Biochemical Pharmacology, Vol. 40, No. 7, pp. 1631–1635, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

INHIBITION OF RAT AND HUMAN GLUTATHIONE *S*-TRANSFERASE ISOENZYMES BY ETHACRYNIC ACID AND ITS GLUTATHIONE CONJUGATE

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(*Received* 28 *March* 1990 *accepted* 18 *May* 1990)

Abstract—Ethacrynic acid, a potent inhibitor of glutathione *S*-transferases (GST), has been shown to enhance the cytotoxicity of chlorambucil in drug resistant cell lines, but a definite mechanism has not been established. Both covalent binding to GST and reversible inhibition of GST have been reported. In the present study no irreversible inhibition was observed: for all rat GST tested, inactivation was complete within 15 sec at 0°, and dialysis of GST after incubation with ethacrynic acid gave complete recovery of enzyme activity for all isoenzymes tested. Moreover, the inhibition was competitive towards 1-chloro-2,4-dinitrobenzene and non-competitive towards glutathione for rat isoenzyme 1-1. Strong inhibition of both human and rat GST of the α-, μ- and π-classes was obtained with ethacrynic acid, while conjugation of ethacrynic acid with glutathione did not abolish its inhibiting properties. For the α-, μ- and π-class I_{50} values (μM) were 4.6–6.0, 0.3–1.9 and 3.3–4.8, respectively for ethacrynic acid, and 0.8–2.8, <0.1–1.2 and 11.0, respectively for its glutathione conjugate. Of all isoenzymes tested the human isoenzyme μ is most sensitive to the action of both ethacrynic acid and its glutathione conjugate.

Recently it has been postulated that glutathione *S*-transferase (GST†) isoenzymes play a role in the intrinsic and acquired resistance to cytostatic drugs [1, 2]. Many electrophilic carcinogens and mutagens were found to be substrates of the GST [3], and also some cytostatics [4]. GST can also bind a wide range of xenobiotics covalently, thus showing a second mode of action by which reactive intermediates may be detoxified [5, 6]. Moreover, GST plays a role in the biosynthesis of eicosanoids, which are involved in several processes related to cell proliferation, cell differentiation, hyperplasia and neoplasia [7]. It has been shown that GST activity is enhanced in human breast cancer cells resistant to Adriamycin® [8], while levels of glutathione have been found to be elevated in arsenic-resistant Chinese hamster ovary cells [9] and cisplatin-resistant rat ovarian cell lines [10]. Inhibition of GST would thus be potentially beneficial in the treatment of tumors.

Ethacrynic acid, a diuretic drug, enhances the cytotoxicity of chlorambucil in Walker 256 rat breast carcinoma cells with acquired resistance to nitrogen mustards as well as in two human colon carcinoma cell lines [11]. Interestingly, ethacrynic acid has been shown to be a very potent inhibitor of rat isoenzymes of GST [6, 12, 13], while it can also decrease glutathione levels due to a conjugation reaction, both spontaneously and GST-catalysed [14]. The nature of the inhibition of GST by ethacrynic acid is not well documentated: uncertainty exists about the reversibility of the inhibition. It has been reported that *in vivo* some covalent binding to rat isoenzyme 3–4 occurs [15], but its relevance to the enzymatic activity was not investigated. Irreversible inhibition would correlate GST activity to *de novo* synthesis of GST. On the other hand, reversible inhibition would relate the inhibition to the pharmacokinetics of ethacrynic acid.

The present paper describes the nature and the concentration dependency of the inhibition by ethacrynic acid, for both rat and human isoenzymes of GST. The inhibition characteristics of the glutathione conjugate of ethacrynic acid were separately investigated, since conjugation with glutathione will almost certainly occur in the target cells.

MATERIALS AND METHODS

Chemicals. Ethacrynic acid (Sigma Chemical Co., St Louis, MO, U.S.A.) was dissolved in absolute ethanol; its glutathione conjugate in 0.1 M sodium phosphate buffer pH 6.5 with 1 mM EDTA (Merck, F.R.G.), both stored (not longer than 3 hr) at 0°.

Synthesis of the glutathione conjugate of ethacrynic acid. One hundred mg of ethacrynic acid in 5 mL of ethanol/water (1:1) was added to 100 mg of glutathione in 5 mL of ethanol/water (1:1) with 10 droplets of a saturated solution of $NaHCO_3$, and stirred at room temperature for 48 hr. The solvents were evaporated and the residue was dissolved in 2 mL of saturated $KHCO_3$, and a solution of 1% H_3PO_4 was added, until the solution became cloudy. The resulting precipitate was filtrated and dried. The identity was confirmed by fast atom bombardment mass spectrometry (m/z = 609.9), the conjugate was more than 99% pure as judged by HPLC-analysis (RP18 hypersil ODS column, elution with a solution of 1% acetic acid in water and a linear gradient from 10 to 100% acetonitrile in 20 min, k' = 4.4).

Purification of GST. Glutathione *S*-transferase isoenzymes were purified from liver and kidney of rats

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† Abbreviations: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

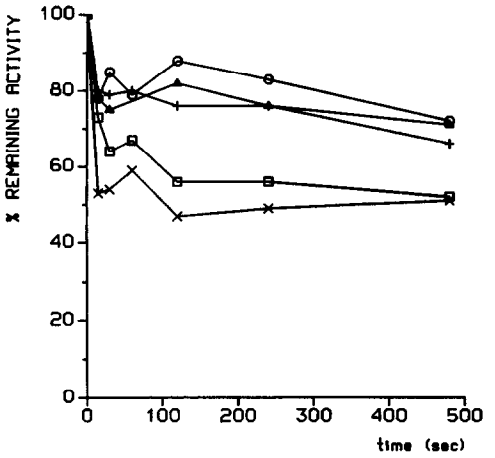

Fig. 1. Time course of inhibition of rat GST isoenzymes 1-1 (□), 2-2 (○), 3-3 (△), 3-4 (+) or 4-4 (×). Five μM ethacrynic acid and 0.25 μM enzyme were mixed at 0°, and a 25-pmol enzyme sample (50 pmol for 1-1) was transferred into a cuvette containing 1 mM CDNB, and 1 mM glutathione was added and conjugation was measured at 340 nm. The results are the average of two incubations.

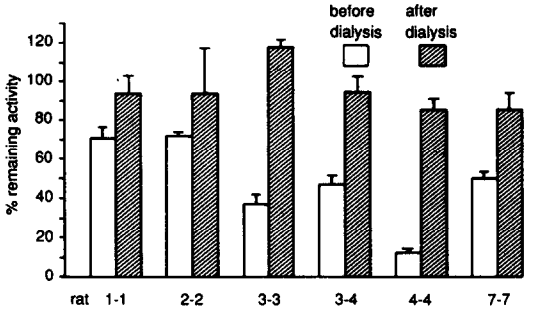

Fig. 2. Effect of dialysis on the remaining activity of rat GST isoenzymes. Five μM enzyme was incubated with 500 μM ethacrynic acid at room temperature, and a 50-pmol (for 1-1, 2-2, 3-3 and 3-4) or a 100-pmol (for 4-4 and 7-7) enzyme sample was transferred into a cuvette with 1 mM CDNB and 1 mM glutathione was added. Approximately 0.7 mL was dialysed overnight at 4° in 400 mL 0.1 M sodium phosphate buffer pH 6.5 with 1 mM EDTA. Controls were treated similarly. Results are expressed as the per cent of control (±SE) of 2–4 incubations, loss of activity during this dialysing procedure was always less than 25%.

[phenobarbital-induced male Sprague–Dawley rats, 15 weeks of age, treated for 7 days with 0.1% (w/v) sodium phenobarbital in drinking water] and human liver and placenta using *S*-hexylglutathione affinity chromatography. Separation of the GST isoenzymes of human liver (B1B2, μ), human placenta (π) and rat kidney (7-7) was achieved with chromatofocusing on a Pharmacia FPLC system equipped with a mono P-column, as previously described [16]. GST isoenzymes of rat liver were separated by ion-exchange chromatography using CM-sepharose fast flow (Pharmacia) [14]. Purity was confirmed by HPLC analysis [17], and isoelectric focusing [16]. Specific activities with CDNB as second substrate (see below) were 38.0, 10.0, 35.1, 32.7, 17.1 and 14.0, respectively, for rat 1-1, 2-2, 3-3, 3-4, 4-4, 7-7 and 19.3, 73.5 and 69.3, respectively, for human B1B2, μ and π.

Incubations. In order to detect a time-dependent covalent inhibition of GST by ethacrynic acid, 0.25 μM GST was incubated with 5 μM ethacrynic acid at 0°: At various time intervals a 0.1-mL sample was transferred into a cuvette containing CDNB and glutathione (both 1 mM) and the inhibition of enzymatic CDNB conjugation was measured at 340 nm (see below).

Reversibility of the inhibition was analysed by dialysing experiments: 5 μM GST was incubated with 500 μM ethacrynic acid for 15 min at room temperature. Activity was determined, and the incubate (approx. 0.7 mL) was dialysed overnight at 4° against 400 mL of 0.1 M sodium phosphate buffer pH 6.5 with 1 mM EDTA, after which the specific activity was determined.

To determine the nature of the reversible inhibition 25 nM rat 1-1 was incubated with 0.3–1.2 mM CDNB and 50 nM rat 1-1 with 0.025–1 mM glutathione with 1.0, 2.5, 5.0 or 10 μM ethacrynic acid in duplicate (for enzyme assay see below).

Fifty nM enzyme was incubated (triplicate) with 0.1, 0.5, 1.0, 5.0, 10 and 200 μM ethacrynic acid or its glutathione conjugate to study the concentration dependency of the inhibition. To determine maximal inhibition, 1/fractional inhibition was plotted versus 1/[inhibitor], for both ethacrynic acid and its glutathione conjugate inhibition was complete. I_{50} values were obtained from plots.

Enzyme assays. The activity of GST with CDNB was determined according to Habig *et al.* [14], in 0.1 M sodium phosphate buffer pH 6.5 with 1 mM EDTA; the conjugation was initiated by adding glutathione in order to minimize effects from the glutathione conjugate of ethacrynic acid on the initial rate of conjugation. To determine apparent K_m and V_{max} with ethacrynic acid, 1.0 μM enzyme was incubated in duplicate with 12.5–200 μM ethacrynic acid and 1 mM glutathione in 0.1 M sodium phosphate buffer pH 6.5 with 1 mM EDTA. The conjugate was determined spectrophometrically at 270 nm (extinction coefficient 5.0 $mM^{-1}\,cm^{-1}$), corrections were made for chemical reactivity. Apparent K_m and V_{max} were obtained from Lineweaver–Burk plots. In all experiments the control samples were treated in the same way. Enzyme concentrations are expressed as subunit concentrations.

RESULTS

Reversible or irreversible inhibition

Two assays were performed in order to discriminate between reversible and irreversible inhibition of GST by ethacrynic acid. Firstly, the time course of inhibition was determined at 25°: inhibition was complete within 15 sec (rat isoenzymes 4-4 25 nM, and 1-1 50 nM both with a 200-fold molar excess). Since the initial rate of the reaction might be very fast, experiments were subsequently performed at 0°. However, for all rat GST isoenzymes tested, inactivation was still complete within 15 sec, i.e. the first time interval measured (Fig. 1).

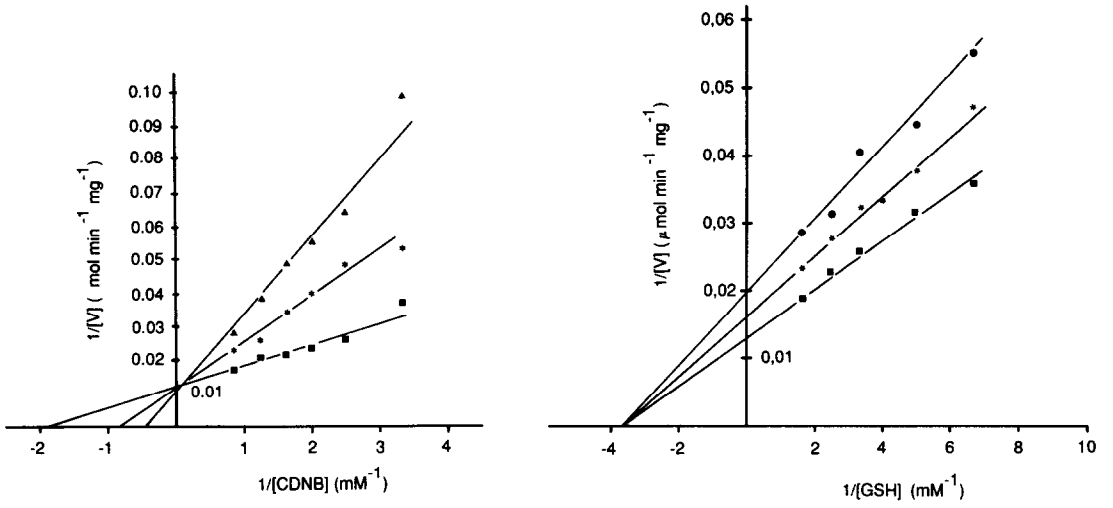

Fig. 3. Lineweaver–Burk plot showing competitive inhibition of rat GST isoenzyme 1-1 towards CDNB by 5 (*) or 10 μM (△) ethacrynic acid (left) or non-competitive inhibition towards glutathione (right) by 1.0 (*), 2.5 (○) μM ethacrynic acid. Control (□). The values are the average of three incubations. Experiments were performed as described in Materials and Methods.

Table 1. I_{50} (μM)* values for the inhibition of the major isoenzymes glutathione S-transferases, of both rat and human, with ethacrynic acid and its glutathione conjugate

	α-class			μ-class				π-class	
	Rat 1-1	Rat 2-2	Human B1B2	Rat 3-3	Rat 3-4	Rat 4-4	Human μ	Rat 7-7	Human π
Ethacrynic acid	6.0	4.6	6.0	1.9	1.9	0.8	0.3	4.8	3.3
Glutathione conjugate	1.2	2.8	0.8	1.2	1.0	1.0	<0.1	11.0	11.0

* The concentration of ethacrynic acid or its glutathione conjugate resulting in 50% inhibition of the enzymic activity (I_{50}) was determined by incubating 50 nM of GST with a concentration range of the inhibitors. For individual values, the coefficient of variation was less than 15%.

Secondly, incubations of GST with ethacrynic acid were performed, after which unreacted ethacrynic acid was removed by dialysis. Although during the incubation the isoenzymes were inhibited up to 90%, the activity of GST was fully recovered after dialysis (Fig. 2): the remaining activity ranges between 85 and 118%, with no significant differences from the controls ($P = 0.05$, Student's t-test).

Nature of the reversible inhibition

To obtain information about the nature of the reversible inhibition, enzyme kinetics experiments were conducted with rat isoenzyme 1-1 and ethacrynic acid. With CDNB as the variable substrate, the apparent K_m increased, while V_{max} did not change (Fig. 3), indicating that ethacrynic acid competes with CDNB. With glutathione as the variable substrate, the apparent K_m remained unchanged while V_{max} decreased (Fig. 3), indicating non-competitive inhibition towards glutathione.

Isoenzyme selectivity of the inhibition by ethacrynic acid and its glutathione conjugate

For both ethacrynic acid and its glutathione conjugate the I_{50} values were determined towards all major human and rat GST isoenzymes (Table 1).

Strong inhibition of both human and rat GST of the α-, μ- and π-classes was obtained with ethacrynic acid, as well as with its glutathione conjugate. Human and rat isoenzymes of the μ-, π-, and α-classes in general showed the same trends of inhibition. There are some interesting differences between the various classes: the glutathione conjugate of ethacrynic acid inhibits the α- and μ-class more strongly than ethacrynic acid itself (with the exception of rat 4-4), while it inhibits the π-class to a lesser extent than ethacrynic acid itself. Of all isoenzymes tested the human isoenzyme μ is the most strongly inhibited isoenzyme, in particular by the glutathione conjugate of ethacrynic acid.

The apparent K_m values of the rat GST isoenzymes with ethacrynic acid were determined. Some correlation exists, between K_m and I_{50} (Table 2), with the exception of rat 7-7.

DISCUSSION

Recently there has been considerable interest in the use of relatively non-toxic inhibitors of GST activity to enhance the effect of cytostatics in the treatment of tumors. The present study indicates

Table 2. Apparent K_m* (μM) and V_{max} (μmol/min/mg) of the major GST isoenzymes of the rat towards ethacrynic acid

	Isoenzymes				
	1-1	2-2	3-3	4-4	7-7
K_m	90	200	50	50	120
V_{max}	0.13	1.33	0.39	0.44	2.50

* For details of the assay, see Materials and Methods.

that ethacrynic acid and its glutathione conjugate are promising *in vivo* inhibitors of both rat and human isoenzymes of GST, especially of human μ. There has been some discussion about the reversibility of the inhibition [15]. *In vivo*, ethacrynic acid was found to bind covalently to isoenzyme 3-4 with a maximum of one molecule per three molecules of heterodimeric rat isoenzyme 3-4, 90 min after injection in rats [15]. After dialysis of rat GST isoenzymes incubated with ethacrynic acid the enzymic action is completely restored, and furthermore there is no time-dependent inhibition: both findings indicate a reversible mechanism of inhibition. This is confirmed by the kinetic data obtained with rat GST isoenzyme 1-1 and ethacrynic acid: competitive inhibition with respect to CDNB and non-competitive inhibition with respect to glutathione was observed, as may be expected for a compound which is an electrophilic substrate of GST. Our results indicate that covalent binding, if it occurs, does not result in inhibition of the enzymic activity. Nevertheless, ethacrynic acid and its glutathione conjugate strongly inhibit rat and human GST. There are some differences between the various classes of isoenzymes, while in general there are only small differences between human and rat GST within the same class. It is interesting that the π-class, which has the highest activity towards ethacrynic acid as a substrate, also is inhibited to the smallest extent by the glutathione conjugate of ethacrynic acid. The high enzymatic activity of the π-class towards ethacrynic acid, could perhaps be explained by the relative affinity of the glutathione conjugate for the enzyme, causing the product to leave the active site relatively quickly. In general, the μ-class is the most strongly inhibited class of isoenzymes, both by ethacrynic acid and its glutathione conjugate, the human μ isoenzyme being even more strongly inhibited than its rat analogs. In agreement with this result, it has been shown that ethacrynic acid inhibits the leukotriene C_4 production in human neutrophils [18]. The human μ isoenzyme is involved in the conjugation of glutathione to leukotriene A_4, resulting in the formation of leukotriene C_4 [19].

Non-toxic concentrations of ethacrynic acid have been shown to potentiate the cytotoxic activity of chlorambucil in Walker 256 rat breast carcinoma cells with acquired resistance to nitrogen mustards, and in human colon carcinoma cell lines [11]. Two hypotheses were formulated to explain the enhanced cytotoxicity. Firstly, inhibition of GST may result in a longer lifetime of chlorambucil, and secondly synergetic effects of ethacrynic acid and chlorambucil on the biosynthesis of prostaglandins may enhance the cytotoxicity [7, 11, 20]. Indomethacin, an inhibitor of GST and an anti-inflammatory drug has been shown to potentiate the cytotoxicity of chlorambucil in CHO cells resistant to nitrogen mustards, while acetylsalicyclic acid, an anti-inflammatory agent, caused no potentiation of chlorambucil cytotoxicity, suggesting that the potentiation is not due to the effects on prostaglandin synthesis [21]. A modest inhibition of GST activity in the nitrogen mustards resistant cell lines has been observed with ethacrynic acid [11]. The actual inhibition of GST by ethacrynic acid and its conjugate may be larger than the observed inhibition, because the intracellular glutathione concentration also decreased [12]. Within 4 hr about 60–70% of a dose of [^{14}C]ethacrynic acid (5 or 50 mg/kg) was excreted into the bile of rats, while approximately 40% was in the form of the glutathione conjugate [22], indicating a high turnover.

Thus, *in vivo* ethacrynic acid temporarily inhibits GST directly; this effect is enhanced by GSH depletion; and lastly the inhibitory effect is prolonged by the glutathione conjugate.

Acknowledgement—This investigation has been supported by the NWO-foundation for Medical and Health Research MEDIGON (Grant 900-521-124).

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