELISA plate. The peroxynitrite treated-fibrinogen (10 nmol of carbonyl groups/mg of fibrinogen) was prepared for use in the standard curve. The linearity of the ELISA method was confirmed by the construction of a standard curve ranging from 0.1 to 10 nmol carbonyl groups/mg of fibrinogen. The amount of carbonyl groups present in fibrinogen after treatment with peroxynitrite (at a final concentration of 1 mM) was determined spectrophotometrically as described by Levine [19].

Production of thiobarbituric acid reactive substances (TBARS) in human plasma

Incubation of plasma samples (treated with $ONOO^-$ alone or with selenium compounds and $ONOO^-$) was stopped by cooling the samples in an ice-bath.

Samples of plasma were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 M HCl and centrifuged at 1200 × g for 15 min. One volume of clear supernatant was mixed with a 0.2 volume of 0.12 M thiobarbituric acid in 0.26 M Tris at pH 7.0 and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured, and the results were expressed as nmoles of TBARS [20].

Data analysis

In order to eliminate uncertain data, the Q-Dixon test was performed. All the values in this study were expressed as means ± SD. The statistically significant differences were assessed by applying the paired Student's t-test.

RESULTS AND DISCUSSION

Peroxynitrite may evoke oxidative/nitrative stress in various cells or tissues. It can induce undesirable effects in biological systems and cause damage to different molecules, including lipids and proteins. Human plasma is rich in proteins and lipids and also contains potential sites for radical formation and destruction. Oxidative damage to plasma proteins mediated by peroxynitrite causes alterations in plasma protein functions. The oxidation of fibrinogen induced by $ONOO^-$ causes changes in its clotting activity [21]. Fibrinogen, ceruloplasmin, transferrin, and plasminogen were identified as nitrated proteins in human plasma from smokers and lung cancer patients [22].

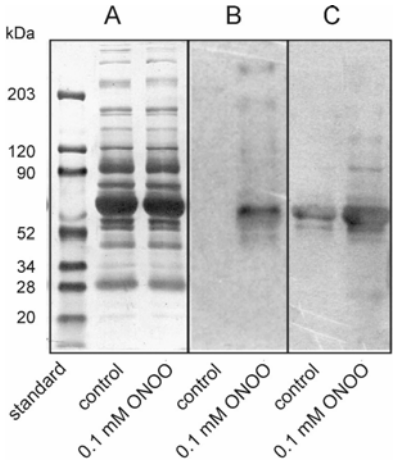

Fig. 2. The effect of peroxynitrite (0.1 mM) on the nitration and oxidation of human plasma proteins. Samples were subjected to SDS-PAGE (5-15%) in the presence of 2-mercaptoethanol and electroblotted to PVDF membranes. A - Whole plasma proteins stained by Amido Black, B - Western blot analysis with polyclonal antinitrotyrosine antibodies, C - Detection of carbonyl residues by Western blot analysis with anti-DNPH antibodies. The results are representative of three independent experiments.

