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INHIBITION OF HEPATIC GLUTATHIONE TRANSFERASES BY PROPYLTHIOURACIL AND ITS METABOLITES

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Abstract—The effects of propylthiouracil (PTU) and its metabolites on the activity of GSH transferases were examined using rat liver cytosol. PTU inhibited the enzyme activity toward both CDNB and DCNB in a concentration-dependent manner. At the concentration of 10 mM, PTU caused 25% inhibition, which was the maximum effect. PTU derivatives such as propyluracil and thiouracil showed the same effect as the parent compound. On the other hand, S-oxides of PTU such as PTU-SO_2 and PTU-SO_3, which were chemically synthesized by the oxidation of PTU, were more potent inhibitors of GSH transferases than the parent PTU. A significant inhibition was observed at a concentration of 0.1 mM of PTU S-oxides. At a concentration of 10 mM the S-oxides caused an 80% inhibition of the enzyme activity. PTU inhibited the transferase activity by competing with GSH but the S-oxides of PTU acted by another mechanism. In contrast to the effect on GSH transferases, PTU-SO_3 had a weak inhibitory effect on GSH peroxidase activity. Thus, oxidation of PTU leads to products which are potent inhibitors of GSH transferases.

Propylthiouracil (PTU)‡, a thiono-sulfur-containing compound, has various toxic properties besides its antithyroid effect [1]. It is proposed that the mechanism by which a number of thiono-sulfur-containing compounds cause toxicities may be due to the metabolism of the thiono-sulfur group [1]. The oxidative metabolites, namely S-oxides of thiono-sulfur groups, are more potent hepatotoxins than the parent compounds [2, 3]. However, little is known about the effect of S-oxides of thiono-sulfur compounds such as PTU on enzyme activity including the hepatic detoxication enzymes.

It has been reported that PTU treatment induces GSH transferases (EC 2.5.1.18) of rat liver cytosol [4], and this induction was specific for ligandin among cationic transferases [5]. GSH transferases are multifunctional proteins serving several roles in detoxication [6]. Various compounds such as steroid sulfates [7], bile acids [8], indomethacin [9, 10], diuretic drugs [11], plant phenol [12] and ciprofibrate [13] inhibited transferase activity, and the isozymes of GSH transferases were inhibited to varying degrees by some inhibitors [7, 10, 13]. In addition, a set of inhibitors caused different effects on different transferase subunits [14]. Furthermore, Yamada and Kaplowitz [15] reported that PTU inhibited GSH transferase activity of rat liver cytosol. We have confirmed that PTU also inhibited splenic transferase activity [16].

In this paper, we have synthesized oxidative compounds of PTU such as PTU-SO_2, PTU-SO_3 and PU which are thought to be metabolites of PTU in the liver and examined the inhibitory action of these compounds on GSH transferases. The results indicate that S-oxides of PTU are more potent inhibitors of the enzyme than PTU itself.

‡ Abbreviations used: PTU, propylthiouracil; GSH, reduced glutathione; PTU-SO_2, 6-propyl-4-hydroxypyrimidine-2-sulfinate; PTU-SO_3, 6-propyl-4-hydroxypyrimidine-2-sulfonate; PU, propyluracil; TU, thiouracil; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; NMR, nuclear magnetic resonance.

MATERIALS AND METHODS

Materials. PTU was purchased from Sigma Chemical Co., CDNB from Aldrich Chemical Co., DCNB from Tokyo Kasei Kogyo, and GSH and TU from Nakarai Chemicals. All other chemicals of highest grade were obtained from commercial source. DEAE-Sephadex A-50 was obtained from Pharmacia.

Synthesis of PTU metabolites. PTU-SO_3 was synthesized by an adaption of the method of Hayatsu and Yano [17]. To a fine aqueous suspension (10 ml) of PTU (170 mg) 50 mM potassium permanganate (40 ml) was added dropwise with vigorous stirring at 0°. The pH of the reaction mixture was maintained at 7.0 by the addition of 1% acetic acid. After the addition of the reagent solution was completed, the reaction mixture was allowed to stand at room temperature for 15 min, filtered, and then lyophilized. The resulting powder was recrystallized from water–ethanol in a refrigerator. The recrystallization afforded fine, white crystals (180 mg, 73% yield) of PTU-SO_3 which exhibited the characteristic u.v. spectrum in 50 mM potassium phosphate buffer, pH 7.4, having maxima at 234 and 265 nm (Fig. 1). The molar extinction coefficients of PTU-SO_3 were calculated to be 7200 and 3000 at 234 and 265 nm, respectively. The NMR spectrum was recorded in deuterated water using sodium 2,2-dimethyl-2-silapentylsulfonate as the internal standard, exhibiting signals at 0.93 (3H, t, $J = 7$ Hz), 1.72 (2H, sextet,

