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A NOVEL BIOLOGICALLY ACTIVE SELENO-ORGANIC COMPOUND—II

ACTIVITY OF PZ 51 IN RELATION TO GLUTATHIONE PEROXIDASE

ALBRECHT WENDEL,* MARTINA FAUSEL, HASAN SAFAYHI, GISA TIEGS and RAINER OTTER
Physiologisch-chemisches Institut der Universität, Hoppe-Seyler-Str. 1, 7400 Tübingen, Federal Republic of Germany

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Abstract—The anti-inflammatory compound 2-phenyl-1,2-benzoisoselenazol-3(2H)-on (PZ 51) catalysed GSSG formation from GSH in the presence of hydroperoxides in an NADPH/GSSG reductase system with the following rates (Δ log GSH/min per molar selenium): 1.1×10^6 with H_2O_2, 1.2×10^6 with butylhydroperoxide, 1.7×10^6 with cumenehydroperoxide. The reaction catalysed by the sulphur analogue of PZ 51 was negligible. Similar results were obtained in a direct assay of GSH-Px activity based on GSH estimation by dithionitrobenzoate. The activation energy of the reaction was determined as 55 kJ/mol·deg in the presence of 30 μmol/l PZ 51 compared to 36.5 kJ/mol·deg obtained in the presence of 1 nmol/l pure GSH-Px isolated from bovine red blood cells. In mouse liver microsomes, NADPH-dependent aminopyrine dealkylation was totally inhibited in the presence of 50 μmol/l PZ 51. *In vivo* experiments with Se-deficient mice showed that the Se-moiety of PZ 51 is not available for the synthesis of the selenoenzyme GSH-Px after dietary treatment or i.p. doses up to 25 mg Se as PZ 51 per kg body wt. After oral administration of labelled PZ 51, unlike with selenite, no radioactivity was incorporated into GSH-Px within 48 hr. The data suggest that several similarities between PZ 51 and the active site of GSH-Px exist, resulting in the capability of the compound to catalyse the GSH-Px reaction. An extracellular pharmacodynamic action of the drug seems likely.

Glutathione peroxidase (EC 1.11.1.9, GSH-Px) represents at the moment the only mammalian selenoprotein with an established stoichiometry and a clearly recognized enzymatic function [1]. It catalyses the following reaction

$$2\ GSH + ROOH \rightarrow GSSG + ROH + H_2O$$

with an extremely high specificity for GSH and almost no specificity for the type of the hydroperoxide [2]. Its complete quaternary structure [3] as well as the amino acid sequence [4] are available. The active site is composed of a selenocysteinyl residue surrounded by aromatic amino acids which are involved in the enzymatic reaction. It was therefore of interest to study the reactivity of a low molecular weight compound which contains some of the features of the enzyme's active site, i.e. a selenium atom in the vicinity of an amidelike N/O arrangement surrounded by aromatic rings.

Similar attempts have been successful with another enzyme which metabolizes activated oxygen species, i.e. copper–zinc-containing superoxide dismutase. Several copper chelates were shown to mimic the enzyme's activity at comparable rate constants *in vitro* [5]. Many of these low-molecular weight copper-complexes are at present under consideration as potential anti-inflammatory agents [6].

Interestingly enough, the selenium compound studied here was found, in initial screening studies, to exhibit *in vivo* anti-inflammatory properties [7]. The toxicity of PZ 51 is extremely low. This prompted us to examine its catalytic properties *in vitro* as well as its biological availability for the selenium moiety of GSH-Px *in vivo*.

MATERIALS AND METHODS

GSH-Px was prepared from bovine red blood cells to apparent homogeneity according to the procedure described in [8]. All organic selenium compounds and sulphur analogues were gifts from A. Nattermann & Cie GmbH, Cologne, F.R.G. Peroxides were purchased from Peroxid-Chemie, Höllriegelskreuth, F.R.G. [^{75}Se]Na_2 SeO_3 was purchased from Amersham Buchler and had a specific radioactivity of 10 mCi per mg selenium. [^{75}Se]-labelled PZ 51 had a specific radioactivity of 7.85 μCi per mg selenium.

