Biochemical Pharmacology, Vol. 34, No. 12, pp. 2157–2161, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

INHIBITION OF PURIFIED RAT LIVER GLUTATHIONE *S*-TRANSFERASE ISOZYMES BY DIURETIC DRUGS*

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(*Received* 18 *September* 1984; *accepted* 21 *January* 1985)

Abstract—Seven soluble rat liver glutathione *S*-transferase isozymes were isolated and the inhibition of these isozymes by selected diuretics was investigated using 1-chloro-2,4-dinitrobenzene as substrate. All isozymes were inhibited to some extent under the experimental conditions used, but there was significant isozyme dependent selectivity of inhibition. The greatest inhibitory effect (over 80%) was found when the phenoxyacetic acid diuretics and indacrynic acid were incubated with glutathione *S*-transferase 3-3, 3-4 and 4-4. The sulphamoylbenzoic acid diuretics, furosemide and bumetanide, were found to have a lesser effect on the isozymes studies. As glutathione *S*-transferase are thought to play an important protective role in the various tissues of animals and man, by catalysing the glutathione conjugation of electrophilic drugs and drug metabolites, their inhibition may be toxicologically important.

Cytochrome P-450-linked drug metabolism can lead to the formation of highly reactive intermediates of drugs and other foreign compounds. The covalent binding of these intermediates to macromolecules can result in necrosis or carcinogenesis [1]. It is thought that the toxicity of many compounds depends on a balance between the activating and detoxication processes. Glutathione *S*-transferase (EC 2.5.1.18) in its multiple forms is believed to be an important detoxication enzyme system, protecting the cell against electrophilic compounds by catalysing the formation of glutathione conjugates [2].

There are several cytosolic glutathione *S*-transferase isozymes, each isozyme having a different substrate specificity [3]. We have previously reported that several diuretic drugs inhibit the unpurified cytosolic glutathione *S*-transferase activity quite strongly [4, 5].

The selectivity of inhibition of glutathione *S*-transferase isozymes may be very important as the inhibition of specific isozymes could lead to increased toxicity of compounds that would normally be conjugated to glutathione by the isozyme in question. In this study we have investigated the effect of several diuretics on purified rat liver glutathione *S*-transferase isozymes to determine the degree of specificity of this inhibition.

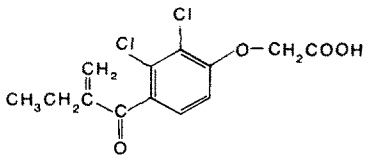
MATERIALS AND METHODS

Reagents. Reduced glutathione was obtained from Pfaltz and Bauer (Stamford, CT); 1-chloro-2,4-dinitrobenzene, 3,4-dichloronitrobenzene, *p*-nitrobenzyl chloride and 1,2-epoxy-3-(*p*-nitrophenoxy)propane, were obtained from Sigma Chemical Co. (St. Louis, MO); bumetanide from Leo Pharmaceutical Products Ltd. (Ballerup, Copenhagen, Denmark); ethacrynic and indacrynic acids from Merck Sharp & Dohme (West Point, PA); furosemide from Hoechst Roussel Pharmaceuticals Pty. Ltd. (Melbourne, Victoria, Australia) and tienilic acid from Smith Kline & French Laboratories (Philadelphia, PA).

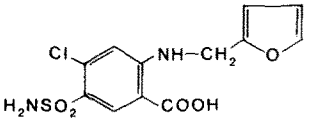
Purification of the isozymes. Male Wistar rat (200–280 g) livers were used as the source of the cytosolic fraction. The cytosolic fraction was prepared by homogenizing the livers in ice cold 0.25 M sucrose to a 15% w/v solution. The homogenate was centrifuged at 12,000 *g* for 20 min. The resulting supernatant was centrifuged at 100,000 *g* for 1 hr. This cytosolic fraction, the 100,000 *g* supernatant, was used for the subsequent isozyme separation carried out by a combination of affinity chromatography and chromatofocusing, essentially by the method of Jensson *et al.* [6] as described previously by Nicholls and Ahokas [7]. The isozymes were identified on the basis of elution profile from the chromatofocusing column and also on the basis of substrate specificity, using 1-chloro-2,4-dinitrobenzene (CDNB), 3,4-dichloronitrobenzene (DCNB), *p*-nitrobenzylchloride and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane as substrates. Methods for these assays were as described by Habig *et al.* [8].

