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A NOVEL BIOLOGICALLY ACTIVE SELENO-ORGANIC COMPOUND—I

GLUTATHIONE PEROXIDASE-LIKE ACTIVITY *IN VITRO* AND ANTIOXIDANT CAPACITY OF PZ 51 (EBSELEN)

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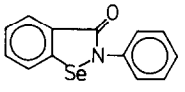
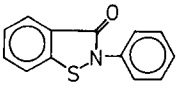
Abstract—A synthetic seleno-organic compound, 2-phenyl-1,2-benzoisoselenazol-3(2*H*)-one (PZ 51), exhibits GSH peroxidase-like activity *in vitro*, in contrast to its sulfur analog, PZ 25. In addition, PZ 51 behaves as an antioxidant shown by a temporary protection of rat liver microsomes against ascorbate/ADP-Fe-induced lipid peroxidation, an effect also elicited by PZ 25 but to a smaller extent. This protection against lipid peroxidation is independent of GSH and of P-450 monooxygenase activity.

Much of the role of selenium in biology can be attributed to the selenoenzyme glutathione peroxidase (GSH Px) [1–3]. Present as selenocysteinyl residue, the trace element undergoes a catalytic redox cycle involving the selenol and selenenic and/or seleninic acids [4, 5]. Since GSH Px catalyses the reduction of a wide variety of hydroperoxides, it together with GSH constitutes a powerful cellular defence system against so-called oxidative stress. As enzyme proteins may not readily reach intracellular target sites [6], the therapeutic use of GSH peroxidase as a tetrameric enzyme could be limited in systemic application, quite apart from potential immunological problems.

The synthesis of seleno-organic compounds which exhibit GSH Px-like activity has led to a series of substances [7], of which we have studied compound PZ51 and its sulfur analog, PZ25, as a reference compound (Scheme I); PZ25 is almost devoid of



PZ 51



PZ 25

Scheme I

GSH Px activity. The present report provides information on GSH Px-like activity *in vitro* and on an antioxidant capacity of these compounds in model systems which is independent of GSH. The latter was studied in the well-characterized microsomal system of lipid peroxidation initiated by ADP-Fe/ascorbate [8]; lipid peroxidation was assessed by the thiobarbituric acid-reactive material test, alkane production, and low-level chemiluminescence.

MATERIALS AND METHODS

Biological materials and chemicals. Liver microsomes were prepared from male Wistar rats fed on stock diet (Altromin, Lage, F.R.G.) and pretreated with phenobarbital (0.1% in the drinking water for 1 week). PZ 51 and PZ25 were kindly provided by A. Nattermann & Co. GmbH (Cologne, F.R.G.). Lyophilized GSH peroxidase (1 U/mg) from bovine erythrocytes was a gift from Prof. A. Wendel. Other chemicals and biochemicals were purchased from Fluka (Buchs, Switzerland), Merck (Darmstadt, F.R.G.), and Boehringer (Mannheim, F.R.G.).

Glutathione peroxidase activity. The assay mixture (700 μl) consisted of 50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM GSH, 1 mM sodium azide, 0.25 mM NADPH, and 1 U/ml GSSG reductase. Absorbance at 366 nm was recorded, and then a sample containing GSH Px activity (usually 50 μl) was added to estimate the sample blank. Subsequently, hydroperoxide (H_2O_2, *t*-butyl hydroperoxide, or cumene hydroperoxide) was added; appropriate blanks were run in the absence of added GSH Px activity and in the presence of hydroperoxide. With no GSH in the assay mixture, there was no absorbance decrease with PZ 51 or PZ 25 in the concentration range studied.

Microsomal incubation. Microsomes were prepared as described in [9] and were stored at −18° before use. Incubations of microsomal fractions (1 mg protein/ml) were carried out under constant oxygenation at 37° in 7.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mM ADP/16 μM $FeSO_4$. Lipid peroxidation was started upon addition of 0.5 mM ascorbate to the above incubation mixture.