Glutathione peroxidase activity was determined either by the reduction of GSSG formed via the NADPH–glutathione reductase system as a continuous indicator system [8] or by the determination of GSH in the reaction mixture at different fixed times by the formation of the thionitrobenzoate from Ellman's reagent [8].

For animal experiments, male albino mice were used which were fed a selenium-deficient diet or the same diet supplemented with 0.5 ppm selenium as sodium selenite (control) for the times indicated [9]. Aminopyrine dealkylation was assayed according to [10].

* Author to whom correspondence should be addressed.

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RESULTS

1. *Model experiments*

In the coupled test system using NADPH/GSSG reductase as an indicator, PZ 51 showed a linear increase in NADPH consumption up to concentrations of 20 μmol/l (cf. accompanying paper by Müller *et al.* [11]). No reaction was observed in the absence of either GSH or peroxide. The formation of GSSG was demonstrated. The following rates were obtained expressed in glutathione peroxidase units Δ log GSH/min per mol/l selenium in the form of PZ 51: 1.1×10^6 with H_2O_2, 1.17×10^6 with *t*-butylhydroperoxide (BuOOH) and 1.7×10^6 with cumenehydroperoxide (CuOOH) in the presence of 1 mmol/l GSH. Under these conditions, pure bovine red blood cell GSH-Px exhibited an activity of 10^{10} U per molar enzyme-bound selenium.

Since we noticed two reaction phases depending on the time of preincubation of the reaction constituents, we recorded the spectrum of the compound upon addition of GSH. Figure 1 shows that besides the aromatic absorption of 280 nm, PZ 51 has a maximum at 340 nm. This seems to be due to the isoselenazol five-membered ring: it is absent in the corresponding Se-methyl compound (crossed spectrum). This finding suggests that the coupled test using the optical determination of NADPH decrease at 340 nm can only be used with special precautions, e.g. a defined preincubation period with GSH. Therefore we tested PZ 51 and several other compounds in a direct GSH-Px assay. In contrast to the coupled procedure, the kinetics of this assay obey pseudo-first-order kinetics with the enzyme (Fig. 2A). The experiment showed that this type of time course is also observed with PZ 51. In the absence of hydroperoxide, essentially no reaction took place as in the blank reaction. It is important

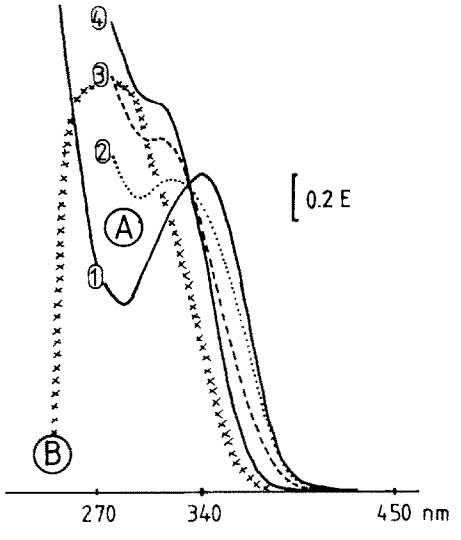

Fig. 1. Optical spectrum of PZ 51 (A) in dimethylsulfoxide before [1] and after addition of an equimolar amount of GSH [2–4]. Spectra were repeated at intervals of 50 sec. $T = 25°$. (B) Represents the spectrum of the Se-methyl derivative of PZ 51.

