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Selenoureas and thioureas are effective superoxide radical scavengers in vitro

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Abstract

Oxygen radicals, such as superoxide radicals, embellishing DNA, protein, lipids, etc., and carrying out the obstacle of the function of a cell is known. It depends for the oxidant level in the living body on the balance of a generation system and an elimination system of oxygen radicals, and research which controls an oxidant level in the living body is briskly done by taking in the substance which eliminates an oxygen radical. We investigated scavenging effects of superoxide radicals by selenoureas and thioureas using a highly sensitive and quantitative chemiluminescence method. At 330 nM, five selenoureas and five thioureas scavenged fractions of superoxide radicals (O_2^-) ranging from 8.4% to 87.6%. Among five *N*,*N*-unsubstituted selenoureas and *N*,*N*-unsubstituted thioureas 1-selenocarbamoylpiperidine and 1-thiocarbamoylpyrrolidine were the most effective scavengers. A possibility that selenoureas could use it as a new superoxide anion-scavenging substance from the result of this research became clear.

Keywords: Selenourea; Thiourea; Superoxide radicals; Scavenging effect; Superoxide anion-scavenging activity (SOSA)

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Introduction

The cells of an organism generate large amounts of reactive oxygen species (ROS) as oxygen metabolites, inevitably resulting in exposure to these injurious chemical species. ROS including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$), cause degeneration of biological macromolecules such as DNA, representing oxidative and sometimes genotoxic stress in vivo (Ramirez et al., 2003; Long et al., 1997). O_2^- is considered to be generated primarily by mitochondria in various cells, and by phagocytes such as granulocytes and monocytes/macrophages (Ricci et al., 2003). Under physiologic conditions, O_2^- is converted to H_2O_2 in hydrophilic solvents such as water by a disproportion reaction (Ueda et al., 1994). In addition, O_2^- can react with nitric oxide (NO) and generate highly toxic ROS including $ONOO^-$ and nitrogen oxides (NOx) (Hu et al., 2002). Thus, elimination of O_2^- is an important biologic need.

Various antioxidant enzymes including the superoxide dismutases (SODs), catalase, and glutathione peroxidase, as well as antioxidant vitamins (C and E) directly scavenge and eliminate ROS. An important antioxidant enzyme, glutathione peroxidase (GPX), contains a selenium molecule in its active domain. GPX effectively scavenges and eliminates H_2O_2 both in vitro and in vivo. In addition, previous studies have shown that selenium compounds such as selenoproteins, protect cells against oxidative stress (Jeong et al., 2002; Taino et al., 2000). Accordingly, a variety of selenium compounds may effectively scavenge and eliminate ROS. On the other hand, certain thioureas that effect the twitch tension of the rat diaphragm upon stimulation of the phrenic nerve-diaphragm also scavenge O_2^- (Crosland, 1995). However, details concerning of the superoxide anion-scavenging activity of thioureas are not known. This study was performed to determine how effectively selenoureas and thioureas scavenge O_2^- in vitro.

Materials and methods

Materials

A cypridina luciferin analogue, (2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a] pyrazin-3-one (MCLA), was obtained from Tokyo Kasei (Tokyo, Japan) for use as a chemiluminescent probe for superoxide radicals. MCLA was dissolved in doubly distilled water, and stored at −80°C until needed. The concentration of MCLA solution was determined by absorbance at 430 nm using an absorbance coefficient value of $\varepsilon = 9600\ M^{-1}cm^{-1}$, as previously described (Kimura and Nakano, 1988). Horse heart cytochrome c (type IV), SOD (from bovine erythrocytes, 3000 units/mg protein), xanthine oxidase (XOD grade III), and bovine serum albumin (BSA, acid-and globulin-free) were purchased from Sigma Chemical (St. Louis, MO). Hypoxanthine was purchased from Wako Chemicals (Tokyo, Japan) and used without further purification. All other chemicals and solvents were analytical grade and used without further purification.

General

Synthetic methods for the production of selenoureas and thioureas

N,N-Dimethyselenourea (4a). HCl (1N; 2 mL, 2 equivalent) in anhydrous diethyl ether was added to *N*,*N*-dimethylcyanamide (0.08 mL, 1 equivalent) of tetrahidroflane (THF) solution (10 mL). The initially

colorless and clear reaction mixture became a milky white suspension in less than 30 s, and then was stirred at 0°C for 2 h. Subsequently, LiAlHSeH (**2**) (1 equivalent) (Ishihara et al., 2001) was added to the reaction mixture. The reaction mixture was stirred further at 0°C for 3 h, and then was extracted with dichloromethane and washed with water. The organic layer was dried over sodium sulfate and evaporated to dryness. The residue was purified by flash chromatography on silica gel with dichloromethane : ether (1:1) to give **4a** (0.105 g; yield, 70%); Mp. 172.2 to 172.8°C; IR (KBr) 3366, 3162, 1551 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 3.18 (6 H, br s, N-CH_3), 7.60 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 37.9, 45.3, 177.7; ^{77}Se NMR (DMSO-d6) δ 230.4; MS (CI): *m/z* = 153 [M^+1]; HRMS (EI) calculated for $C_3H_8N_2Se$ 151.98522; found, 151.98346.