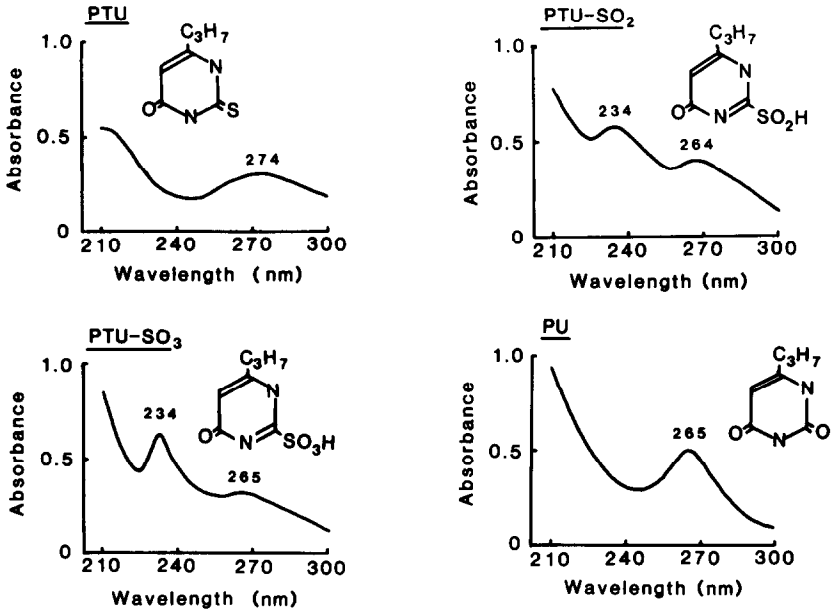

Fig. 1. The structures and u.v. spectra of PTU and its metabolites. The u.v. spectra were recorded in 50 mM potassium phosphate buffer, pH 7.4. The concentration of all compounds was 0.1 mM.

$J = 7$ Hz), 2.62 (2H, t, $J = 7$ Hz) and 6.42 (1H, s) ppm.

PTU-SO_3 was synthesized essentially by the method of Lindsay *et al.* [18] with modification. PTU (1.7 g) was dissolved in 1.0 N potassium hydroxide (30 ml). A 30% solution of hydrogen peroxide was added dropwise to the PTU solution, the temperature being kept at 0°C by using an ice bath. The mixture was kept at 0°C for an additional 10 min and then neutralized by addition of 1% acetic acid. The mixture was washed three times with *n*-butanol to remove unreacted PTU and the undesired by-product PU. All attempts to isolate the unstable sulfinic acid in free form or as ammonium or potassium salts were unsuccessful. Cellulose-DEAE (Baker Flex) thin-layer chromatography, using 0.035 M potassium phosphate buffer, pH 7.4, indicated that trace amounts of PU and PTU-SO_3 contaminated the resulting aqueous solution. Despite this trace contamination, the solution was pure enough for structural assessment by NMR spectroscopy, following reduction of the solution in volume to about 1.0 ml under reduced pressure and dilution with 2 vol. deuterated water containing sodium 2,2-dimethyl-2-silapentylsulfonate as the internal standard: 0.92 (3H, t, $J = 7$ Hz), 1.65 (2H, sextet, $J = 7$ Hz), 2.58 (2H, t, $J = 7$ Hz) and 6.26 (1H, s) ppm. For biological experiments, a further purification was carried out as follows: the aqueous solution obtained by washing with *n*-butanol was applied to DEAE-Sephadex A-50 column (1.5 × 25 cm) which has been equilibrated with freshly prepared 0.1 M ammonium carbonate. The column was eluted with 0.1 M ammonium carbonate at a flow rate of 1.0 ml/min. The effluent was monitored by u.v. absorbance at 260 nm. The purity of unstable PTU-SO_2 in the fraction ranged from 92 to 94% as judged by the recolumn chromatography and the major impurity of the fractions was PU. Since PTU-SO_2 is known to be quantitatively converted to PU by treatment with hydrochloric acid [18], aliquots of the fractions were treated with 0.1 N hydrochloric acid for the spectrophotometric determination of PTU-SO_2 concentration. The u.v. spectrum was recorded in 50 mM potassium phosphate buffer, pH 7.4, and the absorption maxima were seen at 234 and 264 nm (Fig. 1). The molar extinction coefficients of PTU-SO_2 was determined as 7800 and 4100 at 234 and 264 nm, respectively. The yield of PTU-SO_2 was found to be 51%.