Enzyme assays. The inhibition of glutathione *S*-transferase isozyme activities by loop diuretics (Fig. 1) was determined by using GSH (1 mM) and CDNB (1 mM) as substrates. CDNB was chosen as the second substrate because its glutathione conjugation is catalysed to a certain extent by all of the isozymes. The diuretic concentrations used are shown in the results. The assays were carried out using a double beam Hitachi 220A spectrophotometer. In each case

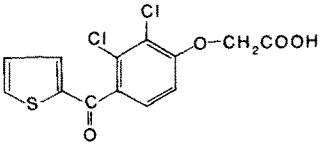
* This work was presented in part at the Annual Meeting of the Australasian Society of Clinical and Experimental Pharmacology, Newcastle, 12–14 December 1983 and the 9th International Congress of Pharmacology, London, 30 July–3 August 1984.



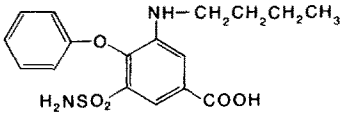
Ethacrynic acid



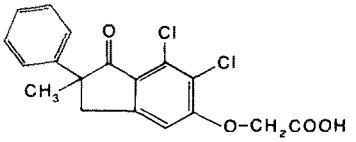
Furosemide



Tienilic acid



Bumetanide



Indacrynic acid

Fig. 1. Structures of the loop diuretics studied.

the reference side of the spectrophotometer was used to compensate for the nonenzymatic conjugation reaction.

RESULTS

Seven basic glutathione *S*-transferases were isolated from the soluble fraction of the rat liver. These were eluted as two distinct groups of three peaks from the chromatofocusing column followed by a seventh isozyme as reported earlier [7]. The first group, group I, in order of elution, was found to be composed of glutathione *S*-transferase (GSH-t) 1-1,* GSH-t 1-2, GHS-t 2-2 (also known as ligandin, GSH-t B and GSH-t AA, respectively). The second group, group II, was found to be composed of GSH-t 3-3, GSH-t 3-4 and GSH-t 4-4 (GSH-t A, GSH-t C and GSH-t D, respectively). The elution pattern was very similar to that reported by Mannervik and Jensson [9] with the exception of peak VII.

The effect of diuretics on the glutathione conjugation of CDNB by purified glutathione *S*-transferase isozymes is shown in Table 1. The diuretics caused substantial inhibition of cytosolic glutathione *S*-transferase. By purifying the basic isozymes we found that specific isozymes were affected to varying degrees. The group II isozymes were very strongly inhibited by the phenoxyacetic acid diuretics, tienilic acid and ethacrynic acid. Indacrynic acid, a structural homologue of tienilic acid, also caused very substantial inhibition of these isozymes. In contrast, tienilic acid and indacrynic acid did not cause extensive inhibition of the other group of isozymes (group I). The sulphamoylbenzoic acid diuretics, bumetanide and furosemide had differing effects. It was noted that furosemide caused a transient inhibition of all glutathione S-transferase isozymes. Figure 2 shows a time course of inhibition with the diuretics, using GSH-t 1-2 and GSH-t 3-4 as representative isozymes of their respective groups. The weakest inhibitor, bumetanide, affected the group II isozymes to a greater extent than group I. The concentration dependence of the inhibition by the diuretics is shown in Fig. 3. The very high absorption due to bumetanide at 340 nm prevented the use of this compound at the highest concentrations. The inhibition of isozyme VII followed the same pattern of inhibition as the group II isozymes, although the degree of inhibition was not as great.