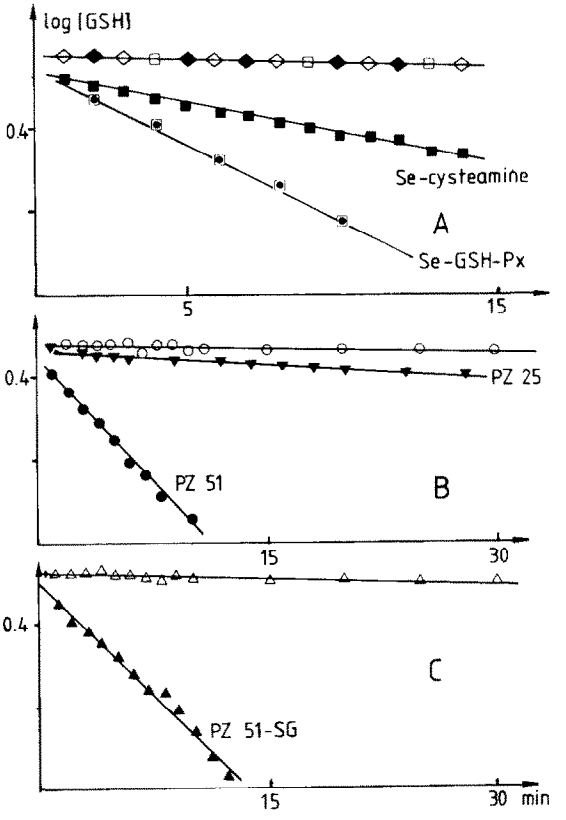

Fig. 2. Direct assay of the reaction of GSH with *t*-butylhydroperoxide in the presence of various catalysts. Conditions: 50 mmole/l potassium phosphate buffer, 1 mmole/l sodium azide, 1 mmole/l EDTA, pH = 7.0; 37°; initial concentrations = 1 mmole/l GSH, 1,2 mmole/l *t*-butylhydroperoxide (BuOOH). Systematic error 5% Ref. [8]. A: ◇ Blank in the presence of dimethylsulfoxide, ◆ 100 μmole/l cysteamine in H_2O, □ blank without GSH-Px in H_2O, ▣ in the presence of 7 nmole/l enzyme-bound selenium at 37°, in 50 mmole/l potassium phosphate pH = 7.0, ■ 100 μmole/l selenocysteamine in H_2O. B: ○ 100 μmole/l PZ 51 − BuOOH in DMSO, ● 100 μmole/l PZ 51 + BuOOH in DMSO, ▼ 100 μmole/l PZ 25 (S-analogue of PZ 51) + BuOOH in DMSO. C: △ 100 μmole/l PZ 51-SG (glutathione adduct) − BuOOH in DMSO, ▲ 100 μmole/l PZ 51-SG + BuOOH in DMSO.

to note that the reaction rates determined in this assay for *t*-butylhydroperoxide are identical to those obtained in the coupled test system. Two pairs of sulphur/selenium analogues were compared: in contrast to cysteamine, selenocysteamine exhibited a measurable "GSH-Px-like" activity (Fig. 2A), similarly the S-analogue of PZ 51, PZ 25, showed little activity compared to the selenium compound (Fig. 2B). When the selenenylsulphide obtained by reacting glutathione with PZ 51 was used in the presence or absence of BuOOH, an identical reaction rate of peroxide-dependent GSH consumption was observed as with the parent compound (Fig. 2C). This suggests that this selenenylsulphide may be formed in an non-rate-limiting step during the reaction cycle.

