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ISOENZYME SELECTIVE IRREVERSIBLE INHIBITION OF RAT AND HUMAN GLUTATHIONE *S*-TRANSFERASES BY ETHACRYNIC ACID AND TWO BROMINATED DERIVATIVES

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Abstract—In the present study it has been shown that ethacrynic acid can inhibit glutathione *S*-transferase (GST) of the pi-class *irreversibly*. [^{14}C]Ethacrynic acid, 0.8 nmol/nmol human P1-1 and 0.8 nmol/nmol rat GST 7-7 could be incorporated, resulting in 65–93% inhibition of the activity towards 1-chloro-2,4-dinitrobenzene (CDNB). Isoenzymes of the alpha- and mu-class also bound [^{14}C]ethacrynic acid, however without loss of catalytic activity. Incorporation ranged from 0.3 to 0.6 and 0.2 nmol/nmol enzyme for the mu- and alpha-class GST isoenzymes, respectively. For all isoenzymes, incorporation of [^{14}C]ethacrynic acid could be prevented by preincubation with tetrachloro-1,4-benzoquinone, suggesting, that a cysteine residue is the target site. Protection of GST P1-1 against inhibition by ethacrynic acid by the substrate analog *S*-hexylglutathione, indicates an active site-directed modification. The monobromo and dibromo dihydro derivatives of ethacrynic acid were synthesized in an effort to produce more reactive compounds. The monobromo derivative did not exhibit enhanced irreversible inhibitory capacity. However, the dibromo dihydro derivative inhibited both human and rat GST isoenzymes of the pi-class very efficiently, resulting in 90–96% inhibition of the activity towards CDNB. Interestingly, this compound is also a powerful irreversible inhibitor of the mu-class GST isoenzymes, resulting in 52–70% inhibition. The two bromine atoms only marginally affect the strong (reversible) competitive inhibitory capacity of ethacrynic acid, with IC_{50} (μM) of 0.4–0.6 and 4.6–10 for the mu- and pi-class GST isoenzymes, respectively.

The glutathione *S*-transferases (GST§) are a multigene family of isoenzymes which catalyse the reaction between numerous electrophilic compounds and glutathione (GSH) [1–3]. In addition, GST can also act as a peroxidase and as a binding protein for a variety of organic compounds [1]. Mammalian cells contain both cytosolic and membrane-bound isoenzymes. The cytosolic GST have been divided into four gene families, the alpha-, mu-, pi- and theta-class [4]. They exist as dimers, with heterodimers occurring within the same class [1].

Considerable evidence indicates that the GST are, in addition to many other factors, involved in cellular drug resistance [5–10]. Drug resistance to cytostatics used in cancer treatment can emerge at the start of the therapy (intrinsic) or as a response to therapy (acquired). GST are implicated in particular in the resistance to alkylating agents such as chlorambucil, melphalan and nitrosoureas, and to redox cycling drugs such as Adriamycin®. Further exploration of the role of GST in drug resistance, and potentially, in modulation of the chemotherapy could be based on selective modifications of the GSH/GST system [7].

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§ Abbreviations: GST, glutathione *S*-transferases; GSH, glutathione; DMF, dimethyl formamide; CDNB, 1-chloro-2,4-dinitrobenzene.

The diuretic drug ethacrynic acid has been shown to be an excellent competitive inhibitor of the GST system: the GST substrate ethacrynic acid depletes GSH and inhibits GST strongly [11, 12]. Moreover, the GSH conjugate formed inhibits GST as efficiently as ethacrynic acid itself [12]. IC_{50} values ranged from 0.1 to 11 μM for ethacrynic acid and its GSH conjugate, and increased in the order mu-, alpha- and pi-class [12]. Tew *et al.* [13] were the first to show that sensitization of cultured tumor cells to alkylating drugs can be achieved by ethacrynic acid, and several similar studies have been reported since [14, 15].