Lipid peroxidation assays. The extent of lipid peroxidation in the above incubation mixture was assessed by the thiobarbituric acid-reactive material test (malondialdehyde accumulation) [10], alkane production [11], and low-level chemiluminescence [12, 13].

Malondialdehyde accumulation was measured at 535–570 nm ($\Delta\varepsilon = 156$/mM per cm) with a Sigma ZWS II Spectrophotometer (Biochem. Co., Munich, F.R.G.). Alkane evolution experiments were carried out in 43 ml-sealed flasks as described previously [14]. The system was calibrated with calibration gas (Messer-Griesheim, Duisberg, F.R.G.) and the amounts of alkanes were calculated using a correction formula for alkane dilution [11] and expressed in pmoles alkane formed per mg protein. Low-level chemiluminescence was measured with an EMI-9658 AM photomultiplier (EMI-Gencom, Plainview, New York, U.S.A.) sensitive in the 350–800 nm range. Assay conditions for chemiluminescence were described previously [14]. Results shown in the figures and the table are representative of three different microsome preparations.

RESULTS

Glutathione peroxidase activity of PZ 51 in vitro

The selenocompound PZ 51 exhibits a much more pronounced *t*-butylhydroperoxide-dependent GSH Px activity than the sulphur analog, PZ 25 (Fig. 1A). Correcting for the blank with only the hydroperoxide present, the GSH Px-like activity of the latter is almost zero. As shown in Fig. 1B, there is a linear dependence on the concentration of PZ 51 until about 20 μM, both with H_2O_2 and *t*-butyl hydroperoxide, and also with cumene hydroperoxide (not shown). The dependence of GSH Px-like activity of PZ51 and GSH Px on the concentration of GSH is shown in Fig. 1C. Whereas PZ51 shows saturation of its peroxidase-like activity beyond 2 mM GSH, GSH Px exhibits a linear response, indicating different kinetic behaviour.

Antioxidant activity of PZ 51 in microsomal lipid peroxidation

The experimental model used to study the effect of PZ 51 and PZ 25 on non-enzymic microsomal lipid peroxidation has been previously described [14]. This model consists in assessing the duration of the induction period or lag phase (τ_0), which normally precedes the initiation of the ascorbate/ADP-Fe-induced microsomal membrane lipid peroxidation.

