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Protection by Organotellurium Compounds against Peroxynitrite-Mediated Oxidation and Nitration Reactions

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ABSTRACT. Diaryl tellurides effectively protect against peroxynitrite-mediated oxidation of dihydrorhodamine 123 (DHR), hydroxylation of benzoate, and nitration of 4-hydroxyphenylacetate (HPA). Bis(4-aminophenyl) telluride offered the most efficient protection against oxidation of DHR induced by peroxynitrite. Protection by this compound was approximately 3 times more effective than that afforded by its selenium analog, bis(4-aminophenyl) selenide, and 11 times more effective than selenomethionine. When peroxynitrite was infused to maintain a steady-state concentration, bis(4-aminophenyl) telluride in the presence of GSH, but neither bis(4-aminophenyl) telluride nor GSH alone, effectively inhibited the peroxynitrite-mediated hydroxylation of benzoate. The inhibition of nitration was most pronounced using bis(4-hydroxyphenyl) telluride, and this compound was *ca.* 3 times more effective than selenomethionine. Bis(4-aminophenyl) telluride also protected proteins in lysates from human skin fibroblasts from peroxynitrite-mediated nitration of tyrosine residues more effectively than selenomethionine. These data establish a potential biological or pharmacological role of organotellurium compounds in the defense against peroxynitrite. BIOCHEM PHARMACOL **55**;6:817–823, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. peroxynitrite; diaryl tellurides; selenomethionine; dihydrorhodamine 123; 4-hydroxyphenylacetic acid; nitrotyrosine

Peroxynitrite is a strong biological oxidant which can be formed *in vivo* and can induce DNA damage as well as initiate lipid peroxidation in biomembranes or low-density lipoprotein. Peroxynitrite also causes tyrosine nitration of proteins and is known to inactivate a variety of enzymes. Furthermore, it has been shown to partake in pathogenesis of atherosclerosis [1]. Low molecular mass compounds such as ascorbate and cysteine have been shown to react with peroxynitrite and protect biomolecules against damage. Recently, it was demonstrated that the selenoprotein glutathione peroxidase (GPx)¶ provides enzymatic defense against peroxynitrite by acting as a peroxynitrite reductase [2].

Ebselen, a selenoorganic compound with GPx-like activity, reacts with peroxynitrite to form the corresponding selenoxide [3] with a second-order rate constant of 2×10^6 M^{-1} sec^{-1} [4], which is *ca.* 100-fold greater than the rate constant observed with low molecular mass compounds such as ascorbate or methionine. Furthermore, ebselen and selenomethionine protect more effectively against oxidation and nitration reactions of biomolecules as well as single-strand break formation in plasmid DNA than does methionine [5, 6]. Previous studies have shown that, like ebselen, diaryl tellurides mimic the role of GPx in the catalytic decomposition of hydrogen peroxide or organic hydroperoxides in the presence of thiols as stoichiometric-reducing agents [7].

Therefore, in the present work we have evaluated the effects of a series of structurally related diaryl tellurides displaying GPx-like activity on peroxynitrite-induced oxidation of dihydrorhodamine 123 (DHR), on hydroxylation of benzoate, on 4-hydroxyphenyl acetate (HPA) nitration, and on the nitration of tyrosine residues in lysates of human fibroblasts. The results indicate that compounds displaying GPx-like activity exhibit biological and potentially therapeutic properties against peroxynitrite.

MATERIALS AND METHODS

Reagents

Selenomethionine and 4-hydroxyphenylacetic acid were from Sigma. DHR was from Molecular Probes, and rhoda-

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¶ *Abbreviations:* DHR, dihydrorhodamine 123; HPA, 4-hydroxyphenyl acetate; GPx, glutathione peroxidase.