Ethacrynic acid can react in a Michael-type reaction with cysteine, and already in 1978, ethacrynic acid was shown to bind to GST [16], in particular to rat GST 3-4 [17]. However this does not result in an inactive enzyme [12]. In general, cysteine modification does not always inactivate the GST, the effects are known to vary for each isoenzyme [18–21]. Since ethacrynic acid and its GSH conjugate are known to act directly as strong competitive inhibitors of GST, the present study was designed to investigate the irreversible inhibitory capacity for individual GST isoenzymes. In addition, two derivatives of ethacrynic acid were synthesized, which would also display alkylating ability, namely the monobromo derivative, which could undergo a Michael-type addition–elimination reaction and the dibromo dihydro derivative which possesses an electrophilic alpha-halogeno ketone moiety.

MATERIALS AND METHODS

Chemicals and synthesis. [^{14}C]Ethacrynic acid was from Amersham (Amersham, U.K.) (15 mCi/mmol). Dibromo dihydro ethacrynic acid was prepared by treatment of ethacrynic acid with bromine as follows: 500 mg of ethacrynic acid was dissolved in 20 mL of methylene chloride in a 100 mL flask, thoroughly flushed with N_2 and protected from light. Under a N_2-atmosphere 68 μL of Br_2 in 20 mL of methylene chloride was added dropwise until the brown color of Br_2 stopped disappearing. The solvents were removed by evaporation. The disappearance of ethacrynic acid was checked with TLC (stationary phase: silica gel; mobile phase: methylene chloride/methanol/acetic acid (50/50/1, by vol.); and detection with a 1% $KMnO_4$/2% Na_2CO_3 solution). The monobromo ethacrynic acid was prepared as follows: 657 mg of dibromo dihydro ethacrynic acid was added to 50 mL of dry dimethyl formamide (DMF). Air was removed under vacuum, and the flask was brought into a N_2-atmosphere. K_2CO_3 (460 mg) was added and the solution was stirred overnight in the dark. K_2CO_3 and DMF were removed by acid extraction: 50 mL of methylene chloride and 200 mL of deionized H_2O were added, while after shaking the pH of the waterphase was adjusted with 1 N HCl to 5–6. The waterphase was further extracted with 25 mL methylene chloride. The combined methylene chloride phases were washed twice with 400 mL of a HCl solution (pH 4–5). The methylene chloride was removed by evaporation until a viscous oil was obtained. TLC (see above) was used to follow the formation of a double bond. Monobromo ethacrynic acid was purified by preparative HPLC, using a Zorbax ODS (250 mm × 21.2 mm i.d.) column, eluted with a gradient of 50–95% in 100 min with a formic acid solution pH 3 (eluens A) and methanol (eluens B), at a flow rate of 4 mL/min, and detection at 270 nm, with injections of 50–100 mg product in ethanol. The eluent was collected on ice, whereafter methanol was removed by evaporation and the residue was rinsed with methylene chloride and dried under N_2. The compounds were pure (>99%) as judged with ^{1}H NMR (see below) and HPLC, using hypersil ODS (100 × 3 mm i.d.) eluted with a gradient of 20–95% in 20 min (for eluens see above), at a flow rate of 0.4 mL/min (k' = 6.2, 7.5 and 8.2 for, respectively, ethacrynic acid, monobromo ethacrynic acid and dibromo dihydro ethacrynic acid). The two bromo derivatives were identified and characterized by ^{1}H NMR (300 MHz) and mass spectrometry. NMR ($CDCl_3$) dibromo dihydro ethacrynic acid (Fig. 1): δ 3.83/4.32 (dd, J = 10.4 Hz, proton *a*), δ 2.10/2.37 (dm, proton *c*), δ 1.10 (t, J = 7.1 Hz, proton *d*), δ 7.67 (d, J = 8.6 Hz, proton *e*), δ 6.80 (d, J = 8.7 Hz, proton *f*), and δ 4.82 (s, proton *g*). Both enantiomers of dibromo dihydro ethacrynic acid are present, as could be derived from the proton signals for proton *a* and *c*, showing double doublets and multiplets. NMR ($CDCl_3$) monobromo ethacrynic acid (Fig. 1): δ 7.05 (s, proton *a*) and δ 2.65 (q, J = 7.5 Hz, proton *c*), δ 1.13 (t, J = 7.5 Hz, proton *d*), δ 7.59 (d, J = 8.5 Hz, proton *e*), δ 6.82 (d, J = 8.5 Hz, proton *f*) and δ 4.81 (s, proton *g*). As expected from the sterical hindrance, presumably only *E*-monobromo ethacrynic acid is present, since the measured δ for proton *a* is closest to the calculated δ (calculated δ = 6.84 and 6.95 Hz, for respectively, *Z*- and *E*-monobromo ethacrynic acid [22]). With mass spectrometry (Finnigan MAT 8200, electron impact) the M^+ (m/z 380) is visible in the spectrum. Moreover, three major fragment ions are present: m/z 321 $[M-CH_2COOH]^+$, m/z 247 $[M-BrHC{=}CC_2H_5]^+$ and a fragment corresponding to m/z 189 $[C_7H_3O_2Cl_2]^+$. The dibromo dihydro ethacrynic acid spectrum was identical to the spectrum from ethacrynic acid with one Br^+-peak extra (presumably by Br^+-abstraction with formation of a double bond, followed by Br^- elimination).

