Antioxidant actions of ovothiol-derived 4-mercaptoimidazoles: glutathione peroxidase activity and protection against peroxynitrite-induced damage

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Abstract 4-Mercaptoimidazoles derived from the naturally occurring antioxidants, ovothiols, were tested for their glutathione peroxidase-like (GSH Px-like) activity and protection against peroxynitrite-induced damage. All the thiol compounds displayed similar significant GSH Px-like activities, which are however weaker than that of the reference compound, ebselen. The inhibitions of the peroxynitrite-dependent oxidation of Evans blue dye and dihydrorhodamine 123 showed that the thiol compounds substituted on position 5 of the imidazole ring were nearly as effective as ebselen while the C-2 substituted ones were less effective. Both assays corroborate the large superiority of mercaptoimidazoles over glutathione as inhibitors of peroxynitrite-dependent oxidation.

Key words: 4-Mercaptoimidazole; Ovothiol; Glutathione peroxidase; Peroxynitrite; Antioxidant

1. Introduction

Biological systems use enzymatic and non-enzymatic antioxidant systems to deal with oxidative stress [1]. Recently a novel class of heterothiols, the 1-methyl-4-mercaptohistidines (ovothiols), has been found in high concentrations in invertebrate eggs [2]. Their antioxidant properties were investigated in vitro. They can replace the function of the enzymic glutathione peroxidase (GSH Px) by consuming hydrogen peroxide and being regenerated by glutathione [3–5]. Radical scavenging abilities were also reported. They act as efficient scavengers of Fremy's salt, Banfield's radical, tyrosyl radical [6], superoxide and linoleate peroxyl radicals [7]. In most cases, their scavenging potency was found to be superior to that of glutathione and puts them together with ascorbate, amongst the most efficient antioxidants from natural origin. These antioxidant properties were related to acid–base and redox properties. 4-Mercaptoimidazoles predominate as zwitterionic structures at physiological pH, exhibiting highly reactive thiolate functions [8]. They reduce free radicals to give thiyl radicals, which are even able to repair vitamin E radicals [7].

On the basis of this natural model, we designed 4-mercaptoimidazoles having various substituents on the imidazole ring [9]. These compounds were shown to be powerful scavengers of hydrogen peroxide, hydroxyl radicals and hypochlorous acid and to inhibit copper-induced LDL peroxidation [10]. Thiyl radicals were characterised upon radical scavenging [11].

Here we report further insight into their antioxidant properties. The most potent compounds as radical scavengers are studied for their activity as GSH Px mimics. The increasing evidence for a role of peroxynitrite in biological processes prompted us to investigate their reaction with peroxynitrite. Indeed, peroxynitrite anion ($ONOO^-$), once protonated under physiological conditions, is well known to rapidly produce very reactive 'hydroxyl radical-like' species, resulting in tissue injuries [12–16]. Several studies have been devoted to characterising the protective effects of antioxidants against peroxynitrite-induced oxidations [17–23]. Two assays were used, the bleaching of Evans blue dye and the oxidation of dihydrorhodamine 123 (DHR 123) to fluorescent rhodamine in presence of peroxynitrite. The established antioxidants Trolox, ebselen and glutathione were used as reference standards for comparative activities.

2. Materials and methods

2.1. Reagents

Chemicals and reagents were of the highest quality available and were purchased from Sigma Aldrich Company. All the 4-mercaptoimidazoles were obtained according to a previously reported method [9]. Stock solutions (0.01 mol/l) were freshly prepared with *N,N*-dimethylformamide (DMF) and buffer solutions with Milli-Q water (conductivity $< 10^{-18}/\Omega$/cm).

2.2. GSH Px activity

GSH Px activity was determined by the reduction of GSSG formed via the NADPH-glutathione reductase system as a continuous indicator system [24]. Loss of NADPH was monitored continuously at 366 nm using a UVIKON 932 spectrophotometer and a molar absorption coefficient of 3300/M/cm. Experiments were conducted at room temperature (22°C) in 50 mM phosphate-buffered saline pH 7.6 containing 0.1 mM EDTA and 1.0 mM NaN_3. Test compound (12.5 μM for ebselen, the reference standard [24] and 250 μM for mercaptoimidazoles), glutathione (1.0 mM), NADPH (280 μM) and GSSG reductase (1 U/ml) were incubated for 4 min to estimate the sample blank and reactions were initiated with H_2O_2 (1.0 mM). Appropriate blanks were also run in the absence of added test compound and in the presence of H_2O_2. The GSH Px activity of ovothiols results from two successive reactions: oxidation of the thiols to the disulphides in presence of hydrogen peroxide and regeneration of the thiols by exchange reactions of the disulphides with glutathione [3–5]. The first step was already studied [10] and here we investigate the second one. For this purpose, disulphide (100 μM) and glutathione (1.0 mM) were incubated for 2 min at room temperature. NADPH (200 μM) and

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GSSG reductase (1 U/ml) were subsequently added. The absorbance decrease at 366 nm was measured and compared to that obtained in presence of glutathione disulphide alone (100 μM). The ratio of both NADPH disappearances gave the amount of glutathione/disulphide exchange.