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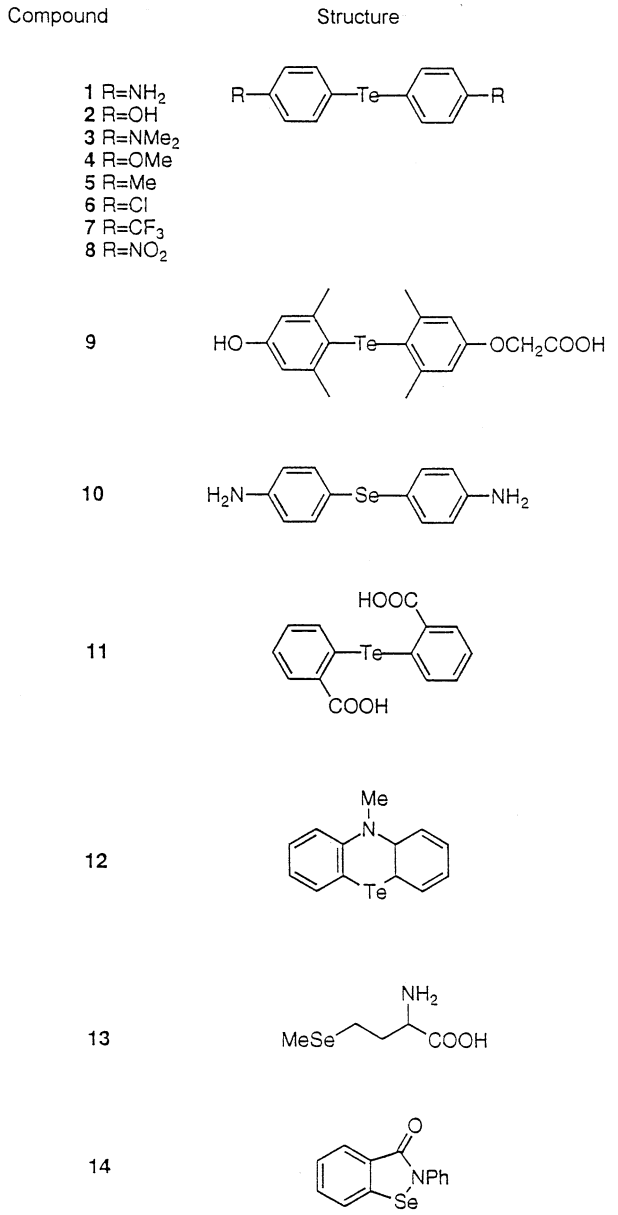


FIG. 1. Structures of the tested diaryl tellurides, diaryl selenide, selenomethionine and ebselen.

mine 123 was from ICN Biomedicals. Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2*H*)-one was kindly given by Rhône-Poulenc-Rorer. Other chemicals and solvents were from Merck.

Diaryltellurides (Fig. 1) Bis(4-aminophenyl)telluride (**1**) [8], bis(4-hydroxyphenyl)telluride (**2**) [9], bis[4-(dimethylamino)phenyl]telluride (**3**) [8], bis(4-methoxyphenyl)telluride (**4**) [10], bis(4-methylphenyl)telluride (**5**) [11], bis(4-chlorophenyl)telluride (**6**) [11], bis[4-(trifluoromethyl)phenyl]telluride (**7**) [9], bis(4-nitrophenyl)telluride (**8**) [12], bis(4-aminophenyl)selenide (**10**) [13], bis(2-carboxyphenyl)telluride (**11**) [12], and *N*-methylphenotellurazine (**12**) [14], were prepared according to methods described in the literature.

4-(Carboxymethoxy)-2,6-dimethylphenyl 2,6-dimethyl-4-hydroxyphenyl telluride (**9**) was prepared from bis(2,6-dimethyl-4-hydroxyphenyl)telluride [15] in analogy to a procedure set out in the literature [16]. Alkylation of the bisphenolic telluride with methyl bromoacetate afforded a monoalkylation product in 38% yield. Lithium hydroxide-induced hydrolysis of the ester afforded the title compound in quantitative yield; [^{1}H]NMR (DMSO-d6) δ 2.23 (s, 6H), 2.27 (s, 6H), 4.60 (s, 2H), 6.51 (s, 2H), 6.66 (s, 2H), 9.5 (s, 1H). Upon heating in a melting point apparatus, the material started to decompose at 140–150°.