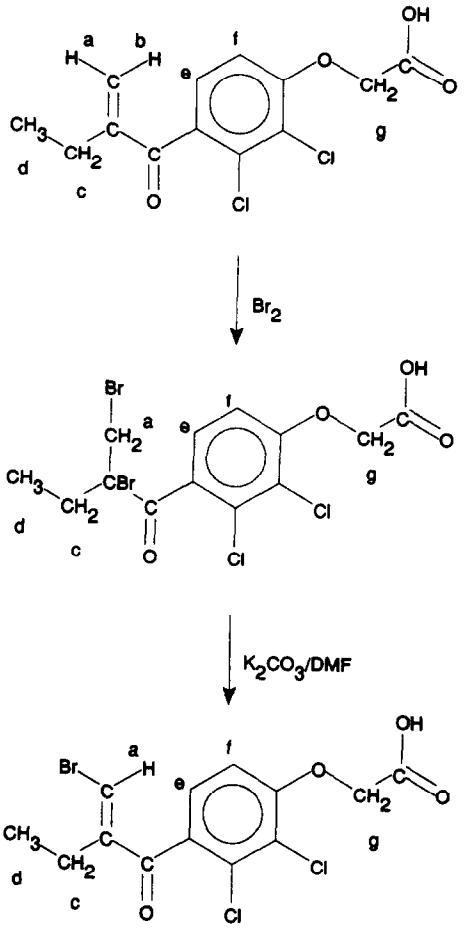

Fig. 1. The structure of ethacrynic acid and its dibromo dihydro and monobromo derivative. The letters at the protons correspond with the NMR data (see Material and Methods).

GST purification and assay. GST isoenzymes were purified from liver, kidney (rat GST 7-7) and placenta (human GST P1-1) using affinity chromatography (*S*-hexylglutathione-Sepharose 6B), as described previously [23]. The separation of the different isoenzymes was achieved by chromatofocusing on polybuffer exchangers (Pharmacia, Uppsala,

Sweden), with PBE 118 for GST 1-1, 2-2, 3-3, 4-4, A1-1, A2-2 and M1a-1a, and PBE 94 for GST 7-7 and P1-1. PBE 118 was equilibrated with 0.025 M triethylamine–HCl (pH 11), and elution was performed with (1:45 diluted) pharmalyte (pH 8–10.5)–HCl (pH 8) (Pharmacia), while PBE 94 was equilibrated with 0.025 M ethanolamine-CH_3COOH (pH 9.4), and eluted with polybuffer 96–HCl (pH 7) (Pharmacia). Purity was confirmed by SDS gel electrophoresis, isoelectric focussing and HPLC analysis as described previously [23, 24]. Protein was determined according to Lowry *et al.* [25] with bovine serum albumin as standard. GST activity was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate [26], specific activity (μmol/min mg) were 54, 22, 44, 16, 17, 31, 22, 150 and 70, respectively, for GST 1-1, 2-2, 3-3, 4-4, 7-7, A1-1, A2-2, M1a-1a and P1-1. All enzyme concentrations were expressed as the concentration of the subunit (M_r: 25,500, 27,500, 26,300, 26,300, 24,800, 25,900, 25,900, 26,700 and 24,800, respectively, for GST subunits 1, 2, 3, 4, 7, A1, A2, M1a and P1 [27]). All steps were performed at 0–8°, with oxygen protection by 0.1 mM dithiotreitol in all steps, with the exception of the chromatofocusing and the final dialysis, which were performed, respectively, with degassed buffer and under N_2.