As described above, PU was obtained from PTU-SO_2 treated with hydrochloric acid [18]. The structure and u.v. spectrum of PU are depicted in Fig. 1.

Preparation of enzymes. Male Sprague–Dawley rats, weighing about 250 g, were used throughout this experiment. The enzymes sources were prepared as described below: isolated liver was perfuged with 1.15% KCl and homogenized with 5 vol. of 0.1 M Tris–acetate buffer, pH 7.4, containing 0.1 M KCl and 1 mM EDTA. The homogenate was centrifuged at 10,000 *g* for 20 min. The supernatant was centrifuged for 60 min at 105,000 *g* to yield a microsomal pellet and cytosol. The cytosol was dialyzed twice against 10 mM sodium phosphate buffer, pH 7.4, and was used as an enzyme source. The microsomal pellet was resuspended with the homogenizing buffer and centrifuged again. The resulting pellet was used as the source of microsomal enzyme activity.

Assay of enzymes. The activity of GSH transferases was determined using CDNB or DCNB as substrates as described by Habig *et al.* [19]. PTU and PTU metabolites exhibit u.v. absorbance at 340 and 345 nm. In order to avoid the interference with the determination of the enzyme activity, a blank which contains no enzyme was also measured. The enzyme activity was determined by subtracting the value obtained in the absence of enzyme from that in

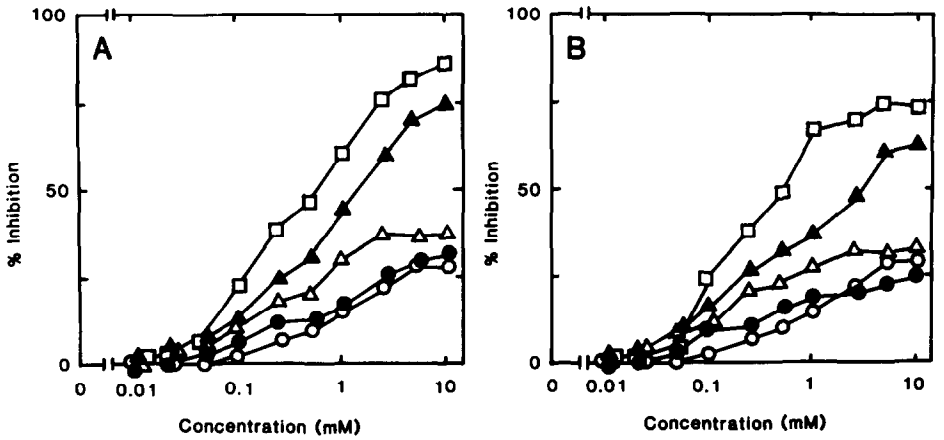

Fig. 2. Effects of PTU and its metabolites on the activity of GSH transferase in rat liver cytosol. The enzyme activity was determined in the presence of PTU (○), TU (●), PU (△), PTU-SO_2 (▲) and PTU-SO_3 (□) using CDNB and DCNB as the substrate in experiment A and B, respectively. The results are the means of values of five experiments and the S.E.M. is less than 10%. Control value in experiment A, 705.6 ± 53.8 nmole/mg/min; in experiment B, 41.5 ± 3.9 nmole/mg/min.

the presence of enzyme. The concentration of PTU metabolites was determined using their molar extinction coefficients. The activities of GSH peroxidase [20] and glucose-6-phosphate dehydrogenase [21] were also determined.

Protein was measured by the method of Lowry *et al.* [22].