The monoclonal anti-nitrotyrosine antibody used in the assay with lysate from skin fibroblasts was a kind gift from Dr. J. Beckman (Birmingham, Alabama). Peroxynitrite was synthesized from potassium superoxide and nitrogen monoxide as described in [17]. H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder. Peroxynitrite was determined spectrophotometrically at 302 nm (ε = 1670 M^{-1} cm^{-1}).

Assay of Peroxynitrite-Mediated Oxidation of DHR

The peroxynitrite-mediated oxidation of DHR was followed as suggested in [18] with minor modifications. Peroxynitrite (1 μM) was added to DHR (5 μM) and the tested compounds in 0.1 M phosphate buffer, pH 7.3, under intensive stirring at room temperature. Fluorescence intensity was measured using an LS-5 fluorescence spectrophotometer (Perkin-Elmer Co.) with excitation and emission wavelengths of 500 nm and 536 nm, respectively, at room temperature. Fluorescence intensity was linearly related to the concentration of rhodamine 123 between 0 and 10 μM. The tested compounds were dissolved in DMSO, which constituted 1% of the final solution tested. The yield of fluorescence intensity from reaction of DHR with peroxynitrite and DMSO alone was set equal to 100%. Data are given as means ± SD.

Hydroxylation of Benzoate Caused by Steady-State Infusion of Peroxynitrite

Peroxynitrite-mediated hydroxylation of benzoate was measured as described [19]. Peroxynitrite was infused with a micropump at a rate of 175 μL/min from a stock solution of 860 μM under constant mixing with a magnetic stirrer at room temperature into a mixture (5 mL) containing benzoate (10 mM) and DTPA (0.1 mM) in 0.5 M potassium phosphate buffer (pH 6.8). Peroxynitrite infusion was performed for 2 min to give a cumulated concentration of 60 μM. The pH in the mixture did not change detectably following the addition of peroxynitrite. The steady-state input concentration of peroxynitrite of 0.8 μM was calculated by using the infusion rate of peroxynitrite (0.5 μM/sec) and its decay rate in phosphate buffer at 25° and at pH 6.8 (0.65 sec^{-1}). GSH, bis(4-aminophenyl) telluride alone or bis(4-aminophenyl) telluride in the presence of GSH was added before peroxynitrite infusion. In control experiments, the peroxynitrite solution was incubated with phosphate buffer at pH 6.8 for 10 min at room temperature

to decompose the peroxynitrite before infusion into the reaction mixture.

Fe(III)/EDTA-Dependent Nitration of 4-Hydroxyphenylacetate by Peroxynitrite

The iron complex Fe(III)/EDTA was prepared by mixing equimolar solutions of iron(III)chloride and sodium EDTA. Peroxynitrite (final concentration 100 μM) was added to 4-hydroxyphenylacetate (HPA, 10 mM) in 0.1 M sodium-phosphate buffer (pH 7.3) containing Fe(III)/EDTA (0.5 mM) and various concentrations of the tested compounds dissolved in DMSO (final concentration 1%) while vortexing. Samples were incubated for 30 min at room temperature. Alternatively, as controls, HPA was added 5 min after peroxynitrite and to buffer alone. The pH was adjusted to 10.0–11.0 with 1 M NaOH before absorbance measurement at 430 nm. The yield of 4-hydroxy-3-nitrophenylacetate was calculated using $\varepsilon = 4{,}400\ M^{-1}\ cm^{-1}$ [20]. The result of the reaction of HPA with peroxynitrite and DMSO alone was set equal to 100%. Data are given as means ± SD ($r = 6–9$).