Labeling of GST by [^{14}C]ethacrynic acid. GST (10 μM) was incubated for 1 hr at 37° with 100 μM [^{14}C]ethacrynic acid (total volume 25 μL) in 0.1 M potassium phosphate buffer pH 7.4, to determine the binding capacity of ethacrynic acid under drastic conditions. The enzyme was precipitated with 0.4 mL of 20% (w/v) trichloroacetic acid and 0.4 mg bovine serum albumin were added to increase the protein concentration and to facilitate the work up procedure. The enzyme was centrifuged 5 min at 10,000 *g*. We carefully washed the pellet three times with ice-cold acetone [with 1% of a 65% (w/v) trichloroacetic acid solution], which removes ethacrynic almost completely from the blank samples. The enzyme was dissolved with 0.1 M potassium phosphate buffer pH 7.4 (three extractions with 0.3 mL) and the sample was screened for radioactivity with 10 mL of scintillation liquid. The recovery of protein was about 80%. These incubations were performed in duplicate. Quinones can label GST very efficiently, presumably by reaction with cysteine residues of GST. The same experiments were performed, after preincubation for 5 min at 25° with 50 μM tetrachloro-1,4-benzoquinone, to compare ethacrynic acid labeling capacity after preincubation with quinones. The time course of the incorporation of [^{14}C]-ethacrynic acid in human GST P1-1 and GST 7-7 was investigated in more detail at 20° by incubating 1.25 μM enzyme with 6.25 μM [^{14}C]ethacrynic acid in 0.1 M potassium phosphate pH 7.4 (total volume 0.16 mL). For washing procedure see above.

Inhibition studies. To correlate inhibition and labeling, the same experimental conditions as described for the labeling experiments were used for determining the enzymatic activity towards CDNB according to Habig [26]. In an independent experiment 1.25 μM GST P1-1 was also incubated for 20 min at 20° with 6.25 μM ethacrynic acid in the presence of 100 μM *S*-hexylglutathione (in triplicate), after which the catalytic activity towards CDNB was determined. In an independent experiment the inhibition of GST 4-4, 7-7, M1a-1a and P1-1 were compared by incubating 0.5 μM enzyme with 10 μM ethacrynic acid, monobromo ethacrynic acid and dibromo dihydro ethacrynic acid for 2 hr at 20° in 0.1 M potassium phosphate pH 7.4. The inhibitor was added, immediately before measuring the activity, to all blank incubations in all time course studies. In an earlier study only 15% irreversible inhibition of GST 7-7 could be detected using the dialysis method [12]. These experiments were performed at a lower pH (6.5) and lower temperature (17°). When these conditions were repeated, using the assay described above, the inhibition found was 38 ± 4.0%. The difference with the dialysis experiment could presumably be attributed to the loss of activity in the blank during the dialysis, which is especially high for GST 7-7. At the present time it is known that protecting cysteine residues of GST 7-7 by reducing agents avoids this drawback of dialysis [28].

IC_{50} (μM) values for ethacrynic acid and its bromo derivatives were determined by mixing 7.5 nM GST with a concentration range of the inhibitors. At least six concentrations (in duplo) were used to determine the IC_{50} values. IC_{50} values were obtained from plots. Maximal inhibition (>95%) was checked using plots as described previously [12].

RESULTS

^{14}C-Labeled ethacrynic acid was used to determine covalent labeling of GST under rigorous conditions (Table 1). Significant labeling of the pi-class GST was observed. The GST of mu-class isoenzymes were also significantly labeled, especially rat GST 3-3 (up to 0.6 nmol/nmol enzyme). Considerably lower labeling of the rat alpha-class isoenzymes was observed. However, significant inhibition of the enzymic activity was only observed in the pi-class (Table 1).