DISCUSSION

Covalent binding and toxicity of a metabolically activated drug, such as paracetamol, is thought to follow the depletion of glutathione to a level which is ineffective in protecting cellular macromolecules [10]. The protection of vital cellular macromolecules could also be reduced if the glutathione conjugation of reactive intermediates was inhibited by means other than glutathione depletion. This may well occur if the glutathione *S*-transferases were extensively inhibited. We found that the phenoxyacetic acid diuretics, tienilic acid and ethacrynic acid inhibited very strongly four of the seven rat liver glutathione *S*-transferase isozymes studied. This inhibitory effect was shared by indacrynic acid, a structural homologue of tienilic acid. Three of the

* The nomenclature is based on that suggested by the Glutathione *S*-Transferase Workshop held in connection with the Meeting on Developmental, Genetic and Environmental Aspects of Drug Biotransformation and Conjugation in Dundee, Scotland, 17 September 1983.

Table 1. Inhibition of glutathione *S*-transferase isozymes by various high ceiling diuretics

		Control	Ethacrynic acid*	Tienilic acid*	Indacrynic acid	Bumetanide†
Cytosol		3.56 ± 0.037 (100)	0.137 ± 0.004 (3.8)	1.55 ± 0.022 (43.5)	1.32 ± 0.012 (37.1)	2.22 ± 0.049 (62.3)
Group I	Peak I	15.76 ± 0.04 (100)	ND (0)	8.66 ± 0.11 (55)	8.56 ± 0.06 (54.3)	12.91 ± 0.07 (81.9)
	Peak II	11.72 ± 0.08 (100)	ND (0)	10.85 ± 0.17 (92.6)	9.79 ± 0.04 (83.5)	10.87 ± 0.08 (92.8)
	Peak III	10.75 ± 0.13 (100)	ND (0)	9.32 ± 0.13 (86.7)	8.86 ± 0.13 (82.4)	10.47 ± 0.13 (97.4)‡
Group II	Peak IV	17.15 ± 0.41 (100)	0.35 ± 0.03 (2.0)	2.96 ± 0.03 (17.3)	2.33 ± 0.09 (13.6)	6.85 ± 0.14 (40.0)
	Peak V	14.14 ± 0.49 (100)	0.28 ± 0.02 (2.0)	1.36 ± 0.02 (9.6)	1.22 ± 0.03 (8.6)	5.80 ± 0.06 (40.1)
	Peak VI	12.73 ± 0.10 (100)	0.32 ± 0.03 (2.5)	1.35 ± 0.04 (10.6)	1.08 ± 0.06 (8.5)	5.63 ± 0.12 (44.2)
Peak VII		4.00 ± 0.06 (100)	0.22 ± 0.02 (5.4)	1.17 ± 0.02 (29.3)	1.25 ± 0.04 (31.3)	2.44 ± 0.07 (61.0)

The GSH transferase activity was measured using CDNB as substrate. The concentration of CDNB, GSH and the diuretics was 1.0 mM, 1.0 mM and 0.2 mM, respectively. The results are expressed as mean specific activity (μmole/min per mg protein) ± S.E. (% control activity remaining). All the activities were statistically significantly reduced by the diuretic drugs compared with the appropriate controls ($P > 0.05$; Student *t*-test) except where indicate by ‡.

* Phenoxyacetic acid diuretics.

† Sulphamoylbenzoic acid diuretic.

ND, no detectable activity under the assay conditions. Furosemide is not included in this table as the inhibition by it was not time dependent.

inhibited isozymes, GSH-t 3-3, GSH-t 3-4 and GSH-t 4-4, form a specific group, composed of 2 subunits in the 3 possible dimeric combinations of 2 homodimers and 1 heterodimer [9]. It would appear that both subunits in this group of isozymes interact with ethacrynic, tienilic and indacrynic acids.

Tienilic acid has exhibited fatal hepatotoxicity [11]. The mechanism of its toxicity has remained obscure. As it appears that tienilic acid is a very strong and selective inhibitor of GSH-t 3-3, GSH-t 3-4, GSH-t 4-4 and GSH-t VII, the possibility of a specific form of drug interaction exists. The inhibition of these isozymes could enhance the toxicity of drugs normally metabolized by the isozymes affected. It is believed that GSH-t 3-3 and GSH-t 3-4 are the isozymes involved in the detoxication of the reactive intermediate of paracetamol.* It is therefore possible that tienilic acid may enhance the toxicity of paracetamol, if administered concurrently.