N,N-Diethyselenourea ***(4b)***. Mp. 121.8 to 122.7°C; IR (KBr) 3340, 3176, 1534 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.27 (6 H, t, *J* = 6.8 Hz, CH_3), 3.49 (2 H, br s, CH_2), 3.97 (2 H, br s, CH_2), 6.48 (2 H, br s, NH); ^{13}C NMR ($CDCl_3$) δ 12.2, 42.9, 51.1, 176.6; ^{77}Se NMR ($CDCl_3$) δ 209.8; MS (CI): *m/z* = 181 [M^+1]; HRMS (EI) calculated for $C_5H_{12}N_2Se$ 180.0165; found, 180.0147.

1-Selenocarbamoylpyrrolidine ***(4c)***. Mp. 215.1 to 215.9°C; IR (KBr) 3292, 3159, 1523 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 1.80 (2 H, m, CH_2), 1.98 (2 H, m, CH_2), 3.26 (2 H, t, J = 6.8 Hz, CH_2), 3.62 (2 H, t, *J* = 6.8 Hz, CH_2), 7.53 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 24.4, 25.9, 47.5, 54.2, 173.6; ^{77}Se NMR (DMSO-d6) δ 245.1; MS (CI): *m/z* = 179 [M^++1].

1-Selenocarbamoylpiperidine ***(4d)***. Mp. 145.0 to 146.2°C; IR (KBr) 3303, 3169, 1522 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 1.47 (4 H, m, CH_2), 1.59 (2 H, m, CH_2), 3.76 (4 H, br s, CH_2), 7.75 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 23.6, 25.3, 176.0; ^{77}Se NMR (DMSO-d6) δ 218.5; MS (CI): *m/z* = 193 [M^+1]; HRMS (EI) calculated for $C_6H_{12}N_2Se$, 192.0165; found 192.01499.

4-Selenocarbamoylmorpholine ***(4e)***. Mp. 207.8 to 209.0°C; IR (KBr) 3316, 3214, 1523 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 3.57 (4 H, t, *J* = 4.4 Hz, CH_2), 3.79 (4 H, br s, CH_2), 7.95 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 65.6, 178.0; ^{77}Se NMR (DMSO-d6) δ 225.0; MS (CI): *m/z* = 195 [M^++1].

N,N-Dimethythiourea ***(5a)***. Mp. 163.0 to 164.2°C; IR (KBr) 3385, 3183, 1542 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 3.11 (6 H, br s, N-CH_3), 7.15 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 181.6; MS (CI): *m/z* = 105 [M^++1].

N,N-Diethythiourea ***(5b)***. Mp. 99.2 to 100.6°C; IR (KBr) 3376, 3192, 1522 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 1.06 (6 H, t, *J* = 6.8 Hz, CH_3), 3.53 (4 H, br s, CH_2), 7.10 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 12.6, 44.5, 179.8; MS (CI): *m/z* = 133 [M^++1].

1-Thiocarbamoylpyrrolidine ***(5c)***. Mp. 197.3 to 199.0°C; IR (KBr) 3316, 3171, 1511 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 1.80 (2 H, m, CH_2), 1.98 (2 H, m, CH_2), 3.26 (2 H, t, *J* = 6.8 Hz, CH_2), 3.62 (2 H, t, *J* = 6.8 Hz, CH_2), 7.53 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 24.6, 25.9, 47.5, 51.4, 178.3; MS (CI): *m/z* = 131 [M^++1].

1-Thiocarbamoylpiperidine ***(5d)***. Mp. 127.4 to 129.1°C; IR (KBr) 3334, 3188, 1510 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 1.45 (4 H, m, CH_2), 1.56 (2 H, m, CH_2), 3.69 (4 H, br s, CH_2), 7.25 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 23.7, 25.3, 48.2, 180.2; MS (CI): *m/z*=145 [M^++1].

