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Novel selenoorganic compounds as modulators of oxidative stress in blood platelets

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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 five new synthesized selenoorganic compounds (2-(5-chloro-2-pyridyl)-7-azabenzisoselenazol-3(2*H*)-one; 2-phenyl-7-azabenzisoselenazol-3(2*H*)-one; 2-(pyridyl)-7-azabenzisoselenazol-3(2*H*)-one; 7-azabenzisoselenazol-3(2*H*)-one; bis(2-aminophenyl) diselenide) on oxidative changes in human blood platelets and in plasma were studied *in vitro* and compared with those of ebselen, a well known antioxidant. Our studies demonstrated that bis(2-aminophenyl) diselenide has distinctly protective effects against oxidative stress in blood platelets and in plasma. It might have greater biological relevance and stronger pharmacological effects than ebselen.

Abbreviations: CGSH, cysteinylglycine; CSH, cysteine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GPx, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; PDI, protein disulfide isomerase; SDS, sodium dodecyl sulfate; TBARS, thiobarbituric acid-reactive substances

Introduction

Platelets are one of the key elements of blood. Their main function is the formation of hemostatic plug or thrombus (Levy-Toledano, 1999; Abrams and Brass, 2001). Platelet activation induced by different agonists plays an important role not only in hemostasis but in atherogenesis and the progression of atherosclerotic lesions (Ryningen and Holmsen, 1999). Antiplatelet therapy may reduce the risk of many cardiovascular and cerebrovascular disorders (Wu, 1996). Compounds that modulate metabolism and activation of blood platelets associated with oxidative stress could have potential benefit in a wide range of vascular diseases (Halliwell, 2000). Selenocompounds, particularly in organic form, may modulate platelet function and metabolism (Soriano-Garcia, 2004). The present study focused on the cellular mechanism of the action against oxidative stress in blood platelets of five novel synthesized selenoorganic compounds:

(1) 2-(5-chloro-2-pyridyl)-7-azabenzisoselenazol-3(2*H*)-one
(2) 2-phenyl-7-azabenzisoselenazol-3(2*H*)-one
(3) 2-(pyridyl)-7-azabenzisoselenazol-3(2*H*)-one

(4) 7-azabenzisoselenazol-3(2*H*)-one
(5) bis(2-aminophenyl)-diselenide

Antioxidative properties of the selenoorganic compounds were evaluated and compared to those of ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one). Ebselen is a nontoxic selenoorganic compound that has been intensively studied during the last decade (Schewe, 1995). Its pharmacological effects appear to be due to its unique antioxidative action in scavenging organic hydroperoxides or mimicking the activities of glutathione peroxidase (GPx) (Sies, 1993). Ebselen is responsible for *in vitro* inhibition of platelet aggregation and has antithrombotic effects attributed to modulation of platelet function, mainly due to inhibition of P-selectin expression on platelet surfaces (Lindenblatt et al., 2003). Ebselen also inhibited the *in vitro* extracellular hydrolysis of ADP and ATP by platelets (Furstenau et al., 2004). Selenoorganic compounds, like ebselen, may have anti-inflammatory and antioxidant effects. The toxicity of selenoorganic compounds is thought to be due to their prooxidant ability for oxidation of thiols with simultaneous generation of superoxide anion, $O_2^{-\cdot}$ (Stewart et al., 1999).

We investigated the effects of selenoorganic compounds on superoxide radical generation and the level of thiols in platelets. We also studied the antioxidative properties of selenoorganic compounds by determination of lipid peroxidation in blood platelets and in plasma.

Materials and methods

Human blood was collected into ACD solution (citric acid–citrate–dextrose; 5:1 v/v); platelets were isolated by differential centrifugation of blood for 20 min at 200*g*, and then platelet-rich plasma for 20 min at 1000*g* to sediment platelets (Wachowicz and Kustroń, 1992). The resulting pellet was resuspended in modified Tyrode's Ca^{2+}/Mg^{2+}-free buffer (15 mmol/L Tris-HCl, 140 mmol/L NaCl, 10 mmol/L glucose, pH 7.4), and the platelets were suspended in the same buffer to the final concentration of 4×10^8 platelets/ml.