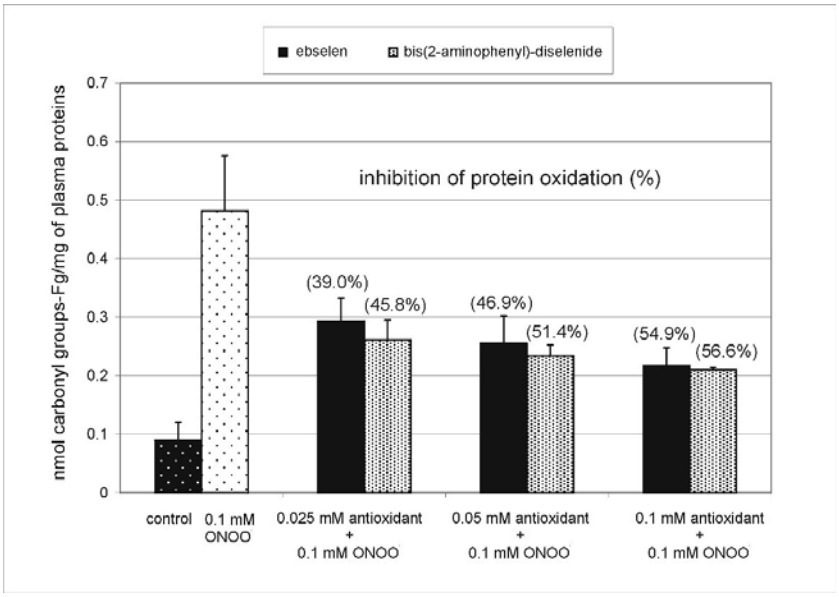

Fig. 3. The effects of ebselen and bis(2-aminophenyl)-diselenide (0.025-0.1 mM, 2 min, 37ºC) on protein oxidation in plasma treated with $ONOO^-$ (0.1 mM, 2 min, 37ºC). The protein oxidation was measured immunologically using the ELISA method. The results are expressed as nmol carbonyl groups-Fg/mg of plasma proteins. The results are representative of six independent experiments, and are expressed as the means ± SD. The effects were statistically significant (Student's t-test; selenium compounds + $ONOO^-$ (0.1 mM) treated plasma *versus* $ONOO^-$ (0.1 mM) treated plasma, $p < 0.05$).

Our studies showed that the incubation of plasma with peroxynitrite (0.1 mM) resulted in an increase in carbonyl groups in plasma proteins, as determined via Western blot analysis (Fig. 2) and the ELISA method (Fig. 3). Two tested selenium compounds diminished the level of protein oxidation induced by $ONOO^-$ (Fig. 3). In the presence of ebselen or bis(2-aminophenyl)-diselenide at a concentration of 0.1 mM, the inhibition of protein oxidation, determined as the level of carbonyl group formation, was about 40% (measured using the ELISA method) (Fig. 3). The effect of the vehicle (0.5% DMSO) under these conditions was negligible.

We demonstrated that the exposure of plasma to peroxynitrite (0.1 mM) resulted in an increase in the amount of nitrotyrosine in the plasma proteins, as determined by a competition C-ELISA method (Fig. 4) and Western blot analysis (Fig. 2). Selenium compounds distinctly diminished tyrosine nitration in plasma proteins (Fig. 4). The used concentration of DMSO (0.5%) had no effect on the level of nitrotyrosine.

The incubation of human plasma with peroxynitrite at a concentration of 0.1 mM resulted in a distinct increase in TBARS (Fig. 5). After 2 min incubation of plasma with $ONOO^-$ (0.1 mM), the amount of TBARS increased to about 125% (Fig. 5). The presence of selenium compounds only slightly protected the plasma

lipids against oxidation induced by $ONOO^-$ (Fig. 5). Ebselen and bis(2-aminophenyl)-diselenide at a concentration of 0.1 mM had very similar protective effects (inhibition about 25%) (Fig. 5).