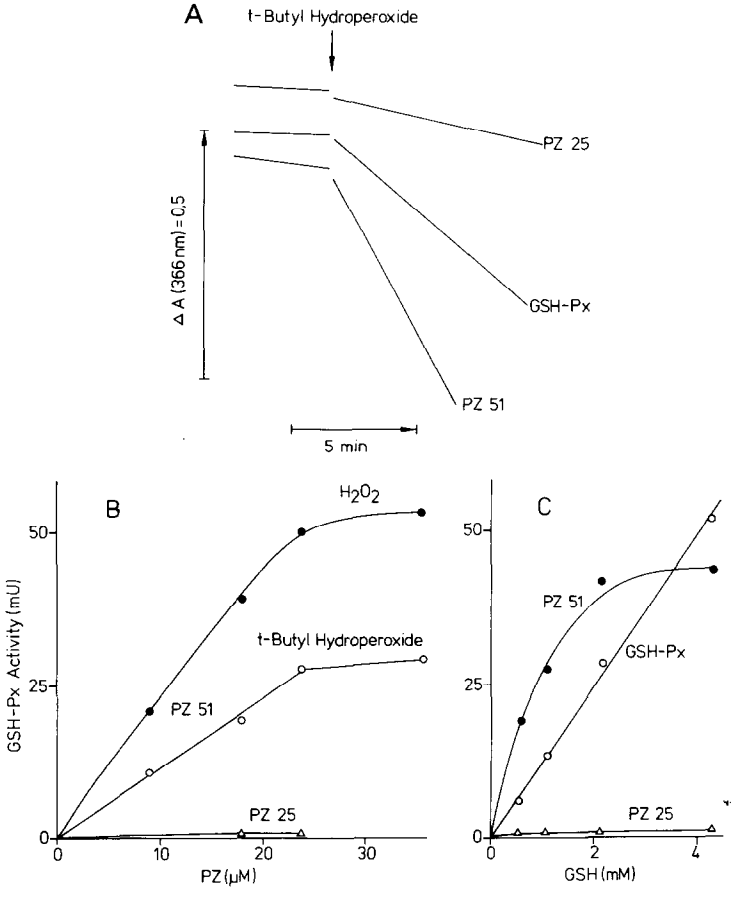


Fig. 1. GSH Peroxidase-like activity of PZ 51. (A) Absorbance at 366 nm in the assay carried out as described in Materials and Methods: note the higher blank with PZ 51 compared to PZ 25 (both 36 μM). The reaction was started upon addition of 1 mM *t*-butyl hydroperoxide. (B) Dependence of GSH peroxidase-like activity of PZ 51 and PZ 25 on concentration of the PZ compound assayed in the presence of 0.5 mM H_2O_2 or 1 mM *t*-butyl hydroperoxide, and 1 mM GSH. (C) Dependence of GSH peroxidase activity and GSH peroxidase-like activity of PZ 51 and PZ 25 on GSH concentration; measurements were carried out in the presence of 1 mM *t*-butyl hydroperoxide.

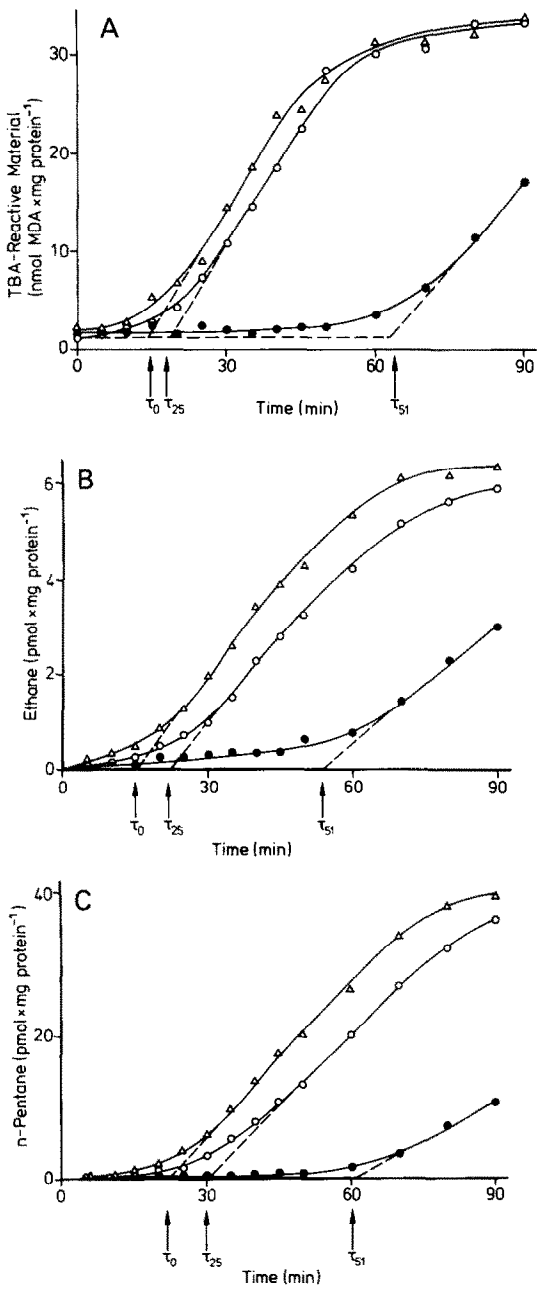


Fig. 2. Effect of PZ 51 and PZ 25 on ascorbate/ADP-Fe induced MDA (A), ethane (B) and *n*-pentane (C) production of microsomal fractions. Assay conditions as described in the Materials and Methods section and in Table 1. Time courses in the absence (△) and in the presence of PZ 51 (●) and PZ 25 (○). τ_0, induction period for control; τ_{51} for PZ 51 and τ_{25} for PZ 25.