The production of superoxide radicals (by superoxide dismutase-inhibitable reduction of cytochrome *c* (Jahn and Hansch, 1990)) was measured after treatment of platelet suspensions with tested compounds at concentrations of 1, 10, 100, and 1000 μmol/L (20 min, 37°C).

To examine the role of selenium compounds in oxidation of platelet glutathione and other low-molecular-weight nonprotein thiols, suspensions of washed human platelets were incubated with selenoorganic compounds at concentrations of 10 μmol/L, at 37°C for 20 min. Protein-precipitating solution (30% NaCl, 0.85% H_3PO_4, 0.2% EDTA) was added to frozen control platelets or selenium-treated platelets. Acid-soluble (glutathione) and acid-insoluble (protein) platelet fractions were separated and then the –SH group content in the fractions was estimated by HPLC (Glowacki et al., 2001) or with DTNB (Ando and Steiner 1973), respectively. HPLC analysis was performed with a Hewlett-Packard 1100 Series system. The assay uses 2-chloro-1-methylquinolinium tetrafluoroborate as a derivatization reagent and trichloroacetic acid as both an ion-pairing reagent and main mobile phase buffer component. Samples were injected using an autosampler into a Waters Nova-Pak C_{18} column. Identification of peaks was based on comparison of retention times and diode-array spectra with the corresponding data obtained by analyzing authentic compounds: glutathione (GSH), cysteine (CSH), and cysteinylglycine (CGSH) (Glowacki et al., 2001). Determination of free –SH groups with DTNB was used in the acid-insoluble (protein) platelet fraction. The pellet (the acid-precipitable fraction) was solubilized in the presence of 10% SDS and free –SH groups were determined with 4 mmol/L DTNB. The absorbance was measured at 412 nm (Ando and Steiner, 1973). A standard –SH curve was prepared for GSH.

To estimate the effects of tested selenoorganic compounds on platelet lipid peroxidation, platelet suspensions were preincubated (20 min, 37°C)

with these compounds at concentrations of 1, 10, 100, and 1000 μmol/L and then incubated with Fe^{2+} (20 min). The level of thiobarbituric acid-reactive substances (TBARS) as the marker of lipid peroxidation was determined (Wachowicz, 1984). The analogous set of experiments was carried out for plasma.

All of the results are representative of six independent experiments, and are expressed as mean $\pm$ SD. The effects were considered statistically significant (Student's *t*-test; control platelets/plasma versus selenoorganic compound treated platelets/plasma) at $p < 0.05$).

Results

The results obtained indicate the stimulatory effects of selenoorganic compounds on the superoxide radical generation. All tested compounds stimulated production of superoxide radicals in blood platelets (Table 1) but only compound no. 5 (bis(2-aminophenyl) diselenide) induced a distinct and dose-dependent increase of $O_2^{-\cdot}$ generation in blood platelets. Other compounds such as ebselen stimulated this process only at high concentrations (Table 1). The results suggest that only very high concentrations of the tested selenoorganic compounds might be responsible for toxicity resulting from their ability to generate free radicals.

We have established that compound no. 5, in contrast to ebselen and its derivatives, induced in platelets a profound increase of the level of low-molecular-weight thiols in reduced forms: GSH, CSH, and CGSH (Figure 1a–c). Other selenocompounds (excluding no. 5) caused a significant increase of oxidized forms of CSH and CGSH, whereas the total pool of these compounds was almost unchanged (Figure 1b, c). All selenocompounds, like no. 5, increased the level of the reduced form of GSH (Figure 1a). In contrast to the action of ebselen, all tested compounds depleted the level of protein thiols (Figure 1d). The level of protein –SH after treatment of platelets with ebselen was almost unchanged (Figure 1d).

Incubation of platelets with selenoorganic compounds (excluding no. 5) did not have any antioxidant effects on the generation of TBARS in these cells (Table 1). Only compounds no. 5 strongly decreased the level of TBARS in platelets in a dose dependent manner (Table 1). Compound no. 1 even had stimulatory effects on TBARS production in both platelets and plasma (Table 1). In plasma, compound no. 5, like no. 4, at low concentration reduced the level of TBARS, whereas ebselen had no effect (Table 1). In the presence of all the tested selenoorganic compounds, lipid peroxidation induced by Fe^{2+} was inhibited in platelets and only slightly reduced in plasma (Table 1).