*4-Thiocarbamoylmorpholine **(5e)***. Mp. 178.9 to 179.7°C; IR (KBr) 3326, 3216, 1509 cm^{-1}; ^{1}H NMR (DMSO-d6) δ 3.56 (4 H, t, ***J*** = 4.8 Hz, CH_2), 3.71 (4 H, t, ***J*** = 4.4 Hz, CH_2), 7.49 (2 H, br s, NH); ^{13}C NMR (DMSO-d6) δ 47.4, 65.7, 181.4; MS (CI): ***m/z*** = 147 [M^{+}+1].

Assay of superoxide anion-scavenging activity (SOSA)

The SOSA of selenoureas and thioureas was measured by a previously reported method (Kimura and Nakano, 1988; Kato et al., 2002; Kato et al., 2003). In brief, the standard reaction mixture contained 10^{-7} M MCLA, 5×10^{-5} M hypoxanthine, XOD (6.5 U), SOD (0.2 to 20 ng / mL) and 50 mM Tris-HCl buffer containing 0.1 mM EDTA (pH 7.8), in a total volume of 3.0 ml, in the presence or absence of various concentrations of the selenoureas or thiourea being tested. Chemiluminescence measurement using a luminometer (Aloka, BLR102) at 25°C was initiated upon the addition of 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride (MCLA) to the standard incubation mixture excluding XOD. Measurement continued for 2 min without XOD and then for an additional 2 min after addition of XOD. A representative example of a measurement of the effect of **5c** on MCLA-dependent luminescence is shown in Fig. 1.

The inhibition (%) of compounds had strong SOSA at 333 nM was also measured at 33.3 and 3.33 nM. The luminescence intensity (count / min) of the solution which does not contain a substance at all, and the solution containing **4c**, **4d**, **4e**, or **5c** was measured, and the inhibition (%) was computed. The inhibition (%) and the concentration of **4c**, **4d**, **4e**, or **5c** were plotted, and concentration equivalent to 50% of rates of inhibition was set to IC_{50}.

This study considered the elimination of superoxide anion generated by XOD by selenamides. Exact SOSA cannot be measured if selenamides inhibit the activity of XOD. We checked that measured electron spin resonance of super oxide anion and selenamides did not inhibit the activity of XOD (Tanigawa, 1990) (data not shown).

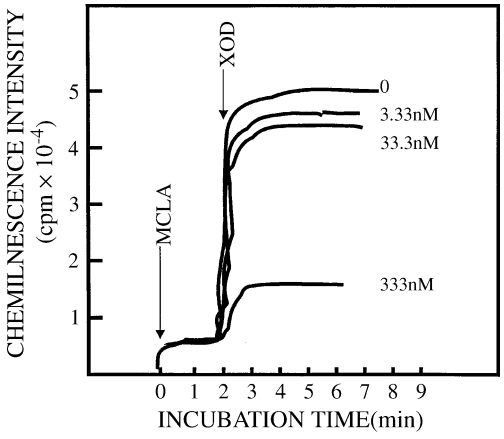


Fig. 1. Dose-dependent effect of 1-thiocarbamoylpyrrolidine (**5c**) on 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a] pyrazin-3-one (MCLA)-dependent luminescence. Incubation conditions are given in the text. Arrows indicate the time at which MCLA or xanthine oxidase (XOD) was added.

Results and discussion

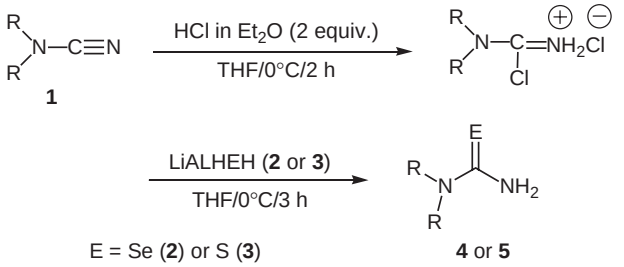
Five *N*,*N*-unsubstituted selenoureas (**4a** to **4e**) and five *N*,*N*-unsubstituted thioureas (**5a** to **5e**), respectively, were prepared by allowing the corresponding cyanamide (**1**) with LiAlHSeH (**2**) or LiAlHSH (**3**) in the presence of 1 N HCl in anhydrous diethyl ether, as shown in Scheme 1. Structures of these selenourea and thiourea derivatives are shown in Table 1.

SOSA of the compounds also studied are summarized in Table 1. Among them, 1-thiocarbamoylpyrrolidine (**5c**) had the highest SOSA at 333 nM (87.6%). The SOSA of 1-selenocarbamoylpyrrolidine (**4c**) and 1-selenocarbamoylpiperidine (**4d**) were 71.1% and 74.7% at 333 nM, respectively. The SOSA of *N*,*N*-unsubstituted selenoureas (**4a** to **4e**) and *N*,*N*-unsubstituted thioureas (**5a** to **5e**) derivatives were evaluated in order to examine the relationship between structure and activity. Except for **4c** and **5c**, selenoureas showed stronger activity than the corresponding thioureas (Table 1).