2.3. Peroxynitrite synthesis

Peroxynitrite synthesis was carried out by modifying the method described by Beckman et al. [25]. Acidified hydrogen peroxide (8.2 M in 1.85 M HNO_3, 6.6 ml) and sodium nitrite (2 M, 6 ml) solutions were drawn into two separate syringes. The contents of both syringes were simultaneously injected into an ice-cooled beaker containing 4.2 M sodium hydroxide (6 ml) through a 'Y'-shaped junction. Excess hydrogen peroxide was removed by MnO_2 treatment. The concentration of the resultant stock was measured spectrophotometrically at 302 nm ($\varepsilon = 1670$/M/cm). The typical yield ranged from 70 to 80 mM.

2.4. Prevention of peroxynitrite-mediated oxidation

Experiments were conducted at room temperature in a 50 mM phosphate-buffered saline containing 0.1 mM diethylenetriaminepentaacetic acid, 90 mM NaCl and 5 mM KCl, pH 7.4. Blanks using DMF alone in the absence of test compound and peroxynitrite allowed to degrade for 5 min in phosphate-buffered saline, pH 7.4, were also run. There was no interference of DMF and degraded peroxynitrite with the oxidative modification of Evans blue and DHR 123.

Peroxynitrite induced the bleaching of Evans blue dye, which was measured at 608 nm ($\varepsilon = 70\,000$/M/cm). Consumption of Evans blue (25 μM) in absence and presence of test compound was measured over a range of peroxynitrite concentration (0–60 μM). Antioxidant activities were determined according to the method reported by Balavoine et al. [22]. The ratios of rate constants k_A/k_{EB} were determined by plotting D_0/D_A against $[\text{antioxidant}]_0/[\text{EB}]_0$. k_A and k_{EB} are the rate constants for reaction of peroxynitrite with the antioxidant and Evans blue, respectively. D_0 and D_A are the stoichiometries for the reaction of peroxynitrite with Evans blue in absence and presence of the antioxidant compound, respectively.

The second method used the oxidation of DHR 123, as described by Kooy et al. [23]. Test compound, DHR 123 (5.0 μM) and peroxynitrite (2.4 μM) were rapidly mixed and fluorescence measurements were performed on a Perkin Elmer LS50 spectrophotometer with excitation and emission wavelengths of 500 nm and 536 nm, respectively. The effects are expressed as the concentration giving 50% inhibition of the oxidation of DHR 123 (IC_{50}).

3. Results

3.1. GSH Px-like activity of 4-mercaptoimidazoles in vitro

The consumption of NADPH upon addition of H_2O_2 in absence of test substance (control) was 13 ± 1 ($n = 4$) μM NADPH/min. There was no influence of the vehicle, DMF. In the catalysed reactions, the following NADPH consumptions (μM/min ± S.D., $n = 4$) were recorded: ebselen [89 ± 5], compound no. **1** [39 ± 2], **2** [48 ± 3], **3** [44 ± 3], **4** [42 ± 2], **5** [39 ± 3], **6** [54 ± 4], **7** [41 ± 4]. Experiments revealed that all the 4-mercaptoimidazoles exhibit significant GSH Px activity (Fig. 1). However their catalytic action (Table 1) is about two times inferior to that of ebselen (% catalysis of 684).

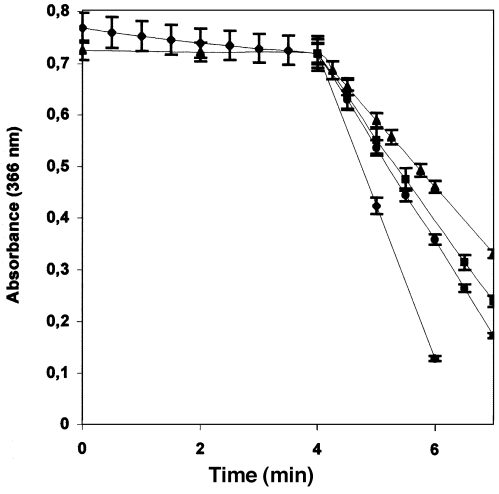


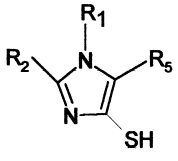
Fig. 1. GSH Px activity of 4-mercaptoimidazoles. Reactions were initiated by addition of 1.0 mM H_2O_2 to the assay mixture containing 1.0 mM GSH, 0.28 mM NADPH, 1 U/ml GSSG reductase and 12.5 μM ebselen (♦), 250 μM compound **6** (●), 250 μM compound **2** (■), 250 μM compound **1** (▲). Results are means ± S.D. of four experiments.