Discussion

Many selenoorganic compounds play an important role in biochemical processes. They may act as antioxidants, enzyme inhibitors, or drugs, but the toxicity some of them is the limiting factor for their use in pharmacology. The biochemistry and pharmacology of stable synthesized selenoorganic compounds are subjects of intense current interest, especially from the point of view of public health (Mouithys-Mickalad Mareque et al., 2004; Soriano-Garcia, 2004).

We have shown that all the tested derivatives like ebselen prevented the generation of oxidized glutathione (GSSG), but only no. 5 (the diselenide) profoundly inhibited formation of oxidized forms of CSH and CGSH (Figure 1a–c). Bis(2-aminophenyl)-diselenide (no. 5) showed the ability to generate superoxide anion $O_2^{-\cdot}$ (Figure 1.5) but simultaneously exerted a powerful antioxidant effect on oxidation of thiols (Figure 1). Ebselen may form adducts with thiols, especially with GSH, and this could be responsible for the lack of the expected effect of GPx-like activity. We did not observe an increase of the level of the oxidized form of glutathione. Disulfides were formed in the cases of cysteine and cysteinylglycine,

Table 1. The effects of selenoorganic compounds on oxidative changes in blood platelets and plasma

Compound	Control [0 μmol/L]	[1 μmol/L]	[10 μmol/L]	[100 μmol/L]	[1000 μmol/L]
			nmol O_2/mg platelet protein		
No 1	0.234	0.089*	0.208	0.316*	0.512*
No 2	0.234	0.084*	0.213	0.316*	0.681*
No 3	0.234	0.173	0.199	0.309*	0.550*
No 4	0.234	0.119*	0.239	0.337*	0.555*
No 5	0.234	0.500*	0.692*	1.048*	1.446*
Ebselen	0.234	0.154*	0.243	0.264	0.482*
			nmol TBARS/mg platelet proteins		
No 1	2.279	3.479*	2.594	3.903*	2.243
No 2	2.273	2.788*	2.667	2.764	2.788
No 3	2.373	2.789*	2.667	2.764	2.788
No 4	2.181	2.254	2.739*	2.739*	2.642
No 5	2.230	1.794*	1.445*	0.776*	0.679*
Ebselen	2.182	2.836*	2.448	2.739*	2.642*
			Control with Fe		
No 1	3.515	2.861*	3.151*	2.909*	2.521*
No 2	3.806	2.473*	2.691*	2.570*	2.376*
No 3	3.564	2.206*	3.418	2.691*	2.690*
No 4	3.709	2.739*	2.909*	3.248*	3.200*
No 5	3.879	3.469*	3.151*	3.006*	2.618*
Ebselen	3.806	2.570*	2.861*	3.321*	2.570*
			nmol ofTBARS/ml plasma		
No 1	1.560	2.267*	2.013*	1.520	1.840*
No 2	1.493	1.640	1.773*	1.613	1.680
No 3	1.293	1.480*	1.453	1.387	1.433
No 4	1.307	1.087*	0.873*	1.060*	1.147
No 5	1.533	1.187*	0.967*	0.967*	1.027*
Ebselen	1.493	1.653	1.560	1.600	1.667
			Control with Fe		
No 1	1.960	1.813	1.613*	1.880	1.613*
No 2	2.107	1.853	1.680*	1.520*	1.413*
No 3	2.000	1.813	1.627*	1.593*	1.467*
No 4	2.133	1.993	1.467*	1.653*	1.607*
No 5	2.093	2.120	1.800*	1.680*	1.533*
Ebselen	1.933	1.747	1.700	1.653*	1.613*

$p < 0.05$.

whereas the total pool of these compounds was almost unchanged (Figure 1a–c). Only no. 5 caused a significant increase in the total pool of low-molecular-weight thiols (CSH and CGSH) in reduced forms concomitant with the reduction of the level of protein –SH groups (Figure 1b–d).