Preparation of Cell Lysates

Human skin fibroblasts were grown to near confluency in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, streptomycin (0.02 g/L), penicillin (20,000 IU/L), and ascorbate (50 mg/L). Cells were trypsinized, washed twice with PBS, pelleted by centrifugation and resuspended in 0.1 M potassium phosphate buffer, pH 7.3. The suspension was kept on ice, sonicated with a probe sonicator (4–5 times for 20 sec) and clarified by centrifugation (10,000 × *g*, 5 min, 4°). The supernatant was kept at −20° until use. Protein concentrations were determined according to Whitaker and Granum [21].

Nitration of Proteins and Detection by Western Blot

Peroxynitrite was injected to a final concentration of 200 μM into a vortexed solution (200 μL) of cell lysate (1 mg protein/mL) containing substances to be tested for their activity in inhibiting tyrosine nitration. After 30 min of reaction at room temperature, 30 μL of each sample were mixed with the same volume of sample buffer (8% (w/v) SDS, 20% (w/v) glycerol, 20% (v/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue, 0.25 M Tris-Cl, pH 8.0) and boiled for 5 min. Twenty microliters of each sample mixture were applied to a 15% SDS-polyacrylamide gel that, after electrophoresis, was blotted onto a nitrocellulose membrane (Hybond ECL nitrocellulose, Amersham) and treated with blocking solution (10% low-fat dry milk in PBS containing 0.05 to 0.1% (v/v) Tween-20 (PBST)) for 1 to 2 hr. Then, it was reacted with a monoclonal anti-nitrotyrosine antibody (1 mg/mL; diluted 1:500 in blocking solution at 4° overnight).

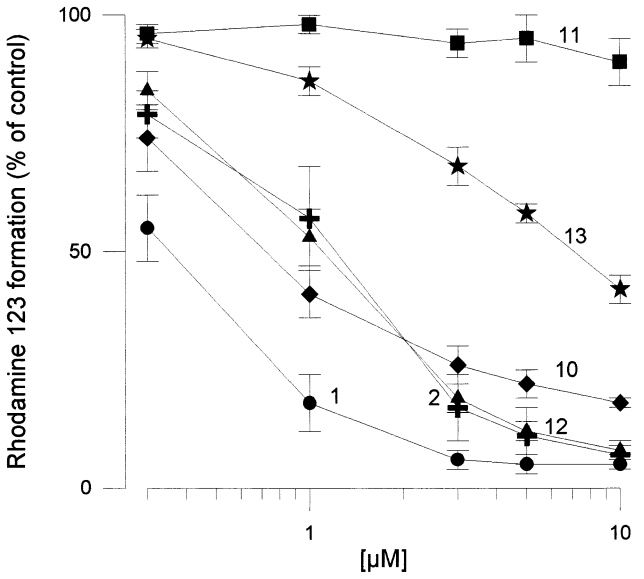


FIG. 2. Effects of tellurium-containing compounds and selenomethionine on rhodamine 123 formation caused by peroxynitrite. Peroxynitrite (1 μM) was added to dihydrorhodamine 123 (5 μM) and the tested compounds in 0.1 M phosphate buffer, pH 7.3, under intensive stirring at room temperature. Data are given as means ± SD ($n = 6–9$).

Following extensive washing with PBST (4 times for 5 min), the membrane was incubated with a secondary goat anti-mouse antibody coupled to alkaline phosphatase at room temperature for 2 hr. Remaining antibodies bound to the membrane after another washing step with PBST (4 or 5 min) were detected using a chemiluminescence substrate (Starlight, ICN Biomedicals).