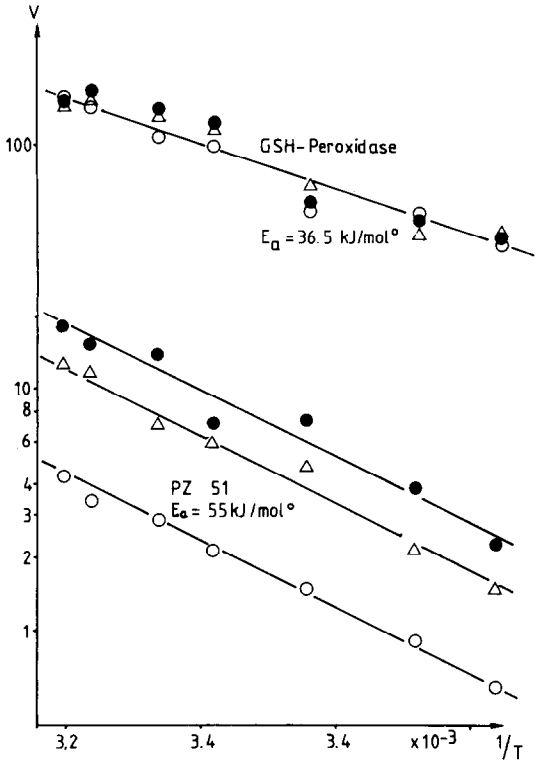

Fig. 3. Temperature dependence of the reactions of GSH in the presence of 30 μmole/l PZ 51 or 1.75 nmole/l pure red blood cell glutathione peroxidase. The selenium compounds were dissolved in ethanol and added to 250 mmole/l potassium phosphate buffer, 5 mmole/l sodium azide, 2.5 mmole/l EDTA, pH = 7.0; to a final concentration of 5% (v/v) of ethanol. The reaction rate of 1 mmole/l GSH with 1.2 mmole/l H_2O_2 (●), *t*-butyl- (△) or cumene- (○) hydroperoxide is plotted in an Arrhenius diagram.

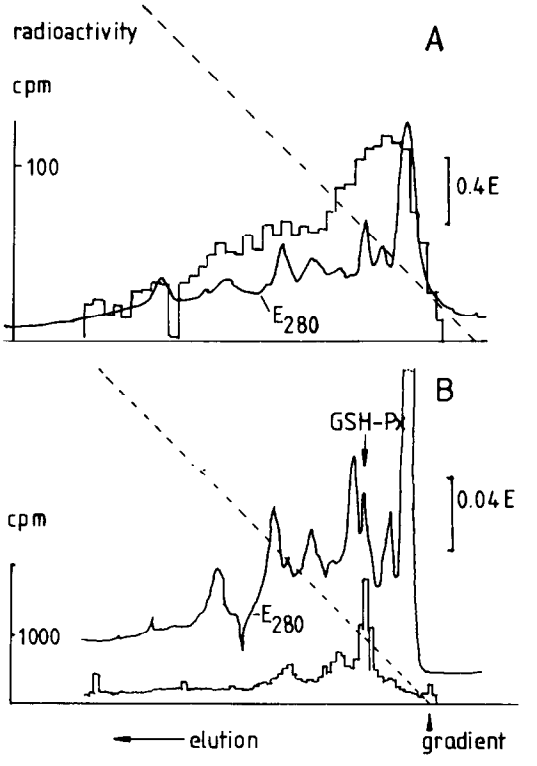

Fig. 4. (A) Incorporation of label into livers, 48 hr after application of 6 μCi [^{75}Se]-labelled PZ 51 (7.5 mg per animal) or (B) 24 hr after i.p. injection of 250 μg/kg [^{75}Se] selenite into male selenium-deficient mice. Cytosol was separated by HPLC on a polyanion Si 17 column of 10 ml and eluted with a linear gradient from 0 to 1 mole/l NaCl in 20 mmole/l Tris–HCl buffer, pH = 7.0. In experiment A, a 10-fold amount of protein was applied compared to B (200 mg protein at 21 mg/ml). Histogram = radioactivity; curve = u.v. adsorption of protein.

Figure 3 shows the temperature dependence of either the PZ 51- or GSH-Px-catalysed reaction of GSH with hydroperoxides. The Arrhenius graph yielded an apparent activation energy of 36.5 kJ/mol·deg for the enzyme-catalysed reaction, a value which is indicative of very good enzyme catalysis. The activation energy needed in the presence of PZ 51 is about 50% higher than for the enzymatic reaction with any of the peroxide substrates. This suggests an effective catalysis also by the model compound.