Oxidation of thiol groups and reduction of disulfide forms of proteins is a dynamic, reversible process that occurs under physiological conditions in cells (Inayama et al., 2002; Martin et al., 2001). The oxidation of protein thiols to mixed disulfides is an early cellular response to oxidative stress (Hansen et al., 2001; Schafer and Buettner, 2001). Protein thiols act as a redox buffer depending on their reactivity with GSH (Inayama et al., 2000). Moreover, the major pathway for

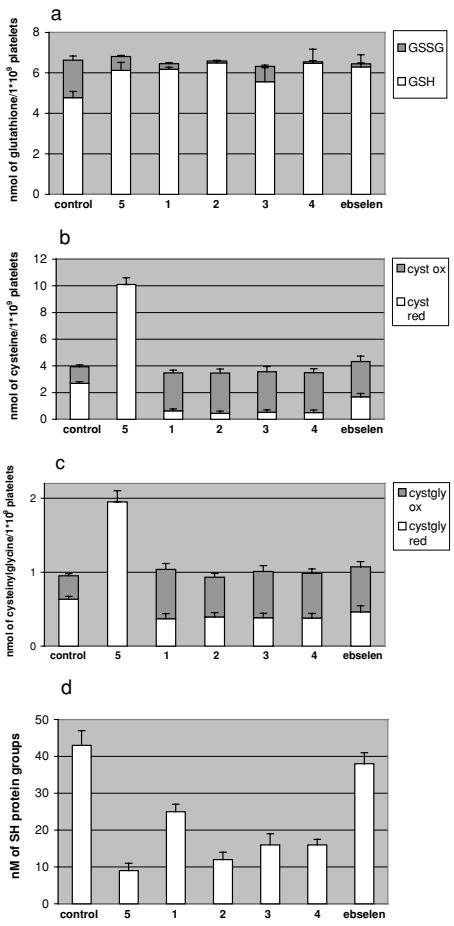

Figure 1. (a) The concentrations of glutathione (nmol glutathione/10^8 platelets) in the platelet acid-soluble fraction after incubation of platelets with selenoorganic compounds (10 μmol/L, 20 min, 37°C); the total pool of glutathione and ratio of GSH to GSSG are presented. (b) The concentrations of cysteine (nmol cysteine/10^8 platelets) in the platelet acid-soluble fraction after incubation of platelets with selenoorganic compounds (10 μmol/L, 20 min, 37°C); the total pool of cysteine and ratio of CSH to CSSC are presented. (c) The concentrations of cysteinylglycine (nmol cysteinylglycine/10^8 platelets) in the platelet acid-soluble fraction after incubation of platelets with selenoorganic compounds (10 μmol/L, 20 min, 37°C); the total pool of cysteinylglycine and the ratio CGSH to CGSSGC are presented. (d) The concentrations of –SH groups (nmol SH protein groups) in platelet protein (acid-insoluble) fraction after incubation of platelets with selenoorganic compounds (10 μM, 20 min, 37°C). (a)–(d) Controls are platelets without selenoorganic compounds. The results are representative of six independent experiments, and are expressed as mean $\pm$ SD. The effects were statistically significant (Student's *t*-test; control platelets versus selenoorganic compounds treated platelets, $p < 0.05$).

signaling in platelets involves, except phosphorylation of proteins, changes in the thiol status due to changes in the redox environment of the cell (Martin et al., 2001). Glutathione is the most abundant intracellular thiol-based antioxidant, prevalent in all living aerobic cells at concentrations far higher than most other redox-active compounds (Hart et al., 2002; Hervig et al., 2001; Kappusamy et al., 2002). GSH functions mainly as a thiol buffer, but it also serves to detoxify compounds (xenobiotics) either *via* conjugation reactions catalysed by glutathione *S*-transferases (GSTs) or directly, e.g., with hydrogen peroxide reaction catalysed by glutathione peroxidases (GPx) (Delton-Vandenbroucke et al., 2001; Desmots et al., 2002; Mari et al., 2002; Nordberg and Arner, 2001). In circulating platelets, above 80% of the glutathione is present in reduced form (Hansen et al., 2001; Hervig et al., 2001). Glutathione plays a critical role in the maintenance of platelet functions by thiol homeostasis and by removal of oxygen free radicals (Kappusamy et al., 2002). The reduction potential in the cell is dependent on the GSH/GSSG (glutathione /glutathione disulfide) ratio and the total concentration of glutathione (Hansen et al., 2001; Desmots et al., 2002; Lee et al., 2002; Mari et al., 2002). The various functions of GSH also include storage and transport of reduced –SH and the signaling of stress (Delton-Vandenbroucke et al., 2001; Schafer and Buettner, 2001)