As encountered for ovothiols, imidazole disulphides are readily reduced by glutathione. At equilibrium, the fraction of exchanged disulphides ranges from 21 to 88% (Table 1).

3.2. Inhibition of the $ONOO^-$-dependent bleaching of Evans blue dye by 4-mercaptoimidazoles

Fig. 2 shows the data D_0/D_A for different concentrations of antioxidant plotted against $[\text{antioxidant}]_0/[\text{EB}]_0$. The slopes of

Table 1
GSH Px activity and protection of test compounds against peroxynitrite-mediated oxidative modification



Entry	R_1	R_2	R_5	Catalysis (%)[a]	Exchange (%)[b]	k_A/k_{EB}^c	IC_{50}^d (μM)
1	$-CH_3$	2-Cl-C_6H_4	-H	300	45.1	13.7 ± 1.1	22.0 ± 1.6
2	$-CH_3$	3-Cl-C_6H_4	-H	369	34.0	15.2 ± 1.2	19.8 ± 1.1
3	$-CH_3$	4-Cl-C_6H_4	-H	338	27.8	15.7 ± 1.0	18.8 ± 1.2
4	$-CH_3$	3-CF_3-C_6H_4	-H	323	21.1	14.8 ± 1.4	20.7 ± 1.7
5	$-CH_3$	-H	2-Cl-C_6H_4	300	54.3	17.3 ± 1.0	4.39 ± 0.34
6	$-CH_3$	-H	4-Cl-C_6H_4	415	88.3	20.0 ± 1.3	2.81 ± 0.14
7	$-CH_3$	-H	2-CF_3-C_6H_4	315	55.0	18.1 ± 1.2	4.05 ± 0.35

[a]The catalyst's percentage increase of the basal reaction rate between GSH and H_2O_2 in the GSH Px activity test was calculated as rate of NADPH consumption+catalyst (μM/min)/rate of NADPH consumption+vehicle (μM/min) × 100.
[b]Fraction of imidazole disulphides (initial concentration 100 μM) reduced to thiols in presence of glutathione (1 mM).
[c]Ratio of the rate constants for the reaction of test compound and Evans blue with peroxynitrite.
[d]Concentration giving 50% inhibition of the oxidation of 5.0 μM DHR 123 in presence of 2.4 μM peroxynitrite.

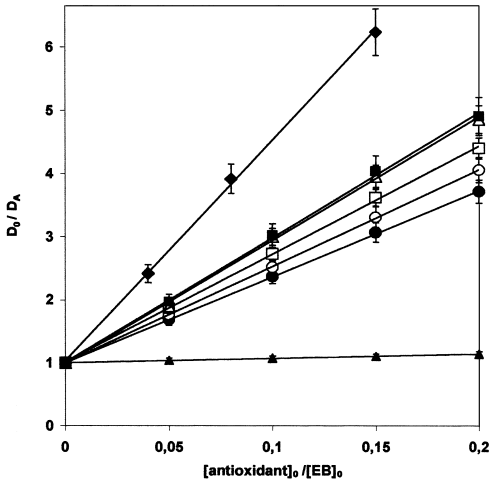


Fig. 2. Plot of D_0/D_A against $[antioxidant]_0/[EB]_0$. Room temperature, phosphate buffer (50 mM), pH 7.4, $[EB]_0 = 25$ μM. (♦) Trolox, (■) ebselen, (△) compound **6**, (□) compound **5**, (○) compound **2**, (●) compound **1**, (▲) glutathione. Results are means ± S.D. of three or more experiments.

the straight lines gave the ratios k_A/k_{EB} (Table 1). As expected, the most active compound is Trolox, which is 35 times more reactive than EB towards $ONOO^-$ ($k_A/k_{EB} = 35.6 \pm 0.9$). Given the standard deviations (Table 1), heterothiols **5–7** substituted on position 5 ($k_A/k_{EB} = 17.0–20.0$) are almost as potent as ebselen ($k_A/k_{EB} = 20.2 \pm 1.8$) while compounds **1–4** substituted on position 2 are a bit less effective. The aliphatic thiol, glutathione, is far less active ($k_A/k_{EB} = 0.68 \pm 0.08$).