Activities of compounds **4c, 4d, 4e,** and **5c** were sufficiently high to suggest further testing; by serial dilution, the 50% inhibitoly concentrations (IC_{50}) for the four compounds were 125 nM, 142 nM, 121 nM, and 123 nM, respectively. IC_{50} of L-ascorbic acid were 227 nM(s), as a result of measuring on the same conditions as the above. Therefore, it was judged that **4c, 4d, 4e,** and **5c** showed practical SOSA.

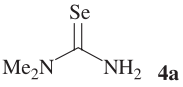
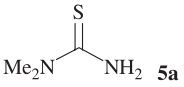
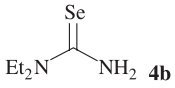
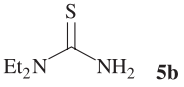
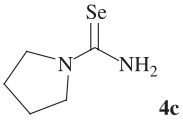
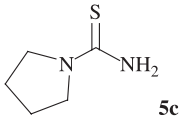
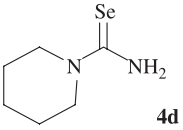
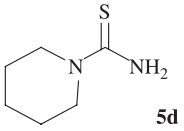
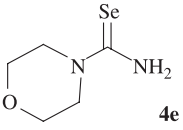
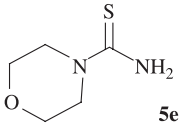
In this study we suspected that these compounds may have the SOSA of selenoureas and thioureas. Among the 10 compounds tested, **4c, 4d, 4e,** and **5c** showed the most potent SOSA. Based on the SOSA results in vitro, we suspect that these compounds prove to have value as therapeutic agents for dismutation of the superoxide anion. This study is the first to demonstrate that selenoureas and thioureas have SOSA, although ureas apart from selenourea and thioureas have been reported to act as free radical scavengers (Sandler, 1984, Yamashita et al., 2000). Our findings represent a beginning for development of potential selenourea and thiourea agents with SOSA.

In an investigation of the effects of selenium in patients with human immunodeficiency virus infection, antioxidant enzyme activity, including that of SODs, glutathione peroxidase, and catalase were evaluated as well as plasma selenium concentrations. GPX activity at baseline was significantly higher in the placebo and selenium groups than in the control group. These higher enzyme activities could be a result of increased synthesis of these enzymes in erythrocyte precursors under conditions of oxidative stress. GPX activity increased significantly between 3 and 6 months after initiating selenium treatment. Similarly, a significant increase in the GSH concentration was observed at 12 months compared with the baseline value after selenium supplementation (Delmas-Beauvieux et al., 1996). Although the generation of a low concentration of superoxide anion in the



Scheme 1.

Table 1
Scavenging activity of several *N*,*N*-unsaturated selenoureas and thioureas on the superoxide anion *at 333 nM*

Entries	Compound	Inhibition (%)	Entries	Compound	Inhibition (%)
1	 **4a**	52.1	6	 **5a**	8.4
2	 **4b**	67.1	7	 **5b**	15.5
3	 **4c**	71.1	8	 **5c**	87.6
4	 **4d**	74.7	9	 **5d**	10.8
5	 **4e**	66.8	10	 **5e**	23.7

human body is useful in biological defenses and intercelluar signal transduction, increases in superoxide anion have been implicated in aging.

Generation of excessive superoxide anion in the human body is countered by an antioxidant enzyme system that includes SOD, GPX, catalase, and GSH. Superoxide anion can cause nerve degeneration (Wrona and Dryhurst, 1998) and heart failure (Ferrari et al., 1989). Further, less SOD activity has been found in blood from patients with thyroiditis, dwarfism, and Turner syndrome than that in samples from healthy persons (Ohno et al., 1991). A drug possessing SOSA could be useful for treatment of these diseases. Indeed, a selenium-containing compound that has been shown to attenuate oxidative stress, ebselen has been studied extensively as candidate drug (Asatryan et al., 2003; Imai et al., 2002; Yoshizumi et al., 2002).

Dimethylthiourea has been shown to ameliorate bronchoconstriction induced cigarette smoke in guinea pigs, and to improve cardiac function in ischemia-reperfusion injury, by scavenging hydroxyl radicals (Matsumoto et al., 2000; Prasad et al., 1994). However, since some thioureas have severe adverse effects such as teratogenicity, safety of compounds intended for clinical use will require careful investigation.

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