RESULTS

DHR Oxidation

Figure 2 compares the results obtained with some of the tested organotellurium compounds (Fig. 1) regarding their ability to suppress the formation of rhodamine 123 from dihydrorhodamine 123 caused by peroxynitrite. The concentration range from 0.3 μM to 10 μM is shown in Fig. 2, and the data for all of the tested compounds at 3 μM are compiled in Table 1. Among the tested *para*-substituted diaryl tellurides, compound **1** [bis(4-aminophenyl)telluride] offered the most efficient protection against peroxynitrite-mediated oxidation of DHR (Table 1, Fig. 2). This compound was *ca.* 11 times more effective than selenomethionine and approximately 3 times more active than compounds **2** or **9** in this capacity. Furthermore, compound **1** was 3 times more active than its selenium analog, compound **10** [bis(4-aminophenyl)selenide]. Compounds **3**, **4**, and **5** showed activity similar to that of selenomethionine (Table 1, Fig. 2). The chloro-, nitro- and trifluoromethyl-substituted analogs (compounds **6**, **7** and **8**) offered only slight protection. In contrast to the heterocyclic organotellurium compound **12**, compound **11** showed no significant activity.

TABLE 1. Effect of organotellurium and organoselenium compounds on the oxidation of dihydrorhrodamine 123, the nitration of 4-hydroxyphenylacetate by peroxynitrite, and a comparison to their GPx-like activity

Compound	Rhodamine 123 formation (% of control) 3 [μM]	Nitration of HPA (% of control) 20 [μM]	GPx-like activity (-fold catalysis)
1	6 ± 2	51 ± 12	20
2	17 ± 7	30 ± 10	14
3	73 ± 13	71 ± 18	1.8
4	59 ± 11	67 ± 10	3.2
5	64 ± 8	64 ± 8	1.5
6	85 ± 4	80 ± 12	n.d.
7	97 ± 2	74 ± 8	1.3
8	83 ± 10	n.d.	1
9	28 ± 6	59 ± 11	n.d.
10	26 ± 4	76 ± 11	1
11	94 ± 3	87 ± 15	n.d.
12	19 ± 3	n.d.	n.d.
13 (Selenomethionine)	68 ± 4	93 ± 19	n.d.
14 (Ebselen)	52 ± 5	95 ± 6	6.3

Experimental details are given in Materials and Methods. The compounds were tested at 3 and 20 μM in the two different assays. Data on GPx-like activity are from Ref. [7] and represent -fold increase over control. Control experiments were done in the absence of the tested compounds and are set equal to 1. n.d., Not determined.

Protection by Bis(4-Aminophenyl) Telluride against Hydroxylation of Benzoate under Steady-State Infusion of Peroxynitrite

Peroxynitrite was infused with a micropump to give a steady-state concentration of 0.8 μM over 2 min. The cumulative peroxynitrite concentration was 60 μM (see Materials and Methods). The peroxynitrite-mediated hydroxylation of benzoate was used as a detector system. Ten micromolar bis(4-aminophenyl) telluride or 60 μM GSH alone had only a small protective effect (Fig. 3). However, bis(4-aminophenyl) telluride in the presence of 60 μM GSH completely suppressed benzoate hydroxylation until 30 μM peroxynitrite had been infused, i.e., within the first minute of infusion.

Thus, the GSH/peroxynitrite ratio necessary for inactivation of peroxynitrite in the presence of bis(4-aminophenyl) telluride is 2/1. These data establish that a diaryl telluride, bis(4-aminophenyl) telluride, inactivates peroxynitrite in a catalytic reaction at the stoichiometry known for that of hydroperoxide reduction.