RESULTS

PTU inhibited the activity of GSH transferases using CDNB as the substrate in a concentration-dependent manner (Fig. 2A) and the enzyme activity toward DCNB was also decreased by PTU (Fig. 2B). At the concentration of 5–10 mM, inhibitory effect of PTU became plateau (about 25%) in the presence of CDNB or DCNB as the substrate. This observation has been compatible with the data of Yamada and Kaplowitz [15]. Figure 2 also shows the effect of PTU analogues such as TU, a PTU compound in which the propyl group is missing, and PU, in which the sulfur atom is replaced by oxygen, on the activity of transferases. TU and PU also decreased the enzyme activity, using CDNB and DCNB, to the same degree as parent PTU.

On the other hand, S-oxides of PTU caused a more potent inhibition of GSH tranferases. As shown in Fig. 2, a significant inhibition was seen at 0.1 mM PTU-SO_2 and PTU-SO_3. At the concentration of 10 mM, PTU-SO_2 and PTU-SO_3 elicited a maximum inhibition of the enzyme activity of almost 80%. S-oxides of PTU also inhibited the enzyme activity using DCNB as the substrate. Thus, it was demonstrated that S-oxides of PTU such as PTU-SO_2 and PTU-SO_3 were potent inhibitors of GSH transferases.

As reported previously [15, 16], PTU inhibited GSH transferases by competing with GSH. The effect of PTU-SO_3 on the kinetic constants of the enzyme was examined (Fig. 3). From this result it

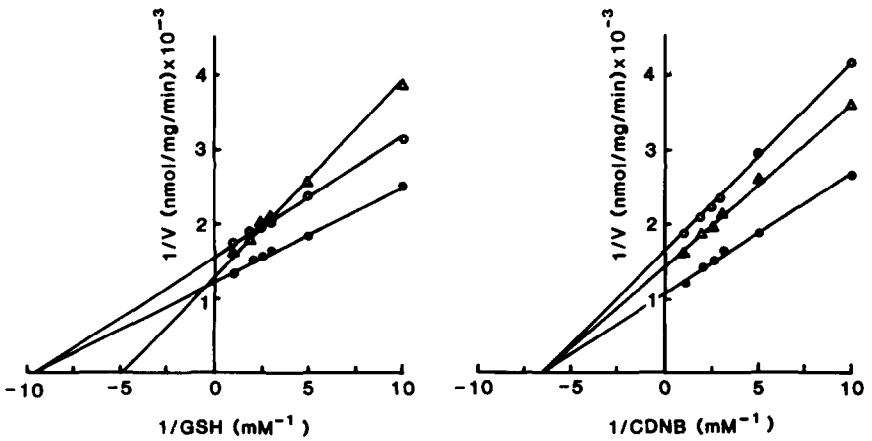

Fig. 3. Double reciprocal plots of hepatic GSH transferases. GSH transferase activity was determined in the absence (●) and presence of 10 mM PTU (△) or 1 mM PTU-SO_3 (○). Values are the means of three experiments and the S.E.M. is less than 10%.

Table 1. Effects of PTU and PTU-SO_3 on microsomal GSH transferase activity

Addition	Enzyme activity (nmole/mg/min)
None	50.9 ± 3.8 (0.0)
PTU (1 mM)	47.8 ± 4.1 (6.1)
PTU (10 mM)	30.6 ± 2.9 (39.9)
PTU-SO_3 (1 mM)	39.1 ± 2.5 (23.2)
PTU-SO_3 (10 mM)	13.6 ± 1.1 (73.3)

The activity of GSH transferase was determined using CDNB as the substrate. Values are the mean ± S.E.M. of three experiments.

Numbers in parentheses are expressed as % inhibition.

was confirmed that PTU increased the K_m value for GSH whereas PTU-SO_3 decreased the enzyme activity without affecting the K_m value for GSH. In the case of CDNB, both PTU and PTU-SO_3 decreased the V_{max} but the K_m value for CDNB remained unchanged.

Hepatic GSH transferase activity is also present in the microsomal fraction [23]. The activity of the microsomal enzyme was decreased by PTU and PTU-SO_3 (Table 1). In the microsomal fraction, PTU-SO_3 also showed a more potent inhibitory effect on the enzyme than PTU. The effect of PTU and PTU-SO_3 on the activities of GSH peroxidase and glucose-6-phosphate dehydrogenase were also determined. PTU had no effect on the activities of GSH peroxidase and glucose-6-phosphate dehydrogenase, and similarly these enzymes were minimally inhibited by PTU-SO_3 (Table 2).