τ_0 reflects, therefore, the occurrence of endogenous protective mechanisms against free radical attack to membrane lipids. This lag phase can be prolonged by antioxidants (τ_x).

PZ 51 (1.6 μM) considerably prolongs the lag phase (τ_{51}) of ascorbate/ADP-Fe-induced lipid peroxidation of rat liver microsomes as measured by malondialdehyde formation (Fig. 2A), alkane production (Figs. 2B and 2C), and low-level chemiluminescence (Fig. 3). A less pronounced effect is obtained with 1.6 μM PZ 25 (τ_{25}), as shown in the same figures. In spite of this prolongation of the induction period, the maximal malondialdehyde and alkane production, and chemiluminescence intensity are similar to control values. The difference between the induction periods in malondialdehyde and alkane (Fig. 2) on the one hand and chemiluminescence (Fig. 3) on the other hand might be due to the slightly different incubation procedures applied. The τ_0 and τ (1.6 μM PZ) values are listed in Table 1 for the parameters of lipid peroxidation. Since *n*-pentane has a greater lipid solubility than ethane, a slightly higher τ_0 value for *n*-pentane was observed.

The τ/τ_0 ratio, which indicates the relative increase in duration of the lag phase in the presence of PZ, would reflect the resistance of microsomes to lipid peroxidation [14]. Figure 4 shows the dependence of the τ/τ_0 ratio on PZ concentration for chemiluminescence measurements and emphasizes the greater efficiency of PZ 51 as compared to its sulphur analog PZ 25. The effect of PZ is dependent on microsomal protein concentration in the assay system (not shown), as already found with diethyldithiocarbamate in the same concentration range [14], and it might involve a specific binding to microsomal components. PZ 51 is also more effective than PZ 25 in preventing the onset of microsomal lipid peroxidation as reflected by alkane evolution (Fig. 5). At 60 min incubation time, ethane, *n*-butane, and *n*-pentane have been measured in the presence of different concentrations of PZ in the incubation mixture. Twofold higher amounts of PZ 25 than PZ 51 are necessary to observe the similar inhibitory effects on alkane production.

It should be mentioned that these effects of PZ

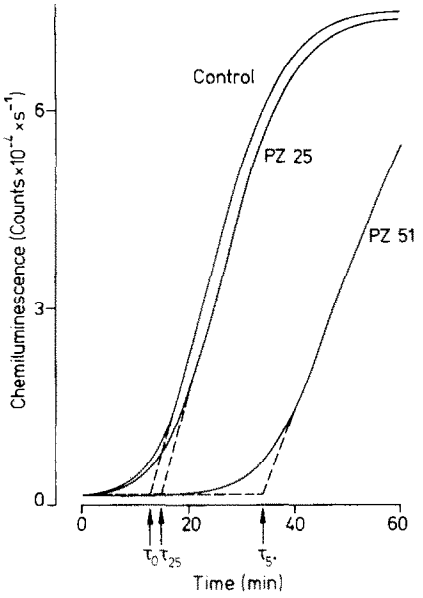


Fig. 3. Time course of chemiluminescence reaction in the absence and presence of PZ 51 and PZ 25. τ_0, τ_{51} and τ_{25} corresponding induction periods. Assay conditions as in Fig. 2.

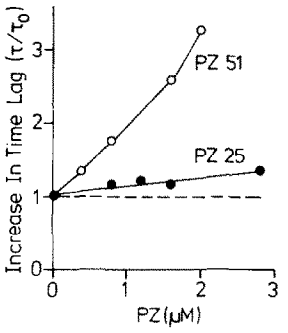


Fig. 4. Effect of different PZ 25 and PZ 51 concentrations on τ/τ_0 ratio estimated by chemiluminescence. Assay conditions as in Fig. 2.