All the tested selenoorganic compounds depleted the level of protein thiols, and ebselen showed the poorest effects (Figure 1d). These results suggest that not only GSH but also platelet protein-bound thiols are highly unstable and may react with selenoorganic compounds. Protein thiols are able to maintain the redox status of cells owing to their cysteine content (Hart et al., 2002; Kappusamy et al., 2002; Malmezat et al., 1998). The concentration of thiol groups in proteins is much greater than that of GSH; protein thiols can be present as free thiols, disulfides, and mixed disulfides when conjugated with glutathione, cysteine, homocysteine, and

γ-glutamylcysteine (GSH is a dominant ligand) (Inayama et al., 2002). Thiol homeostasis determines critical aspects of cell function and cell response (Nordberg and Arner, 2001; Schafer and Buettner, 2001; Malmezat et al., 1998). We noted that the metabolism of platelet thiols was changed after incubation of platelets with selenoorganic compounds. Selenocompounds *in vitro* may cause changes of glutathione peroxidase or protein disulfide isomerase (PDI) activity in platelets. Upon activation, platelets release PDI into the medium (Chen et al., 1995). This enzyme has been shown to be on the external surface of the platelet plasma membrane (Essex et al., 1995). It catalyses the reversible formation as well as the isomerization of disulfide bonds (Huang et al., 1997; Lahav et al., 2003). In metabolism of GSH, the role of platelet glutathione reductase should be taken into account. Other enzymes like glutaredoxin (Grx) and thioredoxin (Trx) present in platelets may also be involved in GSH metabolism (Hansen et al., 2001; Schafer and Buettner, 2001). Glutaredoxin reduces glutathione mixed disulfides such as glutathionylated proteins (Nordberg and Arner, 2001). The thioredoxin system consists of two antioxidant oxidoreductase enzymes: thioredoxin and thioredoxin reductase (TrxR). TrxR reduces disulfide in Trx and several other substrates. Reduced thioredoxin is highly efficient in reducing disulfides in proteins and peptides, including peroxiredoxins and GSSG (Nordberg and Arner, 2001; Schafer and Buettner, 2001).

Selenoorganic compounds have been shown to have antioxidant properties and antitumor activities and may protect against toxic side-effects of drugs (Soriano-Garcia, 2004; Mouithys-Mickalad Mareque et al., 2004). They are much less toxic than the inorganic selenium compounds and are potential therapeutic and chemopreventive agents. Among selenoorganic compounds the most promising drug was ebselen, studies intensively during the last decade. Recently, much attention has been devoted to study of the properties of new synthetic analogues of ebselen—selenides and diselenides (Mouithys-Mickalad Mareque et al., 2004). Ebselen was shown to reduce oxidative stress and some studies suggested that the mechanism of the antioxidant activity of ebselen is related to its ability to reduce peroxides rather than to its radical-scavenging properties (Klotz et al., 2003). We were interested in investigating the antioxidant properties of new synthetic analogues of ebselen. Our data obtained for five derivatives and compared with ebselen indicate that only the diselenide (no. 5) significantly inhibited lipid peroxidation. The antioxidant effect of this compound was even more efficient than that of ebselen.

We tested five analogues of ebselen, determining their effects on blood platelet metabolism. Our study demonstrates that only compound no. 5 (bis (2-aminophenyl) diselenide) prevented the generation of oxidized low-molecular-weight thiols (GSH, CSH, CGSH) in platelets and lipid peroxidation; it might have more biological relevance and even stronger pharmacological effects than well-known compound ebselen. Thus, it may be interesting as a pharmacological tool in cellular model studies.

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