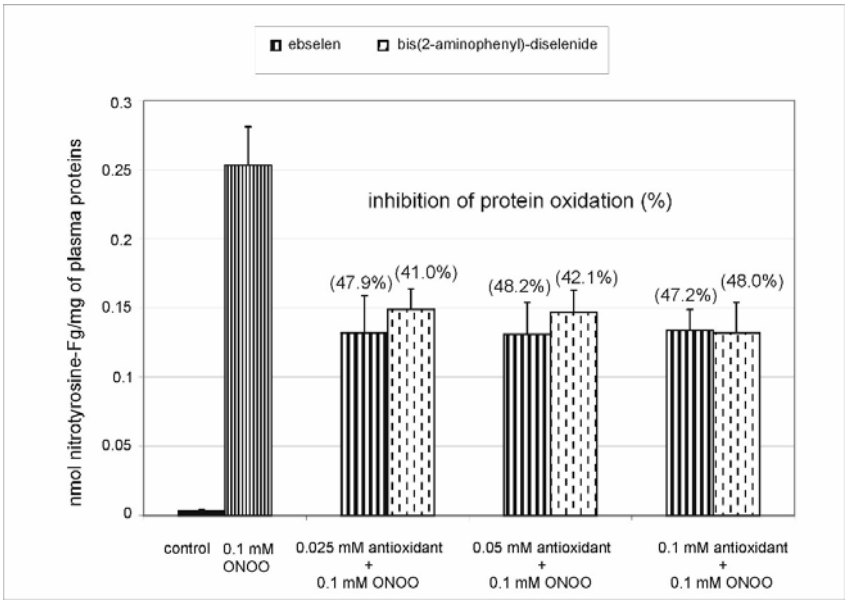

Fig. 4. The effects of ebselen and bis(2-aminophenyl)-diselenide (0.025-0.1 mM, 2min, 37ºC) on the nitration of tyrosine residues in plasma proteins treated with $ONOO^-$ (0.1 mM, 2 min, 37ºC). Human plasma was incubated with 0.1 mM $ONOO^-$ for 2 min at 37ºC. Ebselen and bis(2-aminophenyl)-diselenide at concentrations of 0.025-0.1 mM were added before $ONOO^-$. The level of tyrosine nitration was measured using the C-ELISA method, and the results are expressed as nmol nitrotyrosine-Fg/mg of plasma proteins. The results are representative of three independent experiments, and are expressed as the means ± SD. The effects were statistically significant (Student's t-test; selenium compounds + $ONOO^-$ (0.1 mM) treated plasma *versus* $ONOO^-$ (0.1 mM) treated plasma, $p < 0.05$).

Our earlier [16] and current results demonstrating an increase in both carbonyl group and nitrotyrosine in the plasma proteins, and lipid peroxidation in the plasma treated with peroxynitrite (Figs 2-5) are consistent with results in the existing literature [22-24].

Human plasma is an ideal model system to study the protectory effects of different antioxidants against oxidative/nitrative stress induced by peroxynitrite. The defence mechanisms against $ONOO^-$ action are also very important for the biological functions of human plasma components. The various well-known antioxidants, including deferoxamine, Trolox and ebselen, have

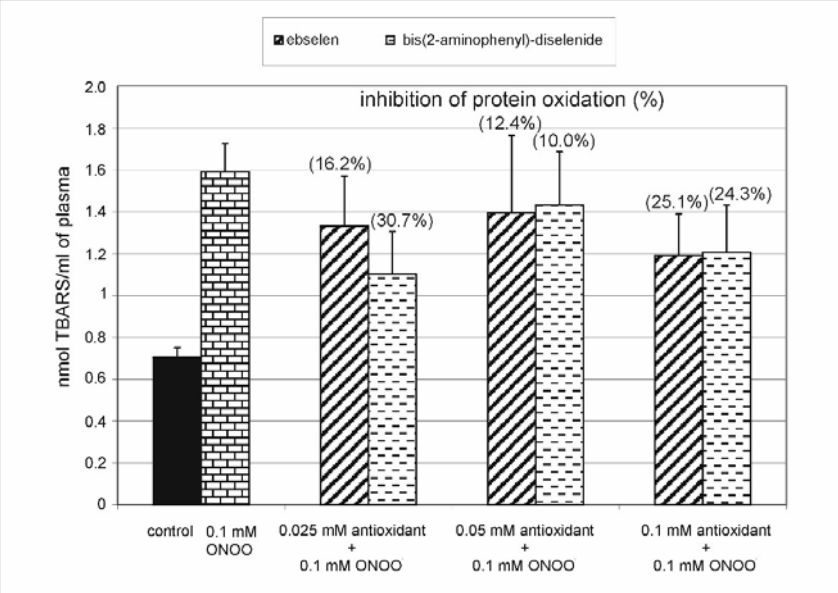

Fig. 5. The effects of ebselen and bis(2-aminophenyl)-diselenide (0.025-0.1 mM, 2 min, 37ºC) on the level of TBARS (marker of lipid peroxidation) in plasma treated with $ONOO^-$ (0.1 mM, 2 min, 37ºC). The results are representative of six independent experiments, and are expressed as the means ± SD. The effects were statistically significant (Student's t-test; selenium compounds + $ONOO^-$ (0.1 mM) treated plasma *versus* $ONOO^-$ (0.1 mM) treated plasma, $p < 0.05$).