Table 1. Induction periods of ascorbate/ADP-Fe induced lipid peroxidation from controls (τ_0), PZ 51 (τ_{51}) and PZ 25 (τ_{25}) determined by MDA, ethane and *n*-pentane production and chemiluminescence (CL) formation

Induction period (min)	MDA	Ethane	*n*-Pentane	CL
τ_0	14	15	22	12
τ_{51}	62	54	61	34
τ_{25}	18	22	30	15
τ_{51}/τ_0	4.4	3.6	2.8	2.8
τ_{25}/τ_0	1.3	1.5	1.4	1.3

Assay conditions as described in Materials and Methods. Controls: 10 μl DMSO. PZ 51 and PZ 25: 1.6 μM in 10 μl DMSO.

on microsomal fractions have been obtained in the absence of GSH in the incubation medium. This was necessary because of the antioxidant capacity of GSH itself [14–16], especially in the concentration range necessary to support the GSH Px-like activity of PZ 51.

The absence of reducing equivalents for the cytochrome P-450 monooxygenase system (as NADPH), as in the present non-enzymatic-induced lipid peroxidation, excludes a possible microsomal-dependent metabolism of the selenocompound, which might divert electrons to other pathways than the lipid peroxidation free radical process.

DISCUSSION

This study presents a synthetic seleno-organic compound, PZ 51, exhibiting a GSH Px-like activity *in vitro*; previous work by Caldwell and Tappel [17, 18] and Yasuda *et al.* [19] were centered on selenium-containing amino acid analogs (see Ref. 20 for a more detailed analysis). In addition, this new compound might display an antioxidant activity through a hypothetical free radical quenching capacity. However, it must be stressed that the term antioxidant is used in the present context as the capacity of retarding the initiation of lipid peroxidation, and it does not imply necessarily a free radical quenching activity.

The sulfur analog, PZ 25, which has no GSH Px-like activity, exerts an antioxidant property only in a higher concentration range. The molecular mechanism of this effect is not clear yet. Because the only difference between the two compounds is the exchange of Se for S, it may be assumed that the different efficiency of these compounds would be closely related to the selenium of sulphur moiety. The hydrophobic nature of these compounds might assure a better interaction with membrane lipids, thus accounting for the low concentration necessary to achieve this protecting effect against lipid peroxidation.

Whether PZ 51 is able to replace GSH Px in rats fed a selenium deficient diet, where GSH Px activity is decreased [17], or to provide an additional peroxidase activity against oxidative stress in rats fed a standard diet remains to be studied. Furthermore, it is necessary to investigate whether the effect of PZ 51 *in vitro* relies more on a peroxidase-like activity or on a more unspecific free radical quenching capacity (cf. Ref. 21).

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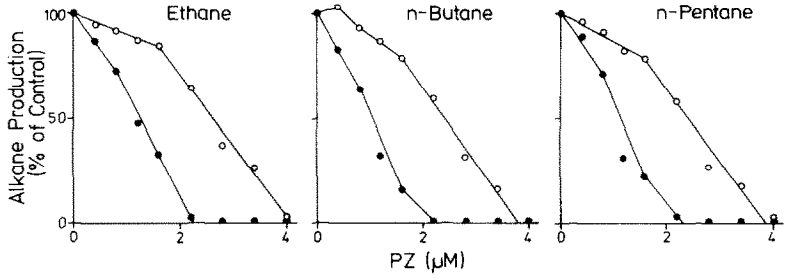

Fig. 5. Effect of PZ 25 (○) and PZ 51 (●) concentration on alkane production. Assay conditions as in Materials and Methods. PZ was dissolved in 10 μl DMSO, controls were with 10 μl DMSO. Control values for alkane production expressed in pmol × mg protein^{-1} in the PZ 25 experiment: Ethane, 6.0; *n*-butane, 1.5; *n*-pentane, 39.8; in the PZ 51 experiment: Ethane, 8.5; *n*-butane, 1.8; *n*-pentane, 51.4. Incubation time 60 min.

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