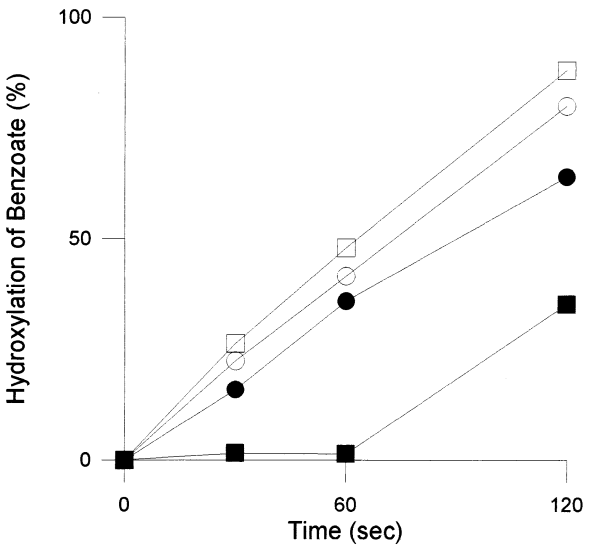


FIG. 3. Protection by bis(4-aminophenyl) telluride against hydroxylation of benzoate caused by a steady-state infusion of peroxynitrite. Peroxynitrite (cumulative concentration 60 μM) was infused at a rate of 175 μL/min over 2 min from a stock solution of 860 μM to yield 0.8 μM steady-state concentration. The reaction mixture (5 mL) contained 10 mM benzoate and 0.1 mM DTPA in 0.5 M potassium phosphate buffer (pH 6.8) (□). The pH in the mixture was not changed following the addition of peroxynitrite. 60 μM GSH (○), 10 μM bis(4-aminophenyl) telluride alone (●), and 10 μM bis(4-aminophenyl) telluride in the presence of 60 μM GSH (■) were added before peroxynitrite infusion.

Nitration of 4-Hydroxyphenylacetate

In analogy to the results presented above, most tellurium-containing compounds were more effective inhibitors of the nitration reaction than selenomethionine (Table 1). The compounds were compared at a concentration of 20 μM in this assay. The inhibition of nitration by compound **2** was the most pronounced among the tested compounds. The IC_{50} for compound **2** was *ca.* 3–4 times lower than that for selenomethionine or ebselen (data not shown).

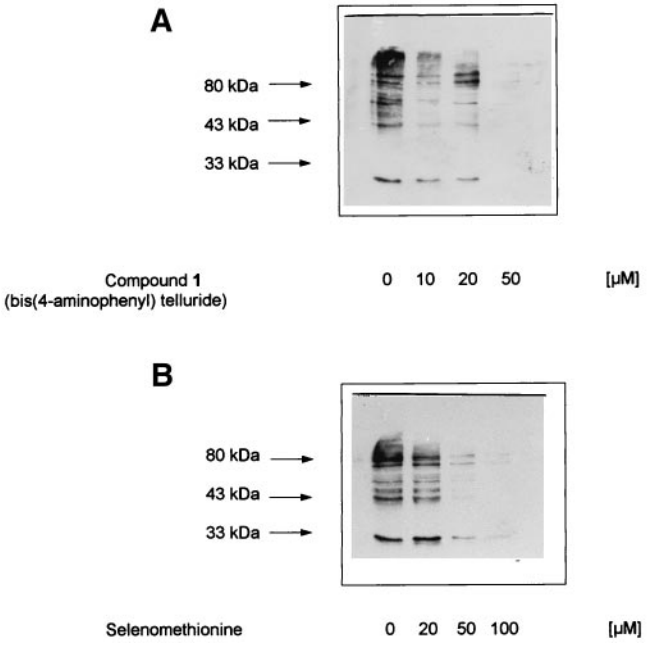


FIG. 4. Effects of bis(4-aminophenyl) telluride (A) and selenomethionine (B) on peroxynitrite-mediated nitration of tyrosine residues in proteins in lysate of human skin fibroblasts.

Nitration of Tyrosine Residues in Lysate of Human Skin Fibroblasts

Figure 4 shows that the protein tyrosine nitration caused by peroxynitrite can be suppressed by the addition of a diaryltelluride, compound **1**, (top), and by an organoselenium compound, selenomethionine (bottom). The onset of a distinct inhibition of peroxynitrite-mediated nitration of tyrosine residues was detected at a slightly lower concentration with compound **1** than with selenomethionine.