been shown to be powerful scavengers against peroxynitrite [25]. The presence of other antioxidants (vitamin C, uric acid, glutathione and other thiols) in the plasma also inhibits peroxynitrite-mediated oxidation. The primary consequence of the interaction of peroxynitrite with low density lipoprotein (LDL) from the plasma is apoprotein damage, and such damaging effects are inhibited by dietary phenolic compounds [26]. The role of different exogenous antioxidants in the defence against $ONOO^-$ action in the human plasma is still unknown. In this study, we examined the defense properties of a novel selenoorganic analogue of ebselen – bis(2-aminophenyl)-diselenide. The antioxidative action of this compound were also compared with the effects of ebselen, which is a typical scavenger of peroxynitrite [25]. Not only ebselen, but also low-molecular-weight selenocompounds such as selenomethionine can be active in protecting several model compounds from being oxidized or nitrated by peroxynitrite [9]. The selenoprotein glutathione peroxidase is able to efficiently reduce peroxynitrite action by preventing the oxidation and nitration of model compounds and the nitration of proteins [27]. A similar mechanism may be applied for selenomethionine [28]. In human plasma, selenoprotein P also protects biomolecules against $ONOO^-$ action [12], suggesting that it may serve as a protector in human blood. The other selenoprotein that has been demonstrated

to be able to reduce peroxynitrite effects is thioredoxin reductase [12]. The results of this study indicate that the tested novel selenocompound, bis(2-aminophenyl)-diselenide, may protect plasma proteins against oxidation and nitration caused by peroxynitrite (or its intermediates) (Figs 3 and 4). Moreover, we observed that bis(2-aminophenyl)-diselenide suppresses peroxynitrite toxicity measured as the level of TBARS – a marker of lipid peroxidation (Fig. 5). Our preliminary unpublished study showed that bis(2-aminophenyl)-diselenide, unlike ebselen and its other derivatives, induced in platelets a profound increase in the level of low molecular weight thiols in reduced forms: glutathione (GSH), cysteine (CSH) and cysteinylglycine (CGSH).

Our data is consistent with the results described by Mouithys-Mickalad *et al.* [29]. Those authors showed the strong inhibitory effect of bis(2-aminophenyl)-diselenide on the lipid peroxidation of linoleic acid compared to the action of ebselen and other selenide derivatives [29]. Many selenoorganic compounds play an important role in biochemical processes. They could act as antioxidants, enzyme inhibitors or drugs, but the toxicity some of them becomes the limiting factor for their use in pharmacology. The biochemistry and pharmacology of stable synthesised organoselenium compounds are subjects of intense current interest, especially from the point of view of public health. Among the selenoorganic compounds, the best known and the most promising drug is ebselen [1, 2]. Recently, much attention has been devoted to studying the properties of new synthetic analogues of ebselen – selenides and diselenides [29]. The results of this study suggest that the novel selenoorganic derivative of ebselen – bis(2-aminophenyl)-diselenide, can scavenge peroxynitrite efficiently; however, its protectory mechanism is not clear.

In conclusion, the presented results indicate that bis(2-aminophenyl)-diselenide seems to have distinctly protective effects against the impairment of proteins and lipids induced by the strong biological oxidant and inflammatory mediator and therefore may be potentially useful in the prevention of $ONOO^-$-related diseases, such as cardiovascular and inflammatory diseases.

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