2. *Physiological experiments*

We examined in several types of *in vivo* experiments whether the selenium moiety of PZ 51 is available for the intracellular selenium pool used for the synthesis of GSH-Px. In a first set of experiments, a

Table 1. Glutathione peroxidase activity of various organs of mice which had been fed a selenium-deficient diet for 3 months before the animals received different dietary supplements of PZ 51 for 6 weeks

PZ 51 (ppm)	Liver	Heart	Spleen	Kidney	Lung	Serum
0	12	33	60	13	58	0.5
0.5	8	26	99	14	35	1.6
1.0	10	27	83	7	34	1.2
2.0	7	37	96	14	36	2.2
4.0	16	29	84	44	24	1.7
Se^-	13 ± 3	42 ± 6	85 ± 18	6 ± 3	51 ± 5	2.3 ± 0.7
Se^+	692 ± 54	80 ± 29	235 ± 17	208 ± 55	188 ± 15	23 ± 7

Data were obtained from organ homogenates and expressed in mU per mg of protein. The PZ 51 values are means of organs pooled from four animals. The control data are means ± S.D. of a group of six animals.

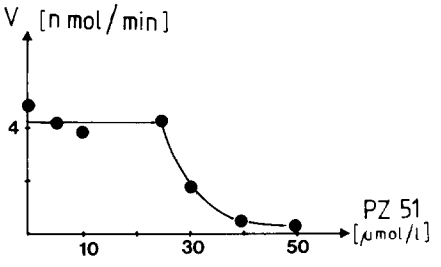

Fig. 5. Inhibition by PZ 51 of aminopyrine dealkylation in mouse liver microsomal suspensions. Conditions: 0.1 mmole/l potassium phosphate pH = 7.4; 5 mmole/l $MgCl_2$, 10 mmole/l aminopyrine, 1 mg/ml microsomal protein, 37°, 5 min preincubation, 10 min incubation.

relatively high dose of Se as PZ 51 (25 mg/kg body wt), was injected i.p. into mice which had been kept on a selenium-deficient diet for 3 months before. Half an hour, 1, 2 and 4 hr after injection the animals were killed and homogenates of liver, kidney, spleen, lung and heart were assayed for the specific activity of GSH-Px using H_2O_2 as well as BuOOH as substrates. In none of the organs was any significant difference found between the Se^- controls and the animals injected with PZ 51 (N = 4 per group, data not shown). However, an approximately twofold increase of serum GSH-Px-like activity was observed 30 min after i.p. injection of the compound to Se^- mice. This observation suggests an extracellular pharmacodynamic principle for the drug.

In a similar type of experiment, using a longer time range of up to 72 hr, 250 μg/kg selenite given to Se^- mice restored about 90% of the control GSH-Px activity, while no effect was observed with a dosage of 250 μg/kg, 2.5 mg/kg or 25 mg/kg Se as PZ 51 over the same time period in total organ homogenates as well as in serum. This means that after 72 hr no significant levels of any GSH-Px mimicking activity could be detected *in situ* within the organs investigated.

After these acute availability experiments, different dietary amounts of PZ 51 were fed over a period of 6 weeks to animals which had been on a Se^- diet for 3 months before. Table 1 shows the GSH-Px-activity determined in total tissue of various organs of mice treated this way. They suggest that dietary supplement of PZ 51 is not sufficient to lead to the synthesis of the selenoenzyme GSH-Px in selenium-deficient animals. Even in the absence of a statistical treatment of the data, these clearly negative findings seem to justify the conclusion that Se in PZ 51 is not bioavailable for utilization in GSH-Px biosynthesis.

In order to finally prove this, radioactivity labelled PZ 51 was administered orally to three Se^- mice. Livers and hearts were removed after 48 hr and first counted for radioactivity. The hearts contained practically no radioactivity while the livers showed labelling. They were pooled and subjected to high performance protein liquid chromatography. Figure 4 illustrates the elution diagrams of animals injected i.p. with [^{75}Se]selenite (B) and [^{75}Se]PZ 51 (A). The results show clearly that no significant increase in specific radioactivity in any of the fractions was observed after PZ 51 injection, while selenite was incorporated into a protein fraction that cochromatographed with GSH-Px. This finding confirms the interpretation of the feeding and injection experiments, i.e. the non-availability of PZ 51-bound Se for selenoprotein synthesis.