DISCUSSION

Organoselenium and organotellurium compounds are readily oxidized from the divalent to the tetravalent state. This property makes them attractive as scavengers of reactive oxidizing agents such as hydrogen peroxide, hypochlorite, and peroxyl radicals, and as inhibitors of lipid peroxidation in chemical and biological systems [7, 22]. As one gravitates down group 16 of the periodic table, the oxidizability of organic derivatives of the elements increases. This trend is reflected in the lower cyclovoltammetric oxidation potentials [9] of tellurium compounds as compared to selenium compounds bearing the same organic substituents. Diaryl selenides or tellurides are an attractive class of compounds for studying the antioxidative properties of organochalcogen compounds. The chalcogen is bonded to carbon via two strong bonds which make the compounds more resistant to photochemical or thermal degradation. In contrast to *dialkyl* derivatives of selenium and tellurium, *diaryl* compounds cannot undergo selenoxide or telluroxide *syn* elimination reactions in the tetravalent state. Thus, the risk of leakage of potentially toxic organochalcogen decomposition products is minimized. By introduction of substituents in the *para* positions of the diaryl chalcogenide, the electron density and reactivity of the heteroatom can be varied over a wide range, with minimal changes in the properties of the compounds. Thus, diaryl tellurides **1** and **3**, carrying strongly electron-donating substituents, would be expected to be the most reactive species towards various electrophiles, whereas compounds **7** and **8**, carrying electron-withdrawing groups, should react much more slowly.

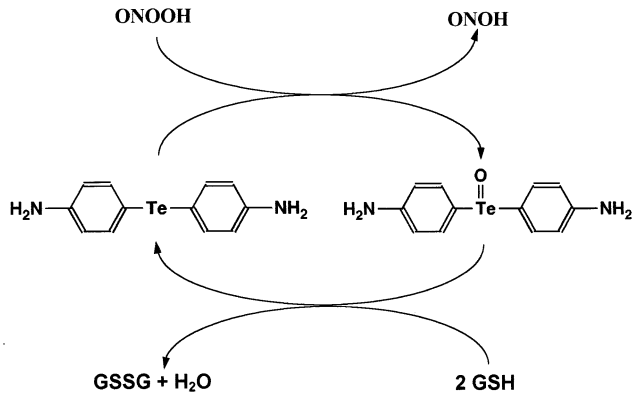
The data reported here demonstrate that organotellurium compounds with glutathione peroxidase-like activity protect against reactions caused by peroxynitrite. Compound **1**, bis(4-aminophenyl) telluride, is more efficient than its selenium analog or ebselen. As mentioned above, the latter reacts with peroxynitrite at a second-order rate constant of $2 \times 10^6\ M^{-1}\ sec^{-1}$ [4]. Compound **1** protects against peroxynitrite-mediated nitration of tyrosine residues in lysates from human fibroblasts, showing high efficiency in the competition with biological antioxidants or proteins capable of reacting with peroxynitrite.

The previously observed glutathione peroxidase-like capacity of diaryl tellurides has been ascribed to the ready oxidation of the heteroatom from the divalent to the tetravalent state (telluroxide) by hydrogen peroxide or organic hydroperoxides. As is also the case with glutathione peroxidase, regeneration of the active species then occurs via thiol reduction with disulfide formation. We suggest that diaryl tellurides act as scavengers of peroxynitrite by an oxygen transfer mechanism similar to that observed with hydrogen peroxide and hydroperoxides:

$$Ar_2Te + ONOOH \rightarrow Ar_2TeO + ONOH$$

The reaction is written here for the acid, but the anion may also react; this requires further study.

As shown in Figure 3, bis(4-aminophenyl) telluride reduces peroxynitrite in a catalytic fashion in the presence of GSH as a stoichiometric reducing agent. This provides additional support for the proposed mechanism (Scheme 1).