The time course of labeling versus inhibition was determined for the pi-class GST, of both human and rat, under milder conditions (Fig. 2). Under these conditions, both human and rat GST isoenzymes of pi-class were inhibited in about 100–130 min to their maximum value, concomitant with maximal labeling of about 0.8 nmol/nmol enzyme. There is a clear correlation between labeling and inhibition. Preincubation of rat GST with tetrachloro-1,4-benzoquinone very efficiently inhibited the incorporation of [^{14}C]ethacrynic acid in all GST isoenzymes tested (Table 1). The incorporation of [^{14}C]ethacrynic acid in GST 7-7 was completely inhibited, while also the incorporation in the mu-class was inhibited remarkably. The competitive inhibitor *S*-hexylglutathione (IC_{50} = 20 μM [2]), completely abolished the inhibition of GST P1-1 by ethacrynic acid (Table 2).

The monobromo and dibromo dihydro derivatives of ethacrynic acid were synthesized in an effort to develop structurally related compounds with increased alkylating ability. Mu- and pi-class GST isoenzymes of both human and rat were incubated with these compounds (Table 3); a preliminary

Table 1. Covalent binding of [^{14}C]ethacrynic acid to rat GST isoenzymes, remaining activity towards CDNB and effect of preincubation with tetrachloro-1,4-benzoquinone (TCBQ) on covalent binding of [^{14}C]ethacrynic acid

Enzyme	Covalent binding (nmol label/nmol enzyme)	% Remaining activity	Covalent binding after preincubation with TCBQ (nmol label/nmol enzyme)
GST 1-1	0.19 ± 0.04	99 ± 11	0.08 ± 0.01
GST 2-2	0.20 ± 0.02	113 ± 6	0.11 ± 0.01
GST 3-3	0.61 ± 0.08	109 ± 12	0.04 ± 0.02
GST 4-4	0.31 ± 0.04	88 ± 14	0.04 ± 0.02
GST 7-7	0.74 ± 0.06	7 ± 1	0.00 ± 0.00

Enzyme (10 μM) was incubated with 100 μM [^{14}C]ethacrynic acid for 60 min at 37° (N = 2), after which the labeling of GST was determined. The labeling was also determined under the same conditions after preincubation with 50 μM TCBQ for 5 min at 25°. The enzymatic activity towards CDNB was determined after incubation of 10 μM enzyme with 100 μM ethacrynic acid for 60 min at 37° (N = 2), and expressed as per cent of control incubation (loss of enzymatic activity in controls <10%, with the exception of GST 7-7: a 20% loss).

Results are means ± SD.

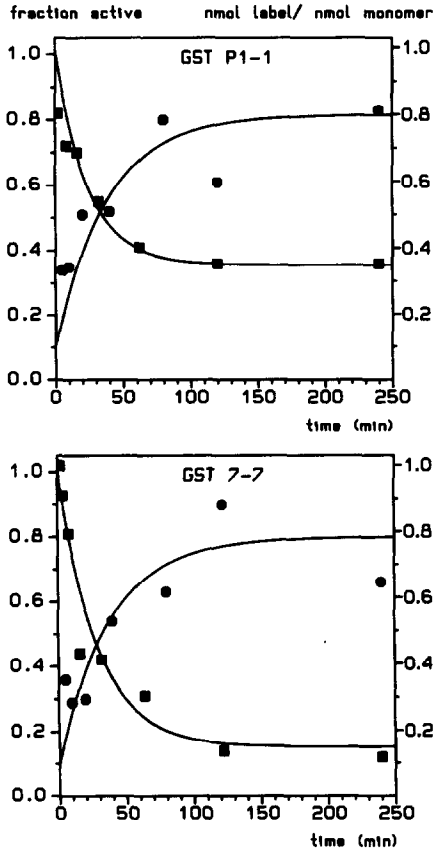

Fig. 2. The time course of the labeling of the GST isoenzymes human P1-1 and rat GST 7-7 was investigated at 20° by incubating 1.25 μM enzyme with 6.25 μM ethacrynic acid, after which the enzymatic activity towards CDNB and the amount of radioactivity incorporated were determined. (■) Remaining fraction catalytically active; (●) nmol [^{14}C]ethacrynic acid/nmol monomer (for experimental details see Materials and Methods).