However, this would not explain the lack of liver toxicity with ethacrynic acid since ethacrynic acid also caused strong inhibition of all the isozymes studied. It is known that ethacrynic acid metabolism involves the glutathione *S*-transferases in 2 ways: (1) catalysis of the glutathione conjugation by GSH-t 1-2 and 2-2 [8], which results in the formation of a mercapturic acid, the major excretion product and (2) covalent binding directly to GSH-t 3-4 [12]. Both of these processes would lead to more effective removal of ethacrynic acid, thus reducing its potential toxicity. Unlike ethacrynic acid, the other diuretics studied are not conjugated with GSH *in vitro* if the cytochrome P-450 system is absent [5].

Examination of the structures of the diuretics (Fig. 1) reveals other possibilities for explaining tienilic acid toxicity. Tienilic acid, in contrast to indacrynic acid and ethacrynic acid, has a two-substituted thiophene ring. McMurty and Mitchell [13] have shown that compounds containing a 2-substituted thiophene ring could, via cytochrome P-450 activation, produce reactive intermediates capable of causing hepatic and renal necrosis. The finding by Zimmerman *et al.* [14] that cellular injury caused by tienilic acid was potentiated by phenobarbitone induction of cytochrome P-450, supports this hypothesis. In addition, a major metabolite of tienilic acid in man was recently identified to be the 5-hydroxy metabolite [15]. The possibility exists for this to have been formed via a reactive epoxide intermediate.

It should be noted that a diacid metabolite is also a major metabolite of tienilic acid [16]. The formation of the diacid involves the removal of the thiophene ring. The metabolic fate of the thiophene moiety, a known hepatotoxin [13], has been neglected. Tienilic acid may induce hepatic damage by (a) inhibition of specific GSH-t isozymes involved in the detoxication of other drugs or (b) formation of a reactive intermediate. Alternatively, should the postulated reactive intermediate of tienilic acid be a substrate for one of the isozymes inhibited by the parent drug, it is possible that enhanced toxicity would result by inhibiting its own detoxication.

The extent of glutathione *S*-transferase isozyme inhibition caused by indacrynic acid parallels that

* B. Ketterer, personal communication.