3.3. Inhibitory effect of 4-mercaptoimidazoles on $ONOO^-$ induced DHR 123 oxidation

The assay performed with DHR 123 revealed the same order of reactivity for the antioxidant compounds (Fig. 3 and Table 1). Trolox and ebselen rapidly inhibit DHR 123 oxidation with half maximal inhibitory concentrations of 1.5 μM and 2.4 μM, respectively. The C-5 substituted mercaptoimidazoles **5–7** with IC_{50} values slightly superior ($IC_{50} = 2.8–4.4$ μM) to that of ebselen are six times more reactive towards $ONOO^-$ than the C-2 substituted ones **1–4** ($IC_{50} = 18.8–22.0$ μM) and largely more protective than glutathione ($IC_{50} = 70.0$ μM).

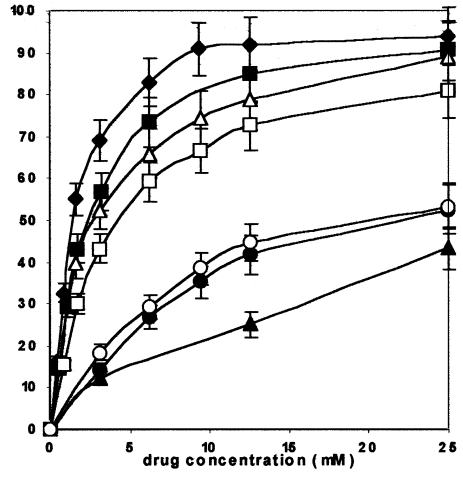


Fig. 3. Effect of antioxidants on peroxynitrite- (2.4 μM) mediated DHR 123 (5.0 μM) oxidation. (♦) Trolox, (■) ebselen, (△) compound **6**, (□) compound **5**, (○) compound **2**, (●) compound **1**, (▲) glutathione. Results are means ± S.D. of four experiments.

4. Discussion

The data reported here demonstrate a significant antioxidant activity for mercaptoimidazoles. These compounds like the natural model, ovothiols, exert GSH Px activity, which is lower than that exerted by ebselen. The catalytic action of all the tested compounds at a 20-fold higher dose is two times lower than that of ebselen. Nevertheless this activity is noteworthy since so far only a few GSH Px-mimicking molecules have been described. This activity results from two consecutive reactions: the oxidation of the thiols by hydrogen peroxide and the reduction of the disulphides by glutathione. Previous experiments showed that the compounds substituted on position 2 are more rapidly oxidised by H_2O_2 than the ones substituted on position 5 [10]. Here we study the second step and show that the C-5 substituted compounds are more efficiently recycled by glutathione than the C-2 substituted ones. The combination of these two effects must explain the similar GSH Px activity obtained for all the compounds (except for the most active thiol, compound **6**, which is the most quickly regenerated compound).

Two assays were performed to evaluate the protection of the thiols against peroxynitrite-induced oxidation. Trolox and ebselen are the most protective agents. Interestingly, compounds **5–7** substituted on position 5 are almost as potent as ebselen. This implies a very rapid reaction with peroxynitrite since the second-order rate constant for reaction of ebselen with peroxynitrite was recently determined to be 2.0×10^6/M/s [19]. Compounds **1–4** with a C-2 substitution are nearly as protective as compounds **5–7** in the Evans blue bleaching assay while they are six times less effective in the second assay using DHR 123.

Mercaptoimidazoles exhibit antioxidant potencies greater than that of the aliphatic thiol, glutathione and can be regenerated by glutathione. However the possible oxidative effect of thiols in presence of peroxynitrite must not be forgotten. Peroxynitrite oxidation of thiols is known to occur, at least partially, through one-electron transfer processes, leading to the generation of thiyl and thiyl-derived radicals [20,26,27], which are able to aggravate the peroxynitrite-induced damage particularly at low thiol:$ONOO^-$ concentration ratios [28]. Of course, this possible effect must be taken into account since thiyl radicals have been characterised upon radical scavenging by mercaptoimidazoles [11]. There is increasing interest in protective effects of sulphur, selenium-containing and phenolic compounds against peroxynitrite-induced damage. The inhibitory effects of the natural sulphur compounds aminoethylcysteine ketimine decarboxylated dimer [29], ergothioneine [17], α-lipoic acid [30], glutathione [20] and ebselen [19,31,32] have been examined in vitro. Recently, the thiol-containing compound penicillamine has been tested as a protective agent for the acute treatment of traumatic brain injury [33]. Some ovothiol derivatives need in vivo experiments and are currently investigated for their neuroprotective and anticonvulsivant properties.

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