DISCUSSION

The toxicity of thiono-sulfur compounds including PTU is thought to be associated in part with metabolism of the thiono-sulfur group [1]. For example, thiobenzamide produces hepatic necrosis and the hepatotoxic effect becomes more pronounced after enzymic conversion to its S-oxides [2, 3]. Therefore, it is postulated that S-oxides of thiono-sulfur-containing compounds are toxic intermediates. Lindsay *et al.* [24] reported that PTU was oxidatively metabilized in the thyroid gland, and the metabolites such as PU, PTU-SO_2 and PTU-SO_3 were detected. The effect of PTU derivatives on thyroid peroxidase activity has been examined. The inactivation of this enzyme by PTU was due to the reaction of the sulfur moiety of the compound with the enzyme [25].

In this experiment, the oxidative metabolites of PTU, namely PTU-SO_2, PTU-SO_3 and PU, were synthesized. The results of experiments with these compounds indicate that the S-oxides of PTU such as PTU-SO_2 and PTU-SO_3 are more potent inhibitors of GSH transferases than PTU. On the other hand, PU, which is PTU with the sulfur replaced by oxygen, and TU, had weak inhibitory effects (Fig. 2). Thus, S-oxidation of PTU was closely related in the inactivation of GSH transferases.

Although it had been reported that PTU was an alternative thiol substrate for the GSH transferases [15], Habig *et al.* concluded that PTU could not replace GSH [26]. However, we confirmed that inhibition of this enzyme by PTU was competitive with respect to GSH in the hepatic (Fig. 3) and the splenic cytosol [16]. Therefore, it is possible that PTU interacts with the thiol-binding site of GSH transferase. Kinetic analysis of GSH transferases revealed that PTU was a competitive inhibitor with respect to GSH, but non-competitive inhibition of GSH was observed with PTU-SO_3. Thus, the mode of action of PTU is different from that of PTU-SO_3. The inhibition of GSH transferases by PTU-SO_3 was non-competitive with respect to both GSH and CDNB, indicating that PTU-SO_3 is unlike other inhibitors of GSH transferases [7–13]. The mechanism of inhibition of GSH transferase activity by PTU S-oxides remains to be elucidated.

When PTU (1.5 mmol/kg) was administered intraperitoneally to rats, the blood level was determined using high performance liquid chromatography as described by Kim [27]. The peak level of 10.5 ± 0.3 μmole/ml (mean ± S.E.M., N = 5) occured at 1.5 hr after the injection and PTU disappeared substantially in 24 hr after the treatment. The peak concentration of PTU is enough to inhibit the activity of GSH transferases. It is likely that PTU inactivate GSH transferases *in vivo*.

In a preliminary experiment, we have detected PTU-SO_3 in the liver of rats treated with PTU (unpublished observation). It is likely that an oxidative metabolism of PTU occurs in the liver. Generally metabolism of sulfur compounds is mediated by cytochrome P-450 and flavin-containing mono-

Table 2. Effects of PTU and PTU-SO_3 on GSH peroxidase and glucose-6-phosphate dehydrogenase activities of rat liver cytosol

Addition	GSH peroxidase (μmol/mg/min)	G-6-P dehydrogenase (nmol/mg/min)
None	0.53 ± 0.02	2.48 ± 0.12
PTU (1 mM)	0.56 ± 0.03	2.53 ± 0.16
PTU (10 mM)	0.54 ± 0.02	N.D.
PTU-SO_3 (1 mM)	0.50 ± 0.02	2.47 ± 0.11
PTU-SO_3 (10 mM)	0.37 ± 0.01	2.55 ± 0.14

The activities of GSH peroxidase and G-6-P dehydrogenase were determined as described in Materials and Methods. The results are the means ± S.E.M. of four experiments.

G-6-P, glucose-6-phosphate; N.D., not determined.

oxygenase. Recently, it appears that the flavin-containing monooxygenase is the principal enzyme catalyzing oxidation of functional groups bearing sulfur [28].

It should be noted that the oxidative metabolites of PTU caused a more potent inhibition of GSH transferases than PTU. In view of the role of the enzyme system in detoxication [6], PTU toxicity may be mediated in part by inhibition of the activity of GSH transferases following metabolic oxidation of the sulfur atom.

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