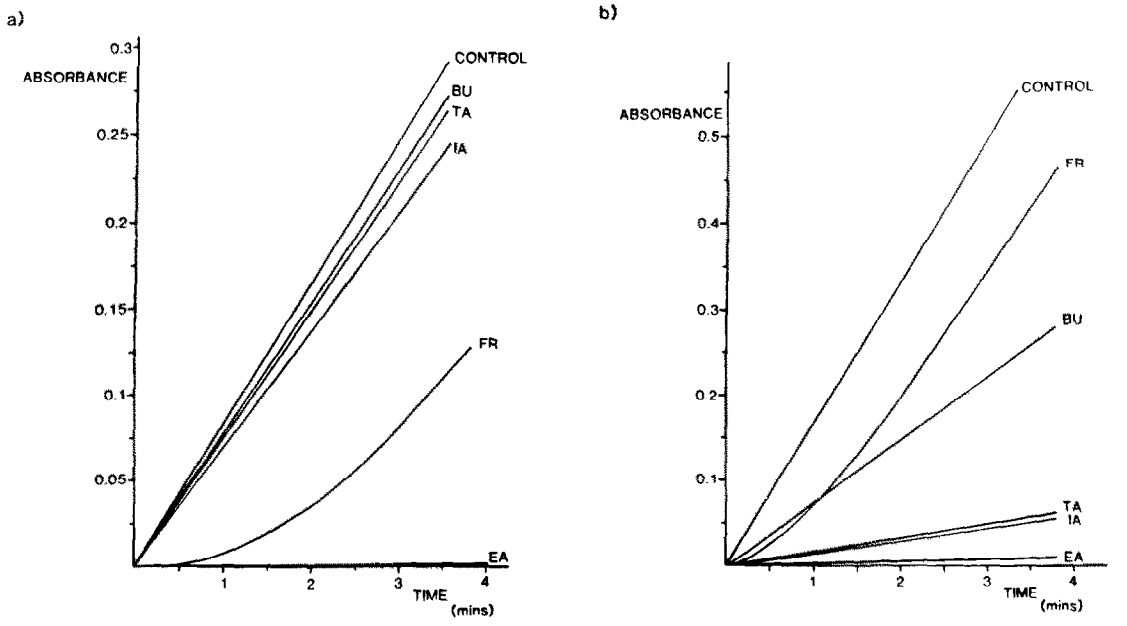


Fig. 2. Time course of conjugation with CDNB catalysed by (a) GSH-t 1-2 and (b) GSH-t 3-4, in the presence and absence of diuretics. The absorbance is measured at 340 nm. EA = ethacrynic acid, FR = furosemide, BU = bumetanide, TA = tienilic acid, IA = indacrynic acid. The following concentrations were used: GSH (1.0 mM), CDNB (1.0 mM) and the diuretics (0.2 mM).

of tienilic acid. If tienilic acid hepatotoxicity was mediated by selective inhibition of the GSH conjugation of other drugs, it is possible that indacrynic acid may share this toxicity. However, if the toxicity is fully dependent on the thiophene ring activation, similar problems would not be expected with indacrynic acid.

The consequences of inhibiting an important protective enzyme system could be toxicologically important. The effect could be similar to that of glutathione depletion. We suggest that interaction of this kind may lead to enhanced toxicity of drugs administered concurrently which are detoxified by the inhibited isozymes. It may be difficult to extrapolate from *in vitro* studies to *in vivo* effects because of lack of information on the intracellular concentrations of drugs. In case of tienilic acid it is known that after ingestion of 250 mg of the drug (normal therapeutic dose), a peak plasma concentration of 22.4 mg/l is reached after 90 minutes [16]. This gives a plasma concentration of 0.07 mmole/l. It has also been found that after administration of ^{14}C tienilic acid to rats, the drug is accumulated in the liver [17]. Therefore it is likely that intracellular concentrations of tienilic acid, comparable to the 0.2 mmole/l used in these *in vitro* studies, are possible.*

Extension of these studies to human tissues would be highly important as multiple GSH-t isozymes are known to exist [3]. In humans, genetic variability in GSH-t has also been reported [18]. The toxicological significance of these findings remains to be completely established and further investigation is currently being undertaken.

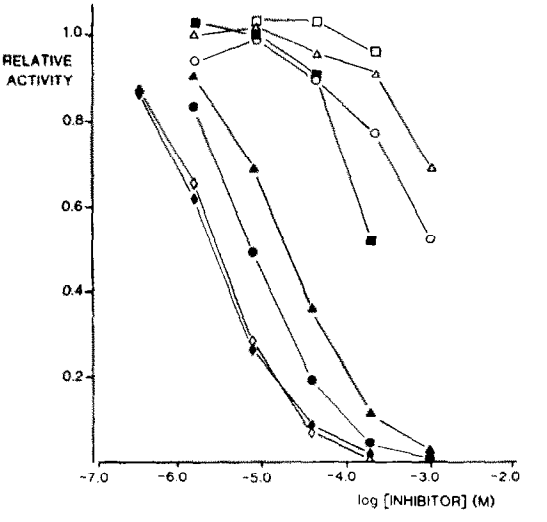


Fig. 3. The effect of concentration (0.032 mM–1.0 mM) of the loop diuretics on the activity of GSH-t 1-2 (unfilled symbols) and GSH-t 3-4 (solid symbols) activity. The concentration of both GSH and CDNB was 1.0 mM; tienilic acid (○, ●), ethacrynic acid (◇, ◆), indacynic acid (△, ▲) and bumetanide (□, ■). Each point is a mean of four determinations. The S.E. of the mean did not exceed 4%.

* It must be noted that the doses of bumetanide are 10 to 100 times lower than those of tienilic acid and therefore it is quite unlikely that the inhibition of GSH-t by bumetanide, observed here, could occur *in vivo*.

Acknowledgements—This work has been supported by the National Health & Medical Research Council of Australia (NH & MRC project grant 830070). We are also grateful for the assistance of Dr. V. Balmer of Smith Kline &

French Laboratories (Australia), Dr. P. W. Feit of Leo Pharmaceutical Products Ltd., Copenhagen, Denmark, Dr. D. Jeremy of Merck Sharp & Dohme (Australia) and Dr. F. Rudolph of Hoechst Roussel Pharmaceuticals Pty. Ltd. (Australia).

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