SCHEME 1. Proposed catalytic mechanism of bis(4-aminophenyl) telluride in the reduction of peroxynitrous acid to nitrous acid (or peroxynitrite to nitrite).

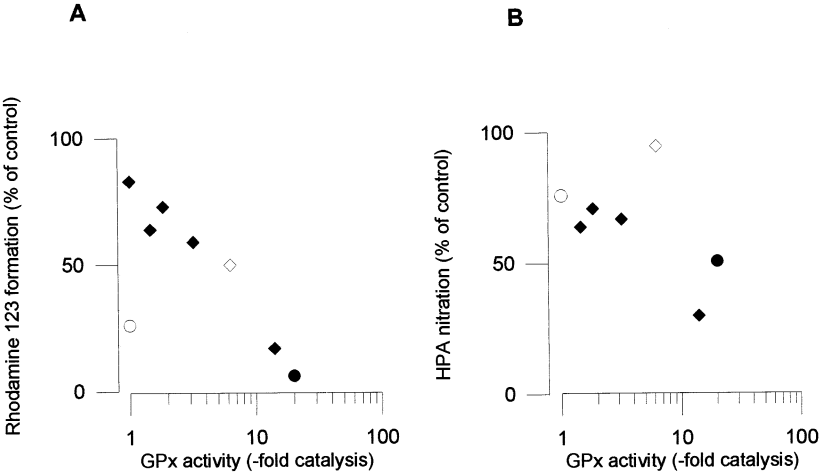


FIG. 5. Correlation of GPx-like activity of diaryl tellurides and organoselenium compounds with their effect on rhodamine 123 formation (A) and on nitration of HPA (B) by peroxynitrite. Compound 1 [bis(4-aminophenyl) telluride] is indicated by (●), its selenium analog compound 10 by (○), ebselen by (◇), and some diaryl tellurides 2-5,8 by (◆). Data from Table 1.

The hydrogen peroxide decomposing capacity of symmetrical 4,4′-disubstituted diaryl tellurides is inversely proportional to their oxidation potentials. A similar order of reactivity is also clearly seen in the inhibition of the peroxynitrite oxidation of DHR, but less so for the nitration of HPA (data not shown). These results suggest that the tellurium compounds act as nucleophiles towards peroxynitrite and that the reactivity is increased by substituents capable of donating electron density to the heteroatom (NH_2, NMe_2 OH). By introduction of more than one of these substituents into each of the two aromatic rings of the diaryl telluride, it may be possible to obtain even more efficient scavengers of peroxynitrite.

The GPx-like behavior of a series of 4,4′-disubstituted diaryl tellurides has previously been assessed by the coupled GSSG reductase assay [7]. As expected, peroxynitrite-mediated formation of rhodamine 123 from DHR in the presence of diaryl tellurides is inversely proportional to their glutathione peroxidase-like activity (Fig. 5A). The correlation between inhibition of HPA nitration and GPx-like activity was not as pronounced (Fig. 5B). Differences in the ranking order for inhibition in the DHR and HPA assay (Table 1) could indicate the involvement of different reactive intermediates in the oxidation and nitration reactions. The diaryl tellurides must compete with Fe(III)/EDTA (which is used as a catalyst in the HPA assay) for peroxynitrite as well as compete with HPA for the nitronium ion. Furthermore, it is not known how Fe(III)/EDTA will interact with the organotellurium compounds; potentially direct reaction products of peroxynitrite with some of the organotellurium compounds have not been searched for in the present study. In addition, the higher concentrations of the sparingly soluble test compounds in the HPA assay are also expected to cause some errors (e.g., compound **2** is significantly more soluble in aqueous medium than compound **3**).

In conclusion, tellurium-containing compounds with GPx-like activity can protect effectively against oxidation and nitration reactions, providing evidence for a possible role of organotellurium compounds in the defense against peroxynitrite.

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