The observation of inhibitory effects of PZ 51 on several peroxidative processes [11, 12] led us to examine the influence of PZ 51 on a basic microsomal reaction. No effect was found on the rate of ethoxycoumarine deethylation by mouse liver microsomes. Figure 5 shows that using the aminopyrine demethylation reaction in aqueous microsomal suspensions, PZ 51 elicits an inhibitory effect which is complete at about 50 μmole/l. It is noteworthy that this effect coincides with the solubility of the compound in water which does not exceed a concentration of 30 μmole/l.

DISCUSSION

The reaction of selenoamino acids with hydroperoxides is a long-known phenomenon. Caldwell and Tappel [13] noticed already 20 years ago the superior activities of these compounds over those of their sulphur analogues. Furthermore, they suggested the reaction of selenocystine with H_2O_2 or organic hydroperoxides to be catalytic [14].

Here we studied the influence of catalytic amounts of an organoseleno compound on the very slow spontaneous reaction of the thiol GSH with hydroperoxides. The catalysis has the following characteristics:

1. PZ 51 present in under-stoichiometric amounts (30 μmole/l) is sufficient to convert 1 mmole/l GSH to GSSG.
2. The activation energy of the reaction is 1.5 times that of the reaction catalysed by the enzyme GSH-Px.
3. Once the selenium in PZ 51 is methylated or replaced by sulphur, the effect on the acceleration of the reaction velocity of GSH and ROOH becomes very poor.

These features allow us to characterize the effect of PZ 51 as a catalytic one, or transduced to a known enzymatic reaction, to speak of a GSH-peroxidase-like reaction. However, the reaction rate in the presence of the compound is several orders of magnitude less than that of the enzyme if expressed in terms of the specific selenium content. Also, on a weight basis, allowing for the 100-fold difference between PZ 51 and GSH-Px, the enzyme catalysis is still more efficient.

The present view of the reaction mechanism of GSH-Px includes an initial oxidation of the protein linked selenocysteine selenol to form a selenenic acid, which can react with a first GSH molecule to form a mixed selenenylsulphide. This intermediate could be split by a second GSH to form GSSG and reduced selenol [1, 3]. In analogy to this reaction cycle and in accordance with previous mechanistic proposals, PZ 51 could enter the catalytic cycle by formation of a mixed selenenylsulphide with GSH with a simultaneous opening of the five-membered

ring (cf. Fig. 1). This initial step may be followed by oxidation of the selenium and its reduction by a second GSH. Via elimination of water, the isoselenazol structure is reconstituted and GSSG formed. Such a reaction mechanism is supported by the catalytic activity of the PZ-GSH adduct (Fig. 2C).

In the second set of experiments aimed to elucidate the possible physiological role of PZ 51, a non-bioavailability for selenoprotein anabolism became evident. Neither after injection nor after short- or long-term administration in the diet, was an enhancement of the extremely low GSH-Px activity in Se^- mice achieved. Similar observations have been made with macrophages from up to 15 weeks Se-deficient mice [12]. This means that it is likely that the anti-inflammatory effect is not directly linked with changes in endogenous GSH-Px levels. On the contrary, an extracellularly mediated pharmacological action as shown for the copper–zinc enzyme superoxide dismutase [15] has to be taken into consideration. It is also feasible that this highly lipophilic compound is preferentially retained in membraneous compartments and there exerts its GSH-Px-like activity, acting either on H_2O_2 or organic peroxides, probably arachidonic acid derived intermediates. Indeed, PZ 51 inhibits both chemiluminescence and prostaglandin E_2 production by macrophages (cf. accompanying paper [12]). A different clue to the biological action profile of the substance may lie in its property to inhibit microsomal dealkylation reactions. This point is not the focus of the present investigation and needs further studies on the different types of microsomal reactions affected.

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