Table 2. Effect of *S*-hexylglutathione on the inhibition of GST P1-1 by ethacrynic acid

Incubation	% Remaining activity
GST + ethacrynic acid	70 ± 8.0
GST + *S*-hexylglutathione + ethacrynic acid	104 ± 6.7

GST (1.25 μM) was incubated with 6.25 μM ethacrynic acid for 20 min at 20° in the presence or absence of 100 μM *S*-hexylglutathione (in triplicate), after which the catalytic activity (expressed as per cent of control incubations) towards CDNB was measured.

Results are means ± SD.

experiment had shown that the alpha-class is either not affected by the dibromo dihydro derivative (GST A2-2) or very inefficiently (GST 1-1, 2-2 and A1-1 only 30–40% inhibition after incubation under very rigorous conditions, results not shown). As was found for ethacrynic acid, the monobromo derivative did not irreversibly inhibit GST of the mu-class, while for the pi-class the monobromo derivative was much less efficient than ethacrynic acid itself. The dibromo dihydro derivative, on the other hand, inhibits both human and rat GST isoenzymes pi-class more efficiently (note that the conditions are slightly different from the ones used in Table 1). Interestingly, this compound is also an irreversible inhibitor of the mu-class GST isoenzymes.

Lastly, the competitive inhibition was determined (Table 3): the two bromo atoms only marginally affect the strong competitive (reversible) inhibitory capacity of ethacrynic acid, only human GST P1-1 is inhibited to a lesser extent.

DISCUSSION

The catalytic activity of the pi-class isoenzymes rat GST 7-7 and human P1-1 was strongly inhibited

Table 3. Irreversible inactivation (expressed as per cent remaining activity) of rat and human GST, and reversible inhibition [expressed as IC_{50} (μM)* values] with ethacrynic acid, and its monobromo and dibromo dihydro derivatives

	Ethacrynic acid		Monobromo		Dibromo dihydro	
Enzyme	IC_{50}	% Remaining activity	IC_{50}	% Remaining activity	IC_{50}	% Remaining activity
GST 7-7	4.8†	29	>50	59	4.6	4
GST P1-1	4.0	19	>50	79	10	10
GST 4-4	0.7	111	>50	110	0.6	48
GST M1a-1a	0.2	127	18	104	0.4	30

The irreversible inactivation was determined by incubating 0.5 μM enzyme with 10 μM inhibitor for 120 min at 20° (N = 2), after which the enzymatic activity (as per cent of control incubations) was determined towards CDNB.

Reversible inhibition was determined by mixing 7.5 nM enzyme with inhibitor, after which the enzymatic activity was immediately determined towards CDNB.

* The concentration of ethacrynic acid or its bromo derivatives resulting in 50% inhibition of the enzymic activity towards CDNB (IC_{50}). For individual values, the coefficient of variation was less than 20%.

† IC_{50} of GST 7-7 from Ploemen *et al.* [12].

Experiments were performed as described in Materials and Methods.

in an irreversible manner by ethacrynic acid. In a previous study, we have shown that ethacrynic acid is also a strong competitive inhibitor of rat and human GST of alpha-, mu- and pi-class, with 50% inhibition at 0.3–6 μM. The inactivation of the pi-class isoenzymes correlates strongly with the incorporation of [^{14}C]ethacrynic acid, with maximal inhibition with incorporation of about 0.8 nmol/nmol enzyme. This suggests that one single amino acid is responsible for the inhibition of GST of the pi-class. Preincubation with tetrachloro-1,4-benzoquinone prevented the incorporation of label completely for GST 7-7, suggesting the involvement of a cysteine residue [29]. The study with *S*-hexylglutathione (a substrate analog, with considerable affinity for the active site of GST [2]) indicates that the modification takes place in the active site. Ethacrynic acid presumably reacts with the highly reactive thiol group in the proximity of the catalytic site of GST 7-7 and human GST P1-1, which has been identified as the cysteine at the 47th position by several groups [30, 31]. This reactive cysteine seems to be conserved within pi-class isoenzymes of various species, e.g. pig and bovine GST pi-class isoenzymes also contain a preferentially modified highly reactive cysteine residue, chemical modification of which leads to enzyme inactivation [32, 33]. These cysteine residues of the pi-class are also unique in their sensitivity to inactivation by oxidation [28] and SH/SS exchange reaction reagents [34], both resulting in (intramolecular or mixed) disulfides. It is widely recognized that oxygen protection is crucial while working with the pi-class isoenzymes to assure that the enzyme has the native form [28, 31].

Significant incorporation of [^{14}C]ethacrynic acid was also observed with representatives of the mu-class GST. In particular the mu-class GST 3-3 and to a lesser extent GST 4-4 were labeled with ethacrynic acid. However, no concomitant loss of catalytic activity was observed. This property is not restricted to ethacrynic acid: e.g. bromobenzene metabolites are also, incorporated in rat GST subunit 1 and 4 without inhibition [35] and studies with iodoacetamide and GST 3-3, showed that only the cysteine at the 86th position was labeled by that reagent without loss of activity [36]. Preincubation of GST 3-3 with tetrachloro-1,4-benzoquinone prevented the incorporation of [^{14}C]ethacrynic acid very strongly, again suggesting the involvement of one cysteine residue, presumably cysteine 86. Interestingly, modification of the enzyme by tetrachloro-1,4-benzoquinone and its glutathione conjugate does lead to inactivation. The question whether this is due to the structure of the reagent, or to the labeling of the other cysteine residues (at the 114th and 173rd position) remains to be answered.

The alpha-class GST isoenzymes are only marginally susceptible to labeling with ethacrynic acid, and tetrachloro-1,4-benzoquinone prevents incorporation of [^{14}C]ethacrynic acid for about 50%, thus indicating that this might be due to non-specific reactions in which residues other than cysteines are involved.

One of the goals of this work was to improve the irreversible inhibitory capacity of ethacrynic acid by newly synthesized derivatives with enhanced chemical reactivity. To this end two derivatives were synthesized, the monobromo and dibromo dihydro ethacrynic acid. The monobromo derivative, with the intact α,β-unsaturated carbonyl bond moiety, irreversibly inhibited pi-class GST to a lesser extent than ethacrynic acid itself. However, the dibromo dihydro derivative which is expected to react at the alpha rather than the beta carbon, inactivates pi-class GST more efficiently than the parent compound. This compound, which undergoes a substitution-type reaction, also irreversibly inhibits GST of mu-class, and to a much lesser extent GST of alpha-

class. The GST A2-2 is the only enzyme not inhibited and is also the only GST isoenzyme which does not contain cysteine residues, suggesting again that the inactivation is a result of cysteine modification. In contrast with the monobromo derivative, the dibromo dihydro compound also retains its strong reversible inhibitory capacity. Thus, this compound combines the properties of a strong irreversible and reversible inhibitor.

Recently, Kuzmich *et al.* [37] showed that ethacrynic acid in cellular systems can induce GST of pi-class at the transcriptional level. Ethacrynic acid is also a substrate for pi-class GST. Thus, presumably, a distinction has to be made between short and longer term effects in the processes induced by ethacrynic acid in a cell. At present, the following train of events seems likely: (i) direct competitive inhibition by ethacrynic acid itself, followed by (ii) GSH depletion with concomitant formation of the GSH conjugate of ethacrynic acid which can result (iii) in a competitive inhibition by the conjugate while (iv) irreversible inhibition can occur at low intracellular GSH concentration, and finally (v) the GST are induced. It will be a challenge to unravel the complex molecular mechanism controlling inhibition and activation in cellular systems and *in vivo*, in this respect in particular the dibromo dihydro derivative